Home Office

Animals (Scientific Procedures) Act 1986

Non-technical summaries for project licences granted January – March 2023 that require a retrospective assessment

Contents

1. A Practical Training Course in Microvascular Surgery 3

2. Assessing the humaneness of spring traps 13

3. Basic research and pre-clinical analysis oftherapies for neuromuscular diseases 21

4. Devising new therapies for multiple sclerosis and small vessel disease 32

5. Disease models of emerging and re-emerging viruses 43

6. Identification of determinants of pathology and protection in infections of barrier organs 52

7. Mechanisms and targets for chronic pain 62

8. Mechanisms underlying abnormal heart rhythm and function 73

9. Molecular control of neutrophils and endothelial cells in inflammation and repair 79

10. Rodent regulatory genotoxicity 86

11. Safety Testing of Chemicals, Plant Protection Products, Biocides and Substances added to Food or Feed Products Using Small Animal Species 94

12. Safety testing of medicinal products using dogs and minipigs 104

13. Regulation of the mucosal immune response during infection with pathogens or pathobionts and inflammation in rodents 115

14. Identification and characterisation of therapeutic targets for cardiac disease 132

15. Identification of New Therapeutic Targets and Investigation of Biological and Synthetic Therapies for Ischaemic Disease 144

16. Development of brain tumour models in mice for tumorigenicity screens and pre-clinical therapeutic trials. 158

17. Safety Testing of Medicinal Products Using Non-Human Primates 169

18. Development and validation of in vivo preclinical models to substantially refine and reduce the number of animals subjected to severe procedures in snakebite envenoming research 181

19. Developing minimally invasive interventional cardiology techniques for studying and treating atrial fibrillation and other arrhythmias in the horse 190

1. A Practical Training Course in Microvascular Surgery

Project duration

5 years 0 months

Project purpose

- Higher education and training

Key words

Microvascular, Surgery

Animal types	Life stages
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.

- Education and training licence

Objectives and benefits

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

What's the aim of this project?

This course has the sole aim and objective of providing suitably skilled surgeons with highest qualityinstruction and technical training to allow them to undertake (or support) microvascular surgery on patients in their clinical practice.

Microsurgery refers to surgery on blood vessels or structures (such as nerves) which is not possiblewith the naked eye, requiring and as such uses magnification to allow the surgeon to achieve a successful outcome.

A retrospective assessment of these aims will be due by 17 July 2028

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 course is a vital component in complex Head and neck reconstruction training

Reconstruction following complex head and neck ablative surgery utilises transfer of required donor tissue(s) with its constituent vascular pedicle (feeding artery and draining venous network) with anastomosis of those vessels to recipient vessels to provide vascularised reconstructions; otherwise referred to as “Free tissue transfer with microvascular anastomosis:. This has become routine practicewithin reconstructive surgery over the last 30 years. In the anatomical area of the Head & Neck, in cases of cancer, congenital malformation and trauma, reconstruction with free tissue transfer has demonstrated far superior functional and cosmetic outcomes than other methods of treatment (Gabrysz-Forget 2019 J Oto HN Surg).

Comparable microvascular anastomoses are required for the repair of complex neurovascular structures as undertaken by Neurosurgeons. This highly specialised, demanding and exceptionally technique-sensitive skill is typically only acquired by experienced neurosurgeons with established clinical practices, frequently in tertiary referral centres. The superficial temporal artery to the middle cerebral artery (STA-MCA) bypass exemplifies a neurovascular/cerebrovascular anastomosis. STA-MCA bypass is a particularly useful technique for the management of complex or giant aneurysms where surgical treatment sometimes requires the sacrifice and revascularization of a main arterial trunk.

Microvascular anastomotic techniques (the surgical connection of arterial and venous vascular networks to provide viable free tissue transfer) are difficult to acquire and involve a graduated learningcurve of practice on prosthetic devices, on chicken/turkey leg blood vessels, on terminally anaesthetised rats and finally performed on human patients under the direct supervision of experienced surgeons/trainers. Acquisition of microvascular reconstruction skills is now considered anessential element of Higher Surgical Training in a number of surgical disciplines (Intercollegiate Surgical Curriculum Programme (ISCP) - OMFS Curriculum 2021; Plastic Surgery Curriculum 2021; ENT Curriculum 2021).

In the UK context, basic training can be obtained in two ways:

Attending practical microvascular courses such as the one (Practical Training Course in Microvascular Surgery) which has been delivered successfully in since 1993

Undertaking research projects that involve microsurgical techniques

Our course has been awarded 37 Continuing Professional Development (CPD) credits by the Royal College of Surgeons of England (representing a significant element of the necessary 50 CPD creditsrequired annually for practicing clinicians) and is an integral component of the a Masters (MSc) in OMFS (20 credits Masters-level credits). Provision of the microvascular training course will be reviewed by the Animal Welfare And Ethical Review Body (AWERB), including detailed course feedback is annually.

Maxillofacial Surgery is concerned with the surgical management of acquired and congenital disordersof the face, jaws and mouth. At present there are approximately 80

Maxillofacial Units in the UK, of which half routinely carry out free tissue transfer procedures. There are 90 career grade doctors training in these units, each of whom are either required to obtain the basic skills necessary for the performance of microvascular anastomosis, or, having previously obtained the skills, have a need to maintain these skills. In many cases, the local unit is not a position to provide either.

In spite of the advances in models that allow trainees to practice operative skills in a simulated surgicalenvironment, such as is the case in laparoscopic surgery or in arterial access proficiency training; thereare no suitable prosthetic devices that replicate vascular microsurgery. The acquisition, development and maintenance of such skills can only be achieved by instruction and practice. A variety of techniques have been used in the past to develop and maintain these skills with variable success (e.g. Chicken legs, placental blood vessels). None of them has provided completely satisfactory. A surgeon needs to practice the key steps, critical for operative success, for example, mobilisation of the artery and vein from surrounding tissues, preparation of the vessel wall with minimal handling and trauma, accurate positioning and tension of sutures, management of vascular leakage (haemostasis), an awareness and appreciation of the problems such as vessel spasm. One is forced to conclude that presently the only truly viable method of assessing the adequacy of microvascular anastomosis is to observe blood flow across the anastomotic site and this inevitably involves living tissue. Training on living tissues (with cardiac output and vascular perfusion) is necessary to acquire technical skills in combination with direct, robust feedback on the adequacy of those skills (microvascular patency).

The critical success of microvascular anastomosis to survival of free tissue transfer in the clinical environment precludes initial training of surgeons until the essential practical skills have already beenacquired – practice and training on animal model prior to commencing training in a clinical setting on patients.

There are no suitable alternatives (either in terms of the animal model or the expertise within the available faculty - all with prior capabilities in the teaching of microsurgery on rats and the clinicalexperience of H&N reconstructive surgery).

Previous experience of running this course for over 30 years supports our understanding that the vast majority of trainees, i.e. more than 95%, gain sufficient skills to be able to perform clinical microsurgicaltechniques under supervision and a number (15-20%) develop sufficient competence to perform them without supervision. 95% are able to achieve a good end-to-end and end-to-side arterial and venous anastomosis using the femoral vessels, and 70-80% are able to produce a successful (patent) interpositional vein graft (Vein graft sited into an arterial system) by the end of the course. This should enable (or support) several units to take on (or continue to employ) microvascular procedures which they could not otherwise consider; which if it were the case could potentially deprive patients of the consequent considerable benefit.

This course provides an essential component in the training of both UK and international surgeons to alevel necessary to sustain ongoing complex microvascular procedures in specialised units.

How will course attendees use their knowledge or skills in their future careers?

All the students are full-time higher trainees on various clinical training pathways. this course and theclinical training supports their development towards becoming competent microvascular surgeons.

The course equips the higher surgical trainee (HST) with the underpinning theory of H&N microvascular surgery and associated reconstruction techniques based on robust evidence and practical demonstration. It facilitates HST development of complex specific surgical skills in Head andNeck Surgery in a safe environment with direct support and immediate guidance and critique.

Without the skills developed in a course such as this, it is unlikely that safe, successful microsurgerycould be practiced - in effect a course such as this will reduce the risk of harm to patients through development of understanding and technical proficiency.

It is worth noting that all trainees will be either registered in higher surgical training programmes in respective surgical specialties (OMFS, Plastic Surgery, ENT and/or neurosurgery) or be establishedconsultants (or equivalent) in those specialties. As detailed below, it is an “entry” requirement for thecourse that participants will provide a formal statement from their respective head of department/specialty indicating that they have the clinical need and ability to develop or maintain microvascular skills.

What are the principal learning outcomes from the course?

The ability to demonstrate critical understanding of the technical set up, vascular preparation, andcompletion of microvascular anastomoses as would be necessary for successful free flap reconstruction in humans.

To understand the sources for technical error in microsurgery and technical means to mitigate theiroccurance.

Each participant should, by the end of the course, have developed the capability to prepare and anastomose vessels with a diameter of less than 1mm. They should be able to critically assess thequality of the anastomosis and have a feel for their aptitude in this area of surgery

How are these learning outcomes important to the people on the course?

These learning outcomes are essential to successful microsurgery in humans.
They ensure the greatest liklihood of successful surgical procedures when undertaken in the clinicalsettting.

Who or what will benefit from the transfer of knowledge, or acquisition of skills that this coursewill deliver?

There are several beneficieries from the course;

- Delegates - as detailed above, the technical surgical abilities and repertoire of the delegates willbe enhanced in such a way as to facilitate successful microsurgery.

- Patients - the impact of failed microvascular surgery for Head & Neck patients is difficult to understate (function, cosmesis and ultimately survival all require successful microvascular freetissue transfer). without success in this area of surgery, outcomes for patients are consistently poor.

- Healthcare providers - successful microsurgery within Head & Neck departments in the UK andinternationally rely on the development of surgeons who possess the necessary understandingand technical abilities

How will you look to maximise the outputs of this work?
This course has now entered its 3rd decade of successful provision with an ever increasing cohort of surgeons worldwide who have benefitted from its education. This course in internationally renowned forits rigour, technical standards and educational benefit.

Each new delegate is able to return to their parent unit and further develop their skills whilst impartingknowedge to others (including their trainees who frequently return following recommendation to complete the course)

Species and numbers of animals expected to be used

- Rats: 65 animals per annum (325 animals in total)

Predicted harms

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

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

The rat femoral and epigastric vessels remain the standard animal microvascular model used widely inthe UK and beyond. They possess many of the features of human microvasculature and in addition, provide exceptional “feedback” as to quality of surgical technique such that errors (which might lead todelayed microvascular failure in patients) are immediately apparent to the operator. The rat femoral and epigastric system reflect comparable vessels in terms of size, calibre and quality to that which is utilised in the human microvascular setting.
Typically, what will be done to an animal used in your project?

Anaesthesia will be induced and maintained using suitable anaesthetic agents.

Blood vessels will be exposed, isolated, divided, and either repaired, anastomosed or grafted.(AC)

In some instance free tissue flaps may be raised on a vascular pedicle which is then divided andre-anastomosed. (AC)

At the end of the procedure, animals will be terminated by a Schedule 1 method. (AC)

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

Each procedure shall be carried out under general anaesthesia (with associated analgesia) which shallcontinue until termination of the animal by a Schedule 1 method. Anaesthesia shall be induced, monitored and maintained by experienced personal licencees. Termination of animals at the end of theprocedure shall be performed by Schedule 1 method by the licensees responsible for induction and maintenance of anaesthesia.

There will be mild stress and discomfort associated with restraint and the initial injection of

the anaesthetic agent. This will be minimised by using experienced licensees for anaesthetic induction. Itis obviously imperative that a suitable anaesthetic plane is maintained throughout the procedure and again those supporting/provising the anaesthetic for the animals will be experienced in such circumstances. The pedal withdrawal reflex, along with respiratory parameters will be assessed regularly to ensure adequacy of anaesthetic depth.

Hypothermia is a fundamental consideration in recovery anaesthesia in rodent surgery but these animals will not recovered. Previous extensive experience has demonstrated that we can comfortably maintain suitable anaesthetic depth without inadvertent loss of animals, without supplementary heatingfor a period of up to 9 hours from induction by providing additional support in the form of heat and fluids. Rats are maintained throughout the procedure on a heated mat with warmed fluids (such ascompound sodium lactate) administered subcutaneously.

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 animaltype)?

The procedure is always conducted under terminal anaesthesia (animal does not recover from theanaesthetic).

End-to-end and end-to-side anastomosis (suturing of blood vessels to allow normal blood flow) will beperformed on the femoral artery and vein and on the epigastric artery and vein.

Not all anastomoses will be technically successful, however, as all animals are terminally anaesthetized, failure of an anastomosis will not cause animal welfare concerns.

Occasionally, due to operative error, haemorrhage will occur; under such circumstances if it is considered that blood loss is excessive the animal will be immediately euthanased. Under no circumstances do the rates ever experience pain or distress. The rats are killed humanely aftercompletion of the training exercise.

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

- Killed

A retrospective assessment of these predicted harms will be due by 17 July 2028

The PPL holder will be required to disclose:

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

Replacement

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

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

It would not be possible to achieve the objectives of the project without this animal model, as there isno alternative for a living vascular system with its dynatic nature and associated clotting profile(s).

A component of replacement has already been included in this course with the use of a prosthetic device (or synthetic blood vessel) to learn, consolidate and assess basic microsurgical skills/suturing.

Attendance and training on a course such as the one proposed here is considered a standard phase inthe learning of microvascular (surgical) techniques that, when applied, result in a successful anastomosis (ie allowing blood flow through anastomised vessels).

As the success of microvascular anastomosis is so important to the surgical patient there is a need foran animal model with flowing blood and natural technical complexities (akin to the human situation) to ensure the surgeons can practice in a situation that the success of the surgery can be fully assessed.

There is no adequate non-living substitute to living blood vessels for learning the critical aspects ofmicrovascular surgery.

Why can’t your aim be met by observing or by participating in ongoing research or clinicalprocedures?

The rat femoral and epigastric vessels remain the standard animal microvascular model used widely inthe UK and beyond. They possess many of the features of human microvasculature and in addition, provide exceptional “feedback” as to quality of surgical technique such that errors (which might lead todelayed microvascular failure in patients) are immediately apparent to the operator. The rat femoral and epigastric system reflect comparable vessels in terms of size, calibre and quality to that which is utilised in the human microvascular setting.

Attendance and training on a course such as the one proposed here is considered a standard phase inthe learning of microvascular (surgical) techniques that, when applied, result in a successful anastomosis (ie allowing blood flow through anastomised vessels). It would not be possible to achieve the objectives of the project without this animal model, as there is no alternative for a living vascular system with its dynamic nature and associated clotting profile(s).

Mere observation would fail to achieve the technical skill, proficiency and surgical capabilities of a microvascular surgeon. It is the physical undertaking of a complex (animal) anastomosis that a) teststechnical ability and b) provides immediate feedback of proficiency - this is witnessed by experiencedtrainers who can instruct where improvement should be made and thus maximise microvascular success.

A retrospective assessment of replacement will be due by 17 July 2028

The PPL holder will be required to disclose:

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

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen 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 tissueand reuse.

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

The numbers of animals used is the minimum required for acquiring the desired surgical skills. Prior tocommencement of procedures on animals, a prosthetic device (synthetic vessel) is used.

A single animal is used per candidate per day ensuring complete utilisation of the resource prior totermination of the animal.
The procedures are carried out under non-recovery anaesthesia so that no animal will experienceadverse effects.

What in silico or ex vivo techniques will you use during training?

A component of replacement has already been included in this course with the use of a prosthetic device (or synthetic blood vessel) to learn, consolidate and assess basic microsurgical skills/suturing.

Will these techniques reduce animal numbers? If so, how?

The use of a synthetic vessel has reduced the number of animals used by one per delegate per course. It has the additional benefit of allowing trainers to witness the capabilities of delegates on thesynthetic construct BEFORE progressing to the animal model. If necessary delegates can continue using the synthetic construct until such time that they demonstrate suitable skills necessary to gain full/maximal benefit from the living model.

What other measures will you use to minimise the number of animals you plan to use in yourproject?

The design of each teaching day ensures that delegates make full use of each individual animal modeland gain maximal technical/microsurgical exposure and training. This includes using both the femoral arteries and both the femoral veins for anastomoses on each animal.

It is expected that trainees will utilise a single rat per day. There may be circumstances where a traineeexceeds training expectations and completes the technical proficiencies/is able to demonstrate outstanding abilities within the 5 day period and therefore would not require an animal for each of the 5days.

Where any trainee is demonstrably slower in their learning of surgical skills/techniques, this can besupported with a return to the prosthetic device (synthetic vessel) to consolidate skills and learningbefore recommencing learning on the animal model.

Evolution in the course has reduced the number of animals used for direct demonstration

purposes. Through the use of videos and simulation, animal numbers have been reduced in the last 3-4 years. Ata maximum, reduction would result in one less animal being required per day (n=5 over the course of the week)

A retrospective assessment of reduction will be due by 17 July 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan 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 welfarecosts (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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rat Femoral Vascular System;

The rat femoral and epigastric vessels remain the standard animal microvascular model used widely inthe UK and beyond. They possess many of the features of human microvasculature and in addition, provide exceptional “feedback” as to quality of surgical technique such that errors (which might lead todelayed microvascular failure in patients) are immediately apparent to the operator. The rat femoral and epigastric system reflect comparable vessels in terms of size, calibre and quality to that which is utilised in the human microvascular setting.

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

Animals utilised provide the optimal vascular model (rat femoral system) both in terms of calibre andcomparablity to human situation. The rat femoral and epigastric vessels are the standard animal model used widely in the UK and beyond.

All animals receive terminal anaesthesia

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

Over the course of 30+ years of running this course, the host BSU team have developed a structuredtechnique for the monitoring of animal welfare during the course. This is supplimented by a strict instruction regarding animal care at the outset of the course for all PIL holders and direct supervisionby experienced surgical trainers with many years of experience of animal (and human) microvascularsurgical instruction. Under no circumstances do the rats ever experience pain or distress.

What published best practice guidance will you follow to ensure experiments are

conducted inthe most refined way?

No evidence exists to support a non-live animal for microvascular training. All attempts to refine theproject and mitigate harm to animals are undertaken as part of the proposed protocols

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

The course directors (inc Home office project licence holder) undertake regular analyses of suitablealternatives for the animal model utilised.

This includes frequent interaction and conversation with similar course directors and a regular literaturereview.

A retrospective assessment of refinement will be due by 17 July 2028

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 forfuture work of this kind? During the project, how did you minimise harm to the animals?

2. Assessing the humaneness of spring traps

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 aimsmentioned in paragraph (b)
- Protection of the natural environment in the interests of the health or welfare of man oranimals

Key words

Spring traps, Humaneness, Spring Trap Approval Order, AIHTS, Wildlife

Animal types	Life stages
Mice	adult, juvenile
Rats	juvenile, adult
Rabbits	adult, juvenile
Grey squirrels	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 orscientific needs it's addressing.

What's the aim of this project?

To produce evidence necessary for the regulation of the Spring Traps Approval Order (STAO), alegislative instrument of the Pests Act (1954).

For any new combination of trap type, trap setting, or target species added to the STAO list, qualifyingspring traps must undergo a quantitative assessment to determine whether they meet the humannesscriteria required. Work under this licence provides the necessary evidence for regulation.

A retrospective assessment of these aims will be due by 31 July 2028

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 advancedor 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?

Ensuring that qualifying spring-traps deliver a rapid and reliable death to wild animal targets and meeta minimum standard of humaneness, avoids the sale and widespread use of relatively inhumane devices. Inhumane spring-traps might otherwise maim wild animals or produce prolonged periods of substantial suffering before death.

By supporting the regulation of spring traps, this work helps substantially reduce the suffering of verymany wild animals which are subject to wildlife management.

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

The principal benefit is robust evidence of the humanness of tested spring-traps, ensuring that trapsfailing the current regulatory standard are not permitted for use where they might cause excessive harm in specific settings or for specific target species.

A secondary benefit occurs by providing manufacturers with assurance that novel designs meet or exceed minimum standards of humaneness for qualifying spring-traps, fostering improvements in theirdesign.

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

Preventing the marketing, sale and use of relatively inhumane spring traps produces significant andimmediate benefits to government, society and the welfare of millions of wild animals which may besubject to wildlife management using spring-traps. These benefits accrue overtime to become substantial.

Wildlife subject to legal management using spring-traps benefit by being protected from the mostharmful and inefficient trap designs which are most likely to produce widespread and substantial unnecessary suffering where more efficient and humane devices are available.

Across all timescales this project supplies the trap licensing authority (Defra in England) and other authorities of the devolved nations of the UK with robust evidence of the humanness of tested spring-traps. In this way work ensures that traps failing the current regulatory standard are not permitted for use where they might cause excessive harm in specific settings or for specific target species. This enables the UK government to maintain environmental standards necessary for its international commitments, as well as permitting all devolved administrations to continue to protect wild animal welfare. These social and economic benefits are central to the establishment of trap testing standards(e.g. Agreement on International Humane Trapping Standards: AIHTS).

By enabling commercial clients to assure the humaneness of novel products we foster innovation in the design of new and yet more humane wildlife management approaches. We achieve this in part by using the experience we acquire undertaking this work and providing feed-back to clients (where theseare trap designers, manufacturers, or suppliers) on how further improvements to welfare might be secured.

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

As a regulatory requirement this work achieves substantial impact, directly informing regulators in all devolved administrations and avoiding the listing of trap designs for species or in settings where theydo not meet the minimum standard of humaneness. As most work is delivered to commercial customers, dissemination of test results is subject to their commercial interests.

Species and numbers of animals expected to be used

- Mice: 50
- Rats: 100
- Other rodents: No answer provided

Predicted harms

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

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

Spring traps generally comprise two physical design elements; a trigger and a killing bar or piston (often powered by a spring) which is designed to strike the animal and produce catastrophic injury sufficient to cause rapid and irreversible insensitivity (unconsciousness) followed by death. Preferred anatomical targets for injury are the brain or neck to produce deaths equivalent to the Schedule 1 methods of cranial depression or cervical dislocation, both so damaging the brain that insensibility anddeath may be instantaneous.

The efficiency of the interaction between trigger position, trigger latency, and the arc and power of strike are complicated by the behaviour of the target animal as it moves through the trap mechanism. This produces variation in strike placement and strike outcome. In some cases this might produce injury but not insensibility or death, or eventual death but after a significant and unsatisfactory period. Whilst trap testing is tangentially concerned with the simple physical characteristics of the trap (trigger speed, striking speed, striking power, clamping force) it primarily measures how variation in wild animalbehaviour may produce ineffective strikes, and result in poor outcomes for target animals. This is why trap setting is important (baited, unbaited, unenclosed, single entrance enclosure, run through etc) as these variations in deployment substantially alter the behaviour of some wild species.

Measuring the consequences of variation in wild animal behaviour in spring trap tests currently requires the use of conscious/active wild animals of the type specified for each STAO listing, e.g. rat, grey squirrel, stoat. This includes the most commonly encountered and anatomically robust individualslikely to be found in deployments in the field, i.e. adults. Thus the use of active adult wild animals is unavoidable.

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

Animals of a target species assessed as suitable for study will be brought in from the wild and held captive for a short period before their use in tests. This can be for as little as a day but may occasionally be longer. Animals are allowed to become familiar with an unarmed trap in its test setting(i.e. in a single entrance enclosure) placed with its enriched enclosure.

The trap is then armed and observed continuously for the duration of the trial. The animal will be allowed to enter the trap of its own accord and once the trap has been triggered, the observer will notethe success of the trap strike throughout the specified trap testing period. If the animal has not becomeirreversibly unconscious before the end of this specified period, it will be promptly euthanised. All euthanasia in this project will be using an approved humane methods.

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

Spring traps may kill instantaneously with successful strikes to the cranium or cervical vertebrae. Alternatively, less efficient strikes may kill within the specified period by other means (e.g. internal haemorrhage, asphyxiation, destruction of limbs or major organs) and in cases of a failed strike, the animal may suffer substantial pain and distress up to the end of the specified period. Animals injured such that they are considered unlikely to become irreversibly unconscious within the specified period (e.g. pinned within the trap) will be euthanised immediately. The trap testing standard currently complies with the Agreement on International Humane Trapping Standards (AIHTS) which specifies different periods for different species, from 45 seconds (stoat) to 300 seconds (a number of species). Animals still conscious at the specified time limit will be promptly euthanised, though most will suffer formuch shorter periods

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 animaltype)?

Severe for all animals

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

- Killed

A retrospective assessment of these predicted harms will be due by 31 July 2028

The PPL holder will be required to disclose:

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

Replacement

State what non-animal alternatives are available in this field, which alternatives you

haveconsidered 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 tests qualifying spring traps on target species to ensure they meet the required minimum humaneness standards before they can be listed on the Spring Trap Approval Order and become legalto use.

As trap testing requires wild animals to behave naturally as they move through the trap we are requiredto use live animal targets.

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

In the UK tests on species listed in international agreements (e.g. AIHTS) require the use of live wildanimal subjects.

Current policy from the regulator is to extend this principle to non AIHTS species, and all trap testingfor qualifying spring traps is required to be done on live wild animal subjects. Computer (simulation)modelling has been discussed and evaluated for this project and is currently used in Canada.
However, it was considered unsuitable for use in the UK.

Why were they not suitable?

Computer (simulation) modelling requires the use of very many live animal tests in order to parameterise and validate useful predictions. The development of a similar models for the UK would require gathering data from a minimum of 35 animals per species for each trap design and setting. Recently in the UK most new traps are novel designs each of which would require the extensive use ofanimals to produce a computer model, and fewer animals are used in a direct test of its humaneness. In addition we note the Canadian computer simulation program does not account for the behaviour of the animal, which is a critical component in the assessment of trap humaneness.

A retrospective assessment of replacement will be due by 31 July 2028

The PPL holder will be required to disclose**:**

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

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen 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 will depend upon which traps are submitted in the next 5 years and for whichtarget species. Estimates of numbers used across predecessor PPLs suggest an

average of 3 trialsper year although this maybe on a range of species.

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

Data from other trap test authorities maybe used as evidence to support the regulators requirement.We always enquire after the existence of such evidence. This may either avoid the use of animals altogether or on occasion reduce the numbers used for UK regulators.

Trials are undertaken in a sequential manner. Trials can be stopped as soon as sufficient dataidentifies a fail or pass. This may be as few as 3 trials to fail and 10 trials to pass a trap.

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

Where trap testing is undertaken to a defined standard (e.g. AIHTS) there is little opportunity to optimise the use of animals. However we note that the strict regulatory use of this project ensures thefate of every animal tested returns inferential value in either supporting the success of a relatively humane trap or ensuring the failure of a relatively inhumane trap.

A retrospective assessment of reduction will be due by 31 July 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan 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 welfarecosts (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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

This trap testing is for regulatory purposes where the traps are assessed against AIHTS standardswhich is to standardised model and method of testing.

Before any animals undergo any trials, the traps are initially given a visual assessment by the PPLh (as expert in trap testing) to identify any flaws that may affect trap success. If any potential design flawis found, then a follow-up discussion would be held with the customer on how to proceed, in order to make the trap more humane before animals are used.

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

The regulator requires us to test traps on the wild target species specified.

Trap testing includes the natural behaviour of the target wild animal species as it moves through the trap we are required to use conscious and active wild animal species. Further wild caught animals have a different muscle, fat distribution and bone density and therefore the power of the trap requiredto meet the standards may be different for wild animals as it would need to be for laboratory bred animals.

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

Wild animals will only be acquired when all preparations for trials are in place to minimise the period animals are help in captivity before being used. Acquisition of wild animals will always form part of thestudy plan and be approved by AWERB. This will include ensuring that methods of capture and transport are undertaken in the most refined manner possible and that all additional licenses and authorities required for the work are in place.

Wild animals will used in a prompt manner ensuring that they are not required to be kept in captivity forlonger than is necessary. Details of the approach used and controls on working will be specified in thestudy plan and agreed with the AWERB.

The substantial differences between wild species, seasonal availability of some species in the wild, differing requirements of traps to be tested, and the length of time wild animals can be kept in captivitywill be discussed with the NVS and NACWO on study by study basis and then specified in each studyplan and agreed with the AWERB.

Husbandry and environmental enrichment requirements will be discussed with the NVS/NACWO/species specialist and specified in each study plan and agreed with the AWERB.

For all trials the animals will be under constant observation from when the trials begin. If an animal receives a 'foul' strike and it is apparent that the animal will not become irreversibly unconscious withinthe specified time, then the trial will be stopped and the animal euthanised promptly.

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

There is no published best practice as to undertake these regulatory tests. As one of the only providers of regulatory trap testing we are conscious that we would define best practice and constantlyseek challenge from AWERB as to how to refine research.

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

I work closely with the establishment NIO, NACWO, AWERB and 3Rs champion to ensure that I stayup to date with advances which may provide refinement in this project, and will be applied where possible.

A retrospective assessment of refinement will be due by 31 July 2028

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 forfuture work of this kind? During the project, how did you minimise harm to the animals?

3. Basic research and pre-clinical analysis oftherapies for neuromuscular 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, ortheir effects, in man, animals or plants

Key words

Muscular Dystrophy, Therapy

Animal types	Life stages
Mice	adult, pregnant, embryo, neonate, juvenile, 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 orscientific needs it's addressing.

What's the aim of this project?

This project aims to assist in the development of better treatments for patients affected by musculardystrophy and other genetic muscle wasting diseases. We intend to primarily study pathogenic, diagnostic and therapeutic approaches in muscular dystrophies.

A retrospective assessment of these aims will be due by 18 July 2028

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 advancedor 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 overriding purpose of this programme of work is to assist in the development and refinement of therapies for neuromuscular diseases. The broad scope of this license reflects a wide range of needs within the neuromuscular scientific community and the diverse interests of the investigators within our institution which focuses on translational research with the aim to better understand the mechanisms ofthe many types of neuromuscular disorders and ways to improve treatment and quality of life for affected patients.

The likely benefits include improved understanding of the pathological processes occurring in genetic neuromuscular diseases and accelerated development of new therapies to treat these diseases. Thesebenefits will translate into more effective treatment for patients with neuromuscular conditions, improving their quality of life and assisting them and their families in dealing with these devastating conditions. In the longer term this will feed through into reduced dependence on state-funded care.

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

At the end of the project we think we will have a clearer idea on the mechanism leading to muscledegeneration in patients with muscular dystrophies and will have identified potential therapeutic compounds for these diseases that can be moved into clinical trials.

We are especially interested in the process of muscle fibrosis and adipogenesis. We have developed aprotocol to isolate fibroadipogenic progenitor cells (FAPs) from fresh muscles of mice and humans. Ourstudies will provide new information on how these cells are activated, how they proliferate and which mechanisms are responsible for their differentiation into fat and fibrotic cells. We think we will be able to identify the exact molecular mechanisms activated in human FAPs, which will help us to identify specific drugs that could potentially block these mechanisms.

We are also interested in testing new treatments for muscular dystrophies, such as genetic therapies, anti-sense oligonucleotides (AONs), nanoparticles, small molecules, pro-regenerative and anti-fibrotic drugs. All these approaches could have an effect on cardiac and skeletal muscle function as well as onthe brain. Our research will also lead to a better understanding of the pharmacokinetic and pharmakodynamic characteristics of these compounds in muscular dystrophies. Ultimately we aim to identify drugs that can be tested in a clinical setting.

We aim to share all our results with the scientific community. In order to do so, we will present our workat conferences and will publish scientific manuscripts. Moreover, we will share the results with patientsand advocacy groups at joint meetings.

The freezing reaction in response to scruffing is very relevant to the human disease, as some patients with DMD suffer from high levels of anxiety and autistic spectrum disorders. We are interested in the cognitive dysfunction seen in this disease and would like to explore this freezing response in more detail to see if this is down to anxiety or to a physiological response due to the firm restraint or possiblya combination of the two. This

work may well identify possible pathways for therapeutic intervention.
Moreover, we would expect this research to be of benefit to the research community working with dystrophin-deficient mice in helping researchers to understand the stress response and how to avoid it.

In summary, we want to contribute to a better understanding of the pathology and pathomechanisms ofneuromuscular diseases. We will do so by investigating the development of fibrosis in muscular dystrophies and by studying cardiac and behavioural changes in a dystrophin-deficient mouse model of Duchenne muscular dystrophy (DMD). Furthermore, we aim to test novel therapeutic compounds fortheir safety and efficacy profiles in mouse models before moving them to a clinical phase.

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

The likely benefits include an improved understanding of the pathological processes occurring in genetic neuromuscular disease and the accelerated development of therapeutic strategies to treat these diseases. We expect that our work will ultimately benefit both patients, their families and otherresearchers.

The results from our studies will also help to improve the experimental design of future investigations,as we are planning to share our results with the scientific community through presentations and publications. The project should also be of benefit for researchers outside our field who work on fibrosis in liver, kidney or lung disease.

We expect that the results from our work will translate into more effective treatments for patients withneuromuscular conditions, improving their quality of life and allowing them and their families to better
deal with these devastating conditions. In the longer term this would also reduce the necessity forstate-funded care.

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

We are well positioned to collaborate with both academic and industrial partners worldwide.

Myself and team members regularly travel to conferences to present their findings, to share ideas andlearn from each other’s positive and negative experiences of techniques.

Though not always easy to publish, it is important to share where possible with colleagues andcollaborators unsuccessful approaches and negative data.

Within our institution, we have many colleagues with varying experience in animal models and socommunication locally is also an essential part of our work.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

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

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

The mouse is the model system of choice in this field due to the high degree of homology in muscle structure and function between rodents and humans. Many aspects of muscle function, such as the role of individual proteins and the structure of the neuromuscular junction, can be effectively modelledin lower vertebrates (such as the zebrafish), invertebrates or even in cell culture. However, when it comes to drug metabolism and efficacy, rodent models are required to be confident that effects can betranslated back to patients. The mouse is the most amenable rodent available, with a large repository of existing control data and well established standard procedures for the measurement of relevant parameters. As such, the mouse is the only model system with both the necessary and sufficient characteristics for the project to meet its aims.

The protocols described for this project are designed to be applied to adult animals. We are not goingto use pups for any kind of experiments. The protocols described here will use animal models of specific muscular dystrophies that are 2 months of age or older. This is based on the fact that many ofthe drugs we will evaluate are more effective when used at early stages of the disease. We will treat 2month old animals for 2 months, expecting to see significant differences between treated and non- treated animals for effective drugs.

On some occasions, animals will be aged in order to obtain a better model of a muscular dystrophy. This is for instance the case for the dystrophin-deficient mdx mouse, the well-established mouse model
for Duchenne muscular dystrophy. These mice do not have major structural changes in its muscles until it is older than 9 or 10 months of age. Exercise regimens using a treadmill or running wheel mayalso be used to worsen the phenotype in order to mimic human muscular dystrophy pathology.
Voluntary wheel running can already lead to more prominent histological features of muscular dystrophy. In the case of treadmill exercise, we will use publicly available standardized operating procedures generated by TREAT-NMD, a global network supporting translational research in neuromuscular diseases, and accepted by the scientific community. We will ensure to apply the treadmill protocols according to the age and severity of the mouse models by adjusting speed and running distances. If necessary, minor encouragement methods such as a touch on the tail may be used for mice that stop running. Dystrophic mice may require more recovery time from treadmill exercise than controls, but based on our experience and the experience of others both the wheel andthe treadmill exercise are well tolerated by mice.

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

Mice will be bred, aged to meet specific experimental needs and may be used in studies where variouspharmaceutical agents are tested.

New drugs (or control substances) expected to modify the disease process in genetic muscle diseaseswill be administered at different doses and by different routes, limited by LASA. We will monitor the mice treated to be sure that the administered drugs don’t affect their wellbeing by using different scoresheets. For new drug studies, where there is no previous data for the application in mice, small pilot studies will initially be carried out to investigate that the drug at the specified dose is well tolerated. In these specific cases, we will check the wellbeing of the mice more often than in the standard protocols, to be certain that we pick up any potential adverse effect.

We are interested in better understanding the behavioural phenotype in mice lacking dystrophin. Theseanimals can develop an adverse effect in response to handling and scruffing. After scruffing, mice appear distressed and breathless and may be relatively motionless for an hour or more. It has been observed by many groups working with mdx mice that ten seconds of scruffing, a commonly used method of restraint for carrying out e.g. ear-clipping or intraperitoneal injections, can lead to this freezing response. Therefore, scruffing of these mice will only be carried out when absolutely necessary and for as short as possible. In order to avoid scruffing, we will use other handling methods,such as tunnel handling, as much as possible.

For behavioural testing such as the Barnes maze and novel object recognition test, tunnel handling orhand cupping will be used. These are not harmful tests, as the mice are being given a safe area to explore whilst we observe their behaviour.
We will use some additional tests to measure muscle strength and function. These tests include the grip strength meter or treadmill exercise. These tests will be performed following standard protocols already published (TREAT-NMD website). Mice can be fatigued after the exercise and may need somerest, but we do not expect animals to have any harm from these studies.

We are also going to analyze the structure of the brain using magnetic resonance imaging, which is a non-invasive technique that could be carried out more than once to study changes in brain over time.

The number of times and precise timepoints in the lifespan of the mice when these studies are going to be carried out is not predefined and will be established based on the information collected from the behavioural tests performed. We may therefore obtain brain images at baseline and again after some months, if we observe changes in the clinical parameters. Mice may undergo up to four MRI scans withat least 2 months in between, therefore mice will fully recover before the next scan. Other imaging methods, if more appropriate for a particular research question, may be explored. Such methods may include computed tomography (CT), positron emission tomography (PET) or in vivo imaging system (IVIS).

For all these procedures, mice will be anaesthetized using a general anaesthetic such as isoflurane and if a contrast agent is to be used, for example gadolinium, the tail vein may be cannulated. During MR imaging, mice will be placed on a sled connected to a warming system in order to maintain the mouse within an appropriate ambient temperature with maintenance anaesthesia. An air pillow placedunder the chest will be used to monitor respiration rate and a temperature probe used to monitor bodytemperature. Following scanning, the mouse will be removed from the scanner and either placed on aheated mat to recover or culled humanely via a schedule 1 method.

Imaging modalities are safe, non-invasive methods conducted under maintenance anaesthesia with fullrecovery. In rare cases, death can occur during the imaging process, which is believed to be caused byadverse reaction to anaesthesia. In order to prevent this reaction, mice undergoing MRI will be monitored for respiration rate and temperature. Anaesthetics will be adjusted when necessary to prevent adverse reactions. Mice will be placed on a heat mat and returned to their cage once they are awake and able to move. Mice will be checked 24 hours after MR imaging to confirm that they are completely recovered.

At the end of studies, mice will be culled and tissues may be collected.

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

Adverse effects will vary between mouse strains though the majority of our mice will be on a mild severity threshold. Our previous experience has shown that most of these mice will be assessed retrospectively to be sub-threshold. For example, despite the fact that dystrophin-deficient mdx micedevelop muscular dystrophy and cardiomyopathy, they generally do not show any severe symptomsand have a normal life expectancy.

There are certain symptoms that each specific strain may develop. This is based on the scientificliterature and our personal experience.

For instance some strains can develop cardiac problems.Other adverse events the mice may experience:

A prolonged freezing reaction has been observed in mdx mice after scruffing

Temporary pain and discomfort in young animals if a tail biopsy is required for genotyping. There are general adverse events related with ageing in mice: dermatitis, greying of hair, hair loss and scruffy fur. Over-grooming may also be observed. If any of these are observed, we will increasemonitoring and if this behaviour exceeds the severity threshold, the mice will be culled.

Aged mice with neuromuscular diseases have a more advanced clinical stage characterized by increased fibrosis in skeletal muscles and sometimes in the heart. Therefore, these animals are proneto develop symptoms related with these histologic changes such as cardiomyopathy, difficulties in running when placed on the treadmill or reduced movement during daily activities.

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 animaltype)?

In our wild type mice, the expected severities will usually be sub-threshold (usually over 50%), these will include the mice which do not receive any treatment or undergo MRI. They may be used for the milder behavioural studies included in this licence, such as the Barnes maze and novel object recognition test. The remaining wild type mice, up to 50%, may be expected to be participating in mild(45%) to moderate (5%) protocols due to drug administration, muscle function tests or MRI where themice will be culled as has been described on each protocol.

Severity of the mice included in this research may vary according to the genetic mutation. The symptoms of the mice that we use are mild or even less, not identifiable after visual inspection (sub- threshold severity almost 50% of the animals). However, with age mice can develop clearer and moresevere muscle weakness and have problems to walk on the treadmill and consequently reduce their daily activity. This being considered, animals will experience mild to moderate severity, 45% of the animals will suffer mild severity and 5% of the animals moderate severity.

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

- Killed

A retrospective assessment of these predicted harms will be due by 18 July 2028

The PPL holder will be required to disclose:

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

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered 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 considered using non-sentient alternatives, such as cell cultures, echinoderms (echinodermsare a type of marine invertebrate such as sea urchins or starfish), sponges or hydra to our mutant mouse models, but at present this is not possible as none of these animals models reproduce the same histological and functional pathologies as patients with the muscular diseases. The main non-
animal alternative to these studies is to use cells in culture. This is unsatisfactory for a number ofreasons. Most cell culture systems consist of a single population of cells which are identical.

Specifically, we work with myoblasts, a type of muscle resident stem cell, that are in charge of regenerating damaged muscle fibers. Myoblasts in culture can be induced to fuse and form myotubes,the fusion of several myoblasts represent the in culture counterpart of muscle fibers. However the limited size and structure of muscle fibres in culture do not recapitulate the anatomical and functional structure of muscle fibers. Muscle is a complex syncytial organ consisting of multiple cell types including myoblasts, muscle stem cells, fibroblasts, endothelial cells and neurons. The complete arrangement of all these cell types and systems is required to form functional muscle and cell culture systems or tissue preparations are not capable of delivering this at present. Furthermore, skeletal muscle has several interactions, notably with motor neurons via the neuromuscular junction, with the blood vessels and with cells of the immune system, which cannot be modelled in culture systems. Forthese reasons, cell culture models are only suitable for initial pre-clinical drug screening and basic scientific questions regarding myoblast and cardiomyocyte function (cardiomyocytes are the cells of the heart muscle). We aim to use cell cultures for these purposes wherever possible but cannot envisage completely replacing animal experiments at the present time.

Where possible we utilise the least sentient model organism. For our purposes this equates to the zebrafish, where it is possible to examine the consequences for muscle of various genetic manipulations and pharmacological interventions. However, the structure of fish muscle differs markedly from mammalian muscle, limiting the applicability of this model. Furthermore, this model isnot acceptable for bio-distribution and pharmacodynamic studies (assessing the biochemical and physiological effects of drugs) which are required before novel treatments can be applied in patients.Since this is our direct goal, there is no choice but to use a mammalian model system. These experiments can be undertaken using mouse models based on the close similarity between mouse and human muscle in terms of biochemistry, physiology and pathological states.

Mouse models for neuromuscular diseases are well developed, with a large body of control data and well-established standard assays for pathology and muscle function testing. From a regulatory point of view, experiments in mouse models are sufficient for progression of therapies through the pre-clinical phase to translation into clinical use. As such, we conclude that mouse models are both necessary andsufficient for the purposes that we have outlined.

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

We are developing good cell culture models to test some of our hypotheses. We have already well established 2D culture models of myoblasts or fibro-adipogenic precursor cells (2 types of cells presentin skeletal muscles) and we are using them to test new therapeutic compounds. Moreover, we are setting up 3D cell culture models. We do not yet have co-culture models that could be used in research. We are also starting collaborations to develop software that could predict response to a certain drug in vitro.

Why were they not suitable?

For many or our research questions a single population of cells in isolation is not an appropriate modelto address the complex pathomechanisms of muscular dystrophies. We are nevertheless progressing in finding new better cell culture-based methods to replace animals, for example we are working on 3D cultures of muscle cells and have started testing drugs in these new models. However, these culturemodels are developed with one cell type, and we are starting to co-culture two cell types. Skeletal muscles contain several cell types, including muscle fibers, vascular cells, nerves, and fibroblasts.
Each of these cells release factors that influence other cells present in the muscles. This complex network of cells is something that cannot yet be reproduced in vitro. If we want to test the efficacy of anew treatment, we can perform some preliminary experiments in vitro, but need to move in vivo and test it in murine models to confirm safety and efficacy. So far there is no cell culture model or softwarethat can reproduce the complexity of a living organism.

A retrospective assessment of replacement will be due by 18 July 2028

The PPL holder will be required to disclose:

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

Reduction

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

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

We estimated the number of animals based on our planned experiments over the next 5 years. Basedon our experience and the results from previous studies using new

treatments or behavioural modifications in the same mouse strains, the minimum number of animals per group was 10. This is the number that we need to achieve statistical significance and to reduce the risk of background variation as an explanation of the findings obtained. For example, when we test a new treatment in murine models, we need almost 40 animals (10 healthy, 10 non-treated affected mice, 10 treated at lower doses and 10 at higher doses). We add 10 animals that could be used if animals die during the procedure. To arrive at 50 animals per experimental group, we need to breed almost 150 to 200 animals. Moreover, we need a minimum number of animals to maintain the colony.

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

Each experiment is designed with great care, reference to previous experiments and what we havelearnt from our successes and mistakes.

Literature reviews, advice from colleagues and the use of websites such as:

https://www.nc3rs.org.uk/experimental-design

https://www.nc3rs.org.uk/experimental-design will be utilized when planning experiments, specifically https://www.nc3rs.org.uk/experimental-design-assistant-eda

https://www.nc3rs.org.uk/experimental-design-assistant-eda may be used for deciding on appropriate group sizes.

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

We will undertake the following measures

Efficient breeding: We will work closely with the colony manager/team to establish the most efficient breeding program. We will follow the recommendations included in the document “Efficient Breeding of Genetically Altered Animals” published by the Home Office. We will perform breeding calculations before we plan our experiments and intend to only produce the numbers of animals that we need. The number of breeding pairs to be set up varies for one strain to another. Control and youngmice have a mean of 7 to 10 pups, mdx and GAA mice have 5 to 7 pups and sarcoglycan-deficient mice have 4 to 8 pups.

Use of cell cultures whenever possible: we are setting up new 3D culture models that could substitute animal experimentation in some circumstances and therefore reduce the number of animalsneeded for research.

Small pilot studies may be used to test the robustness of protocols before embarking on studieswith larger numbers of mice.

Tissue archive: When experiments are completed and mice are culled, tissues which are considered useful for current or future experiments will be collected. The maximum output from everyanimal will be considered on a case by case basis

A retrospective assessment of reduction will be due by 18 July 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan 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 welfarecosts (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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mouse models of neuromuscular diseases that are diseases involving the nerves and the muscles characterized by progressive muscle weakness. Some of our mouse models, for example themdx mouse, generally have a mild phenotype and do not suffer any adverse effects with a near normallifespan.

Methods to investigate these diseases will include grip strength measurements, a non-invasive technique where mice are given the opportunity to hold on to a wire mesh and their tail is pulled backwith slight force in order to measure the strength of muscles.

Mice may undergo non-invasive behavioural testing for example using the Barnes maze test. In this test, the mice are given a safe area to explore and their behaviour and memory are monitored for finding a hidden box to hide in. This is preferential as it is less stressful for the mice than the significantly more challenging Morris water maze, where mice are placed in an opaque bath of waterand their ability to locate a submerged platform to rest on is measured.

Other methods will include MRI, where mice are anaesthetised prior to undergoing preparation andimaging. If no further information is required from the mouse, scanning will be performed under terminal anaesthesia.

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

I have long-standing experience working with zebrafish and where possible, we will use zebrafish models (under separate licence authority) rather than mice. However, the structure of fish muscle differs markedly from mammalian muscle. In addition, this model is not suitable for many of our studieswith new drugs such as bio-distribution and pharmacodynamic studies which are crucial to find out the optimal doses to see an effect of a drug in a new organism. Such studies are required before novel pharmacological compounds can be taken to clinical trials.

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

Mice are checked daily by animal care staff. Concerns will be raised as necessary with PIL holders,NACWOs, NVSs and myself.

Where possible, i.e. in all maintenance and experimental handling (except in protocol 4,

where we specifically plan to scruff mice to study the stress response), mice will undergo low stress handling by using cupping or tunnel handling. Following experimental procedures, mice will be monitored using appropriate score sheets until sufficient recovery is made. Animal care staff will be notified to make anyadditional checks required. In procedures that may cause pain, any suffering will be assessed and if appropriate soaked diet, extra bedding, and advice for appropriate analgesia will be sought from the NACWO or NVS.

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

RSPCA and LASA, 2015, Guiding Principles on Good Practice for Animal Welfare and Ethical ReviewBodies. A report by the RSPCA Research Animals Department and LASA Education, Training and
Ethics Section. (M. Jennings ed.)

From the RSPCA guidance, we do not expect our animals to experence severe suffering: https://science.rspca.org.uk/-/severe-suffering

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

Our group maintains close links with the animal facility and members of my research team regularly attend their meetings. We find the NC3Rs website an excellent resource and liaise with colleagues toshare ideas. We hold regular meetings to discuss our animal work and how we can optimise experiments whilst following the 3Rs. We also follow the Home Office guidance and attend regularly, webinars organized by them or by other organizations about novelties in this field.

A retrospective assessment of refinement will be due by 18 July 2028

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 forfuture work of this kind? During the project, how did you minimise harm to the animals?

4. Devising new therapies for multiple sclerosis and small vessel 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, ortheir effects, in man, animals or plants

Key words

Multiple sclerosis, Cerebral small vessel disease, Neuroinflammation, Hypoxia, Cerebral blood flow

Animal types	Life stages
Rats	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?

Our aim is to reduce the suffering of people with diseases such as multiple sclerosis and small vesseldisease. We seek to understand the biology of these diseases in order to develop new, clinically relevant therapies to protect from these disorders.

A retrospective assessment of these aims will be due by 16 July 2028

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 proposed programme of research addresses the pathophysiology (i.e. understanding the mechanisms that are responsible for causing structural damage (pathology)) and therapy of inflammatory diseases of the brain, spinal cord and nerves in the arms and legs. These diseases cause severe and often permanent neurological symptoms. Neuroinflammation is a broad term that includes not only the classical inflammatory diseases multiple sclerosis and Guillain-Barré syndrome,but also small vessel disease and the neurodegenerative diseases (e.g. Alzheimer's and Parkinson'sdiseases). Our research is relevant to all these disorders.

The disorders cause inflammation and reduced blood flow within the brain, and this causes severe symptoms (including blindness, paralysis and numbness) and damages brain tissue causing dementia,amongst other problems. The mechanisms responsible are not known, but our research aims to discover the mechanisms so that we can develop new therapies to protect patients.

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

In the short term, our output will be the introduction of a valuable new model of secondary progressivemultiple sclerosis that has revealed not only when the damage responsible for the relentless advance of disability occurs, but also how to prevent it. Our ongoing research also provides an increased understanding of how neurological disability arises in the major neurological diseases. In the medium
term, our understanding of the causes and consequences of prolonged energy deprivation will indicatenovel therapeutic strategies for protection that will be tested in the laboratory within the period of this licence. The research will add to our existing strategies new ways to tackle neuroprotection in multiplesclerosis (MS), and to extend these strategies to other neurological disorders, including dementia and neurodegenerative disease.

Our research is literally changing the neurological research community's understanding of multiple sclerosis (MS), and we are introducing new approaches to therapy that are likely to persist and become established adjuncts to existing therapies. This research will also build on our recent findingsthat indicate an effective strategy to reduce dementia due to prolonged high blood pressure. Our research findings are, of course, published in the scientific and medical literature.

Who or what will benefit from these outputs, and how?
In the short term, laboratory and clinical researchers will benefit from a greater understanding of howinflammation and cerebral blood vessel disease cause short and long term damage to the brain, andthrough analysis of novel therapeutic interventions we will learn how to prevent this damage.

We have created models that aid in the refinement of protocols, including the only model of the type ofmyelin (nerve fibre insulation) loss that occurs in early MS lesions, and the best model of slowly progressive neurodegeneration that occurs in MS. These advances reduce the number of animals experiencing the severe model of experimental autoimmune encephalomyelitis (EAE) typically used inMS research.

We will gather data using spatial transcriptomics (a method that reveals the expression of genes in allthe different cells in a tissue while retaining the spatial relationships between the cells) in our models,and these data will be uploaded to public repositories so that other researchers may benefit.

With our collaborators, the work in this project will be used to develop techniques to understand disease mechanisms in awake patients, using non-invasive methods that are entirely pain free. Thuswe are using methods (magnetic resonance imaging (MRI) and near infrared spectroscopy (NIRS)) that are translatable to investigations in patients, and that can aid diagnosis and monitoring of therapeutic regimens.

In the long term, patients will benefit from therapies that protect brain tissue from causing symptomsand suffering.

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

We have strong collaborations with engineers to make devices to measure brain oxygenation, with physicists to make a device (a new type of ophthalmoscope) that can detect even subtle deficits in theoxygenation of the retina, and with clinicians to translate these new developments to study disease in patients. The new methods allow us to monitor the value of existing and novel protective therapies in patients. Data obtained from our spatial transcriptomic studies, and similar studies, are shared publicly, as noted above. Our findings are presented at specialised and major conferences, and published in the open access scientific literature. Pathology specimens are shared upon reasonable request. We also communicate our findings to patients at local and national conferences organised forpatient participation.

Species and numbers of animals expected to be used:

- Rats: 4000

Predicted harms

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

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

The diseases that we study are experienced by adult humans. Several selectively bred rat strains have been developed over the last 10 years that recapitulate and mimic different pathologies of neuroinflammation and small vessel disease with accuracy. Our research uses the most appropriateanimals for the research question we are studying. Individual experiments are usually completed within a month. We only use rats of a developmental age that is reflective of the onset and development of the relevant disease in humans.

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

For models of MS we mainly use a model where the disease is induced by immunisation (via subcutaneous injection) and autoimmunity rather than by surgical methods, and so these experimentsdo not involve surgery. Many people believe that MS is caused by a similar autoimmune activation of the immune system. Immunised animals are observed

over the following weeks (typically 2-3 weeks) while walking around to detect any deficits in their hindlimb or tail function. These deficits usually start10 days after immunisation, and since we are mainly interested in stopping further advance of the deficits, the animals typically do not experience any deficits for more than a few days. In the early days of deficits the animals usually only experience a slightly floppy tail and weakness in their hindlimbs, but if the disease advances to impair their ability to reach food and water, they are fed wet mash and water (e.g. hydrogel) on the floor, and by hand if necessary. Experiments involving progressive MS employ a lesion induced by microinjection into the spinal cord under general anaesthesia. This model only involves tail and hindlimb weakness. The therapies we use are mainly administered by adding them to the food, or by raising the amount of oxygen the animals breathe.

Rarely, therapies are administered by injection, typically subcutaneous or less commonly byintraperitoneal or intravenous injection.

Animals with a model of small vessel disease have the disease as a consequence of inherently raisedblood pressure, which is also the main cause of the disease in humans. Nothing is done to promote the high blood pressure, but the animals have previously been selectively bred for this characteristic. High blood pressure in rats, like high blood pressure in humans, does not cause any symptoms, at least at the early to medium stages.

All animals are typically housed in groups in standard cages. At the end of experiments all animals arekilled under general anaesthesia, because we typically want to take tissues after perfusion fixation under general anaesthesia.

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

Animals with a model of MS are likely to have hindlimb and/or tail weakness or paralysis, with deficitsstarting around 10 days after immunisation and usually expressed for only a few days before they improve. We are mainly interested in the very early consequences of inflammation in the nervous system, and at this stage the animals either do not show any symptoms, or only very mild symptoms.Therapeutic intervention is expected to reduce symptoms, and certainly not expected to cause furthersymptoms.

Any painful procedures (e.g. surgery if required) are, of course, performed under general anaesthetic. Demyelinating lesions, as in MS, are not usually painful in patients, and animals do not appear to be in pain either. Animals are more likely to lose sensation, resulting in numbness of parts of their hind limbsand tail, rather than experience pain.

Raised blood pressure does not cause any symptoms or discomfort in the animals, just as it causes nosymptoms or discomfort in humans, at least in the early and moderate stages. After a long time (e.g. 6months), the high blood pressure can weaken blood vessels and reduce blood flow and then animals can get cognitive (dementia-like) deficits, as occurs in older people, and they might get a stroke, again like humans. Animals that experience such severe symptoms are promptly 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 animaltype)?

Study 1. Breeding and maintenance of selectively bred animals. 95% Mild, <5% moderate.

Study 2: Terminal examination. 100% non-recovery as rats will only undergo terminal anaesthesia.

Study 3: CNS lesion (excluding EAE). 100% moderate as animals experience an intraspinal microinjection under general anaesthesia, with subsequent tail and hindlimb weakness. Weakness is expressed on days 2 and 3 after lesion induction, after which the animals recover for a month or so, and then the symptoms slowly recur over the lifetime. The hindlimb weakness is never severe, and thehindlimb strength is always sufficient to support the body weight and allow walking. In a typical experiment, hindlimb weakness that is visible to an observer is mainly only experienced by lesioned byplacebo-treated ('control') animals that are used to demonstrate disease, as a comparison to show the beneficial effects of animals we treat with our experimental therapies.

Study 4: Experimental autoimmune encephalomyelitis (EAE). 10% Mild, 60% moderate, 30% severe. EAE can cause severe symptoms as it progresses, but we aim to terminate our experiments before severe symptoms develop. Thus, most animals (70%) under this study will experience only mild or moderate symptoms, such as hindlimb and tail weakness. The weakness/paralysis usually only lasts for a few days, partly because the animals spontaneously recover after a few days, and partly because
it is only the onset of disease that we are mostly interested in studying, and so most animals are terminated within 2-3 days of disease onset. The weakness is expected to be painless (and animalsshow no signs of being in pain), as it typically is in humans with MS. Some animals (less than 10%)may experience more severe symptoms such as hindlimb and tail paralysis. Such animals are promptly terminated.

Study 5: Spontaneously hypertensive rats (SHR). 80% Sub-threshold, 20% moderate. Animals typically do not show any symptoms or discomfort, in common with humans with hypertension. After along time (e.g. 6-12 months), animals can get cognitive (dementia-like) deficits, as occurs in older people, and they might experience a stroke. Strokes in both animals and humans frequently cause either no symptoms, or only mild symptoms: animals with moderate symptoms are promptly terminated. Typically, half the animals under this study are on neuroprotective therapy (a therapy thatwe can directly apply to patients) and such treated animals have never experienced any neurological deficit from strokes in our experience over 7 months of age.

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

- **Killed**

A retrospective assessment of these predicted harms will be due by 16 July 2028

The PPL holder will be required to disclose:

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

Our research has shown that understanding the pathophysiology of the inflamed nervous system, particularly with regard to oxygenation and mitochondrial function, absolutely requires a functioningimmune system, cardiovascular system, and pulmonary system. With current technology and understanding it is impossible to replicate the interaction of these systems in a physiologically meaningful way outside the body.

In particular, our experiments indicate that a key factor in causing symptoms is an inadequate supply ofoxygen to the inflamed tissue, and this can only be studied in the brain with a functioning blood system. Our aim is also to test novel therapies, and to do so we need a functioning blood supply to deliver the therapies, just as they would be delivered in patients. We also aim to improve function in the animals and patients, and to assess this we need to be able to watch how well the animals can walk along, before and after the medicine being tested has been given. It is therefore unfortunately necessary to use animals in the proposed research.

Other experiments study the consequences of a lifetime of raised blood pressure. It is not currentlypossible to achieve these requirements in cultures or computers.

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

We have considered the use of computer simulation, tissue culture, and ex vivo preparations.

Computer simulations of neuroinflammatory lesions are not yet realistic because we remain ignorant ofmost of the key values necessary to mimic the environment within such lesions. We use tissue culture to study the properties and behaviour of certain immune cell types (e.g. T cells and microglia), but suchpreparations cannot mimic the complex environment of a neuroinflammatory lesion because they lack the crucial ingredient of an intact blood supply. We employ ex vivo preparations when possible (e.g. excised peripheral nerves), but mitochondrial function is strongly affected by the concentrations of oxygen and nitric oxide, and it is impossible to reproduce meaningful concentrations of these agents invitro because their concentrations within inflamed lesions are not known.

We also need to see how the ability of animals to walk around is improved by the drug therapy. It istherefore unfortunately necessary to use animals in the proposed research.

Why were they not suitable?

Non-animal alternatives do not have a functioning vasculature and immune system, and models of neuroinflammatory lesions in culture are not closely comparable to those in humans. It is not realisticto study the effects of medicines intended to improve disability in humans using non-animal systems.

A retrospective assessment of replacement will be due by 16 July 2028

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is thereanything 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 statistical power analysis to calculate the minimal number of animals required to achieve experimental aims. Professional statisticians are at hand within our department and within the Institute, and they are consulted as necessary to ensure that our research employs sufficient animalsto make statistically significant findings, but no more animals are used than required. Animals are randomly assigned to experimental groups, and 'blinding' of investigators performing judgement tasks,to avoid bias, is of course routine.

Numbers chosen reflect the minimum numbers required for initial pilot studies and subsequent largerstudies, for individual projects, in order to achieve statistical significance. Our individual experimentstypically employ 8-20 animals per group.

We optimize our breeding strategy to produce minimal numbers of animals.

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

We ensure our study design employs the best technical approaches to maximize the scientific outcomeof our work while ensuring the least number of animals are used and that experimental bias is minimized. Experiments are designed with reference to the ARRIVE guidelines.

We routinely employ the NC3R's Experimental Design Assistant to assist us in designing experiments and in calculating the number of animals required for statistically significant findings. In each experiment we obtain the maximal observations from each animal, such as studying them behaviourally during the experiment, correlating these findings with serial physiological observations using, for example, near infrared spectroscopy to measure brain oxygenation, blood pressure, multispectral imaging to measure retinal oxygenation), and correlating all these recordings with detailed microscopic examination of the affected tissues after death. These processes are reflective ofthe clinical scenario.

In some experiments we require histological examination of the tissues, in which case serial observations of the same animals are not possible, but mostly we employ serial observations to reducethe numbers of animals involved. The electrophysiological examinations are minimally invasive (temporary insertion of hypodermic needle electrodes under anaesthesia) so animals can be examinedserially. With retinal examination it is possible for repeated examination, as the process is non- invasive.

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

We routinely use pilot studies to assess the feasibility of new study protocols or new hypotheses. These pilot studies usually employ very small numbers of animals, and allow

us to examine and testmultiple parameters within individual test subjects. We find such studies invaluable to our research.

We have significant experience in the induction of experimental lesions, minimising experimental variation, and will employ this experience to ensure that minimal numbers will be used. We also employ small animal MRI using a 9.4T scanner, which allows the same lesions to be monitored over time, rather than in different animals. We are also able to ensure that animals with experimental autoimmune encephalomyelitis (EAE), for example, can not only be used for in vivo or MRI imaging, but following perfusion fixation their tissues can be used in the several histological studies ongoing inour laboratory. In addition, high volumes of tissue are generated from individual experiments as we harvest as much tissue as possible (e.g. spinal cord, brain, optic nerve, liver, kidney, sciatic nerve, muscle etc), allowing us to build up an indispensable bank of tissue. This bank of tissue is sharedbetween projects within the group, and also with internal and external collaborators.

Our ability to make and correlate multiple observations from each animal is a powerful strategy toreduce the number of animals used.

A retrospective assessment of reduction will be due by 16 July 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan 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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Animal suffering will be limited in our studies by our strict monitoring of animals to ensure that they remain within the severity limits of their associated protocols, which have been designed to minimizetrauma and suffering.

We apply well-established protocols that have been repeated over many years of use, ensuringstandard operating procedures and technical competence and standardisation.

We also apply state of the art approaches that take into account relevant developments in the literatureand the knowledge of competent researchers, with guidance from the NVS to ensure most refined models and methods are used.

To minimise stress whenever possible animals will receive environmental enrichment and be housed ingroups. Anaesthetic and analgesic regimes will be used to minimize pain wherever appropriate.

We will employ rats in the proposed research, and there is substantial evidence that findings made inthese species can be meaningfully translated to the care of patients in the clinic. Much of our preliminary data have also been obtained in rats.

We employ several animal models of multiple sclerosis and small vessel disease. The most commonlyused model of multiple sclerosis around the world is known as experimental autoimmune encephalomyelitis (EAE), and we sometimes use this model. EAE in rats, particularly in DA rats, has been described as closely reflecting the spectrum of pathology in MS (Storch et al., Brain Pathology, 1998). It is necessary to use EAE because regulatory authorities require demonstration of the efficacyof therapies in this model: EAE is arguably the closest model to MS. Indeed, clinical trials directly based on data obtained in rats with EAE have proven to be a successful translation to human disease,providing very significant protection of vision, establishing the value of rats in MS research. The modelis also widely used and a potential therapy has to be demonstrated in this model to gain scientific credibility.

Multiple sclerosis is typically not painful, especially in younger adults, and rats with EAE similarly show no signs of pain. Rather, multiple sclerosis and EAE tend to remove sensation, resulting in numbness. We are interested in developing medicines to treat symptoms at their immediate onset, and so we haveobtained all our observations within a few days of the onset of any symptoms, and the experiment is then terminated.

Inflammation within the CNS is normally hidden from view, but inflammation of the retina (CNS tissue)can be monitored in the intact eye via the pupil, just as in humans, and we employ retinal examinationas a non-invasive avenue to events hidden within the brain.

Another model has been developed in our laboratory and at its most severe the animals can have weak hindlimbs and tail, but the hindlimb weakness is typically so slight that it requires study of videosto detect it.

Our model of small vessel disease involves rats with inherently raised blood pressure (hypertension).High blood pressure in rats does not cause any symptoms, just as high blood pressure causes no symptoms in humans, at least in early and moderate ages. In late life the raised blood pressure can cause strokes, and these can cause symptoms, although many strokes in rats (and humans) do not cause symptoms. In fact, at least a third of people over 70 have had at least one silent stroke (i.e. thepatient is unaware and the stroke is detected upon routine scanning later in life). If strokes occur that do cause symptoms, the animals are promptly euthanised.

There is a range of animal models available for research into small vessel disease, and the main one chosen for our studies is the spontaneously hypertensive rat. This rat is one of the best existing modelsof human hypertension and cerebral small vessel disease. Examination of these rats allows us to study cerebral blood flow and tissue oxygenation, together with therapeutic studies to prevent cerebral pathology. We typically administer medicines by adding them to the diet, to avoid stress due to injections. Use of this model is consistent with published guidelines to improve translation from pre- clinical to clinical studies by using a model with many existing co-morbidities.

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

We use adult animals because most experiments last for weeks while the lesions (which mimic the lesions in multiple sclerosis) develop and are treated. Immature animals would not be representative as their brains are not fully developed and thus they do not have an

adult vasculature and oxygen consumption, which are important considerations for our experiments. The brains of neonatal animalsare much more tolerant of inadequate oxygenation than adult animals, and so they do not provide meaningful observations. Multiple sclerosis affects adult humans, and it is necessary to study adult animals for comparable observations.

Rats have a brain with a metabolism comparable with that of humans, and we study the changingenergy balance of the brain as it undergoes inflammation, demyelination and degeneration. Non-mammalian animals do not reflect this biology. Mammals are also required because we are developingmedicines for humans, and the medicines require testing in mammals.

We are unable to use terminally anaesthetised animals because the experiments last for weeks, and itis not feasible to study anaesthetised animals for such periods.

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

Our experiments aim to develop medicines to prevent symptoms, and so treated animals typically haveonly mild, if any, symptoms. Untreated animals typically only show any symptoms for a few days at most, as they are terminated once the beneficial effects of the treatments in the treated animals have been demonstrated by comparison. Experiments in our MS research that employ EAE do not involve any recovery surgery, and similar lesions in patients are typically not painful. In other experiments, if recovery surgery is involved, pain resulting from the surgery is controlled with analgesics. The animalsdo not display evidence of suffering, and similar lesions are typically not painful in patients. In experiments where we induce inflammation in a specific region of the brain and spinal cord, we choose“clinically silent” locations that do not cause any symptoms.

General anaesthesia is used for all procedures that may be painful, such as surgery, and electrophysiological examination (although such examinations are conducted without anaesthesia in patients). We employ state-of-the-art methods for our investigations to ensure the optimal data, just as are employed in the clinic, and we also closely monitor the physiological condition of the animals underanaesthesia. Notably, where possible we employ non-invasive methods similar to those employed in the clinic, such as small animal MRI, to examine lesions over the course of disease. This not only reduces animal numbers but it also allows lesions to be examined using the same technology as is used in patients, providing findings that are often directly applicable to findings in patients examined in the same way.

All experimental animals are examined at least daily. Animals with EAE are examined twice a day between days 8-14 post-immunisation, when symptoms can advance rapidly. Examination of animals includes study of not only whether there is evidence of a neurological deficit (e.g. limb weakness), but also whether there is evidence of more systemic problems such as weight loss and signs of discomfortor abnormal behaviour (listless, aggressive, sedated, hunched, fearful, piloerect, ‘hiding’ in corners).

Examination for pain includes evidence of spontaneous vocalisation, aggressive upon handling, hunched, piloerect, or scores on the grimace scale (Sotocinal SG et al. (2011). The Rat Grimace Scale. Molecular Pain 7: 55.)). Animals giving cause for concern may be discussed with the named veterinary surgeon (NVS), and will be killed if they exceed the severity limits assigned for their protocolstep.

Animals will always be able to reach water and food, so these will be provided within reach, includingmashed food, if indicated. Animals will also be fed and watered by hand if indicated.

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

All our experiments follow the ARRIVE 2.0 guidelines.

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

We keep a close eye on the NC3Rs website (https://www.nc3rs.org.uk/) and are also routinely updatedabout changes and best practice by the Biological Unit at our institution. We also attend seminars/workshops hosted by NC3Rs.

A retrospective assessment of refinement will be due by 16 July 2028

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 forfuture work of this kind?
- During the project, how did you minimise harm to the animals?

5. Disease models of emerging and re-emerging viruses

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, ortheir 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 aimsmentioned in paragraph (b**)**

Key words

Arbovirus, Robovirus, Therapy, Pathogenesis, Modelling

Animal types	Life stages
ice	adult, juvenile
Rats	adult, juvenile
Guinea pigs	adult, juvenile
Hamsters (Syrian) (Mesocricetus auratus)	adult, juvenile
Ferrets	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?

To develop specialised animal models of arthropod-borne viruses (arboviruses), rodent-borne viruses(roboviruses) and other emerging and re-emerging zoonotic viruses of consequence.

A retrospective assessment of these aims will be due by 20 August 2028

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?

Transmission and pathogenesis of viruses that cause severe disease in humans is often poorlydefined, largely due to the difficulty of studying pathogens of high disease consequence in the laboratory setting.

Development of robust animal models that directly compare to human disease will enhance our understanding of human pathogenesis and can be used for intervention (therapeutic and prophylactic)studies.

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

Information generated from infection models will determine the stages and situations where other susceptible animals - including humans - may be at risk of infection. The data will inform public healthbodies and provide advice on how to avoid exposure to zoonotic pathogens.

By understanding whether exotic arboviruses can be maintained and transmitted by UK arthropod vectors (ticks and mosquitoes), information for future UK government policy and resilience planningcan be provided.

A successful model for intervention testing could lead to the prevention of severe disease or reduction/delay in the onset of clinical signs in susceptible animals including humans. This informationcan be taken forward for subsequent human clinical trials.

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

Public health bodies will be better informed to provide advice on how to avoid exposure to zoonoticpathogens. Understanding of new viruses transmitted by UK ticks and mosquitoes can allow futureresilience planning.
Progression of therapeutics and interventions (vaccines) towards clinical trials and licensing will benefitclinicians, patients and animals.

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

All collaborations (either new or maintained from previous licensed studies) will ensure we keep pacewith relevant scientific developments, avoid duplication and use current best practice in experimentaldesign of animal studies.

Importantly this work also underpins a UK capability and maintains a unique level of

expertise that canbe rapidly called upon in times of emergency, for example during the COVID pandemic and more recently the monkeypox outbreak which were each declared as Public Health Emergencies of International Concern (PHEICs).

Our group actively publishes and disseminates its work in open access journals and scientific meetings. Dissemination of all data - both successful or less so, (For example, when the WHO declared the Zika virus outbreak a Public Health Emergency of International Concern (PHEIC), our group published the first data on Zika virus (ZIKV) susceptibility of IFN-a/bR-/- mice through a publiclyaccessible website before it had been accepted for print publication. This ensured that groups across the world could use an appropriate murine strain to facilitate intervention testing. It also contributed tominimising the numbers of mice used). will allow for refinements in further projects which may in turn lead to a reduction of animals used.

Species and numbers of animals expected to be used

- Mice: 3500
- Rats: 600
- Guinea pigs: 800
- Hamsters (Syrian) (Mesocricetus auratus): 900
- Ferrets: 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.

Use of resources such as literature and our knowledge gained in previous studies and previous licences, will indicate which species will be most relevant for the studies in this project. The preferred models will use a natural host and reservoir where possible. When this is not possible or is unknown, pilot studies will be conducted using a hierarchy of species that will include mice, rats, hamsters, guinea pigs and ferrets to provide insight into the most clinically relevant model. Prior to any studies using therapeutic agents and/or pathogens, prior knowledge will be researched for information on variables such as determination of dosage. In addition, pilot studies will be performed when evaluatinga novel virus.

Mice with genetic alterations in their immune pathways will also be utilised. For the majority of virusesthat will be used in this project it is our experience that genetically altered mice are likely to provide a more useful model of disease then wild-type mice because of a more consistent susceptibility to infection and progression to a defined humane endpoint. Studies will be designed using the most applicable species.

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

Typically, animals will have their temperature monitored and recorded during the study from an implanted chip. This may be repeated in a different area if the implanted chip fails, but the likelihood ofthis happening is low.

During intervention testing animals will typically experience mild, transient pain and no

lasting harm from administration of substances using standard routes (intravenous, subcutaneous, intraperitoneal, intranasal, intramuscular, intradermal, oral). Vaccines may need to be given up to four times. Antivirals(and some less stable therapies) may need to be given up to four times a day but for no more than three weeks, depending on the pharmacokinetic properties of the compound concerned. Most other therapies will be administered once before and/or up to three times after challenge. Regular pre-and post-interventions saliva and/or blood may be taken. Total volumes of blood in one sample and/or across multiple sampling points will adhere to guidance from Wolfensohn and Lloyd (4th edition).

Animals will be monitored for clinical signs to ensure minimal pain, suffering and distress.

Animals will be culled by a Schedule 1 method or exsanguinated under general anaesthesia without recovery at specified time points in general, no longer than six weeks post-final treatment but some may be longer,or once humane endpoints have been met.

During infection modelling, animals will be challenged directly by intradermal, subcutaneous, intramuscular, intraperitoneal, intravenous injection, by attachment of infected vectors (including but not limited to ticks and mosquitoes), intranasal or aerosol route, or indirectly by introduction of naïve animals to groups of infected animals and allow to interact. To allow vector attachment, a small sectionof fur may be removed to allow feeding or the attachment of a patch or holding device to be sealed onto the skin of the animal to contain the vectors. Sedation may be used to allow exposure of animal and mosquito feeding. Regular pre-and post-interventions saliva and/or blood may be taken. Total volumes of blood in one sample and/or across multiple sampling points will adhere to guidance from Wolfensohn and Lloyd (4th edition). Animals will be culled by a Schedule 1 method or exsanguinated under general anaesthesia without recovery at specified time points in general, no longer than six weeks post-final treatment but some may be longer, or once humane endpoints have been met.

Clinical signs will be monitored to ensure minimal pain, suffering or distress and humane endpoints willbe clearly defined by a set of clinical signs (significant weight loss, temperature deviations, neurological signs).

During efficacy testing all procedures from intervention testing and infection modelling may beperformed.

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

The adverse effects of novel interventions are largely unknown but would not be expected to cause continued pain nor negative effects. The route of administration (injection, oral, inhalation) may causeshort term discomfort and/or local inflammatory responses and as such are likely to cause adverse effects. These will be counteracted by the use of suitable analgesics where necessary.

Disease and challenge models whereby the animals are administered with a virus by different routes (injection, oral, inhalation) will have aspects of severe disease side effects as these animals are likely to develop disease over the course of the study. However, due to the nature of these studies all clinicalsigns observed will be recorded on a clinical score sheet with a set of pre-defined criteria of what is and is not acceptable during the studies. Any clinical scores that exceed the criteria – or specific clinical signs that may cause enhanced pain/distress - will result in the animal reaching a humane endpoint with that

animal removed from the study and euthanised using a Schedule 1 method.

During studies when the severity is likely to increase, or when there is a likelihood of a humane end point being reached, the frequency of observations and recordings will be increased to ensure pain and distress is kept as low as possible and animals are removed from the study to ensure no animalsuffers any unnecessary pain or 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 animaltype)?

- Infection models: 80% moderate, 20% severe for all species (mice, rat, guinea pig, hamster, ferret).
- Intervention characteristic testing: 80% mild, 20% moderate for all species (mice, rat, guinea pig,hamster, ferret).
- Intervention testing: 80% moderate, 20% severe for all species (mice, rat, guinea pig, hamster, ferret).Vector competence: 80% moderate, 20% severe for all species (mice, rat, guinea pig, hamster, ferret).

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

- **Killed**

A retrospective assessment of these predicted harms will be due by 20 August 2028

The PPL holder will be required to disclose:

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

Computer models and laboratory cell-based models are not useable in these studies due to the complexity of the studies and the therapeutic/vaccine licencing regulation requirements for animalstudies prior to human clinical trials.

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

Published data on similar models, human or epidemiological data will be sought to replace potentiallyunnecessary early pilot studies.

Certain aspects of how therapeutics may act chemically can be assessed using computer modelling, which may enable unsuccessful candidates to be removed from studies prior to animal modelling. Thiswill be employed wherever possible. It cannot be employed for all candidates but will replace the needfor some animals.

In early studies it may be possible to use lab-based cell assays to test novel therapeutics and vaccinesprior to using animal models. Where these are available, they will be used to remove early non- successful candidates prior to any animal modelling.

Why were they not suitable?

Cell based laboratory assays are not complex enough to align closely with animal models. Due to regulatory requirements for use of therapeutics/vaccines in humans, animal models are still needed.

Many of the viruses in these studies are novel and cause severe disease in humans. There is limiteddata available and therefore limited to no computer models nor lab-based cell assays for these pathogens.

A retrospective assessment of replacement will be due by 20 August 2028

The PPL holder will be required to disclose:

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

Animal numbers have been estimated based on information from previous licences and published dataalluding to numbers of animals required per test group. In the past group sizes of no less than 6 have provided the quality of results required. In early modelling studies, the levels of dose for each virus will need to be assessed for novel pathogens prior to use in intervention testing. Where models/data are available for similar viruses, these will be used to potentially reduce the numbers of groups required.

Where possible genetically modified animals will be used to reduce variability within group and reducethe sample size required.

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

Use of an experimental design tool (e.g.: The NC3R's experimental design assistant) for each studywill be employed to ensure the minimal numbers of animals may be used in each procedure.

Pilot studies will be performed to assess variability and use of a particular species, especially when emerging viruses with limited animal model data are being assessed. This will ensure that follow on studies are only performed once a suitable model is established. Data from pilot studies will inform on suitable group and experimental unit size in

subsequent studies. Factorial experimental designs will beused to allow the most information from a single study with minimum numbers of animals used.

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

Lab based studies using cell culture or ex vivo studies using material from tissue banks will be performed wherever possible to reduce animal numbers. Where vaccines/therapeutics show little effectin the lab it may be possible to eliminate them from animal studies.

A further way to reduce animal numbers will be in study sampling. Providing a procedure (ie: bloodtaking), does not cause prolonged or unnecessary pain and distress, and it is beneficial to do so, sampling one animal multiple times over the duration of a study will reduce numbers, rather than culling a separate animal at each required time point.

Immunocompromised mice will be used in several studies as wild-type rodent reservoirs will not alwaysexhibit significant pathogenicity to viral infection. Where possible, wild-type mice will be used, but where immunocompromised/modified mice are to be used, they will be sourced from specific accredited breeders. The animals will have undergone minimal adaptation to maintain the clinical relevance of the models.

Study details are made available to other groups such that tissues not required in our study can bemade available to other groups for use in similar research.

A retrospective assessment of reduction will be due by 20 August 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan 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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

The most relevant animal model that will provide the most robust scientific data will be used throughoutthe studies to ensure repeated experiments and increased numbers of animals will not be required.
Where possible genetically modified animals will be used to reduce variability within group and reducethe sample size required.

The use of clinical score sheets and increased observations during the studies ensures the severity isreduced as far as is possible and potential pain/distress is limited.
If a procedure would benefit from anaesthesia beforehand or subsequent pain relief (which won’t affectscientific results nor cause the animal more distress than the procedure itself),

this will be performed. The potential welfare effects of the anaesthesia itself will be accounted for (regular checks to prevent hypothermia and dehydration).

Where possible, the routes of administration and the equipment used for administration will be altered to ensure the minimal impact on the animal (eg: some treatments will be better tolerated when injectedrather than orally administered and this will be accounted for when planning specific treatment studies).

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

Mammalian species are required due to the complexity of their immune systems and the ability to produce scientifically relevant results. Animals at an immature life stage do not directly compare tohuman models and are therefore inappropriate models to use.

Where possible anaesthesia will be used to relieve any discomfort that might arise, however, due tothe nature of the viruses being studied, it is not possible to alleviate all levels of discomfort.

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

Some viruses covered in the proposal may never previously have been used in a specific model or infectious route. Many viruses can cause severe disease in humans and so may also have severe effects in animal models. However, whilst the clinical signs seen in humans may be clearly defined there is a possibility that disease progression in an animal model may be different and that treatment regimes may also affect the expected course of disease. Thus for these studies it may be impossible to eliminate all potential severe aspects but every effort will be made to refine end points by increasingmonitoring frequency and by continuous refinement of the clinical scoring system such that humane intervention can be applied at the earliest possible stage. The clinical score system implemented is constantly refined based on previous experience and knowledge in prior studies. It has been shown, that by increasing the observations up to six times per day, animal suffering can be limited and, in many cases, where humane endpoints can be applied, adverse effects can be considered as moderate. The euthanasia criteria in adult animals used in this licence for all scientific procedures using infectious organisms includes immobility and neurological indicators (e.g., repetitive or unusual movement). Immediate euthanasia criteria also include 20% baseline weight loss for more than 24 hours.

If a procedure would benefit from anaesthesia beforehand or subsequent pain relief (which won’t affectscientific results nor cause the animal more distress than the procedure itself), this will be performed. The potential welfare effects of the anaesthesia itself will be accounted for (regular checks to prevent hypothermia and dehydration).

Refinement of procedures to reduce the number of animals and the pain/distress will be applied wherethey can be. Suitable analgesics will be sought following procedures where the likelihood of pain is increased. All methods will be discussed with the NACWO and NVS to ensure the best approach is taken.

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

Publications from the NC3R's and the Laboratory Animal Science Association will be used

tocontinually refine and enhance animal models.

The PREPARE guidelines will also be followed when planning new and updating existing models.

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

I am signed up to the NC3R's newsletter to keep me regularly updated on any changes. We regularlycheck information on NC3Rs website. We also have regular updates and meeting with our animal support team, NACWO's and NVS to ensure we have the latest information and are following new advances.

A retrospective assessment of refinement will be due by 20 August 2028

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 forfuture work of this kind? During the project, how did you minimise harm to the animals?

The applicant receives regular update on 3Rs via the NC3Rs resource updates and web tools(https://nc3rs.org.uk/3rs-resources)

6. Identification of determinants of pathology and protection in infections of barrier organs

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Lung infection, Gut Infection, Immune response, Tissue damage and repair, Immunopathology

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?

The overall aim of this project is to identify determinants of severity in the lung and gut that wereinjured as a result of infection, with two separate but closely linked aims:

First, to identify immune-mediated mechanisms of pathology and protection in viral infections such asinfluenza or COVID-19, and bacterial infections caused by Streptococcus pneumoniae, Yersinia pseudotuberculosis or Yersinia enterocolitica.

And **second**, to study requirements of epithelial differentiation and repair in health and disease, insteady state and following infections, immune responses or pollutant exposure.

A retrospective assessment of these aims will be due by 16 August 2028

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 advancedor 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?

Epidemics and pandemics of both respiratory and enteric infections pose a major global health burdenand can bring societies to a worldwide standstill. It is unknown why some people suffer severe symptoms but others only mild or no symptoms when infected with the same pathogen. Understandingthis better is the first step to make predictions about disease outcome and to develop host-directed therapies that may even have a protective effect across several types of infection (known as pandemicpreparedness). The immune response to infection is complex and involves many cell types residing in and moving between several organs. Only experiments in live animals can reproduce this complexity and allow us to understand infection better.

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

We aim to push the boundaries of our understanding of host determinants of severity in lung and gut infections. The new information will be made widely available, in scientific publications, press releases,at scientific conferences and through outreach activities to a wider lay audience. We aim to identify interindividual differences that explain different disease outcomes, and environmental factors that change course of disease. We will thereby contribute to deeper knowledge of how infectious pathogens and the host response to them influence severity of infectious disease. Building on this newknowledge, we also expect to identify novel targets for immunotherapy of infectious disease to be tested in preclinical models and, eventually, in clinical trials.

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

While our work is basic research, it has strong translational potential, due to the fact that all infectious settings we work on closely mirror relevant clinical situations of high public health impact. This applies to influenza, COVID-19, other respiratory viral and bacterial infections, and influenza-bacterial or influenza-viral coinfections, known to be among the most severe complications of influenza. In particular, the coincidence of influenza and SARS-Coronavirus-2 in the winter season is cause of greatconcern. We also seek to understand mechanisms underlying susceptibility to gut infections, and complications arising from resulting enteric pathology in different patient groups. Our study of zoonotic foodborne Yersinia enteropathogens in mice reflects natural human infection where the pathogen disseminates from the gut to deeper tissues such as spleen and liver, and provides a model very similar to typhoid-like syndrome observed after salmonella infection. Thus, studying these infectious agents will facilitate understanding on intestinal barriers, gut tissue immunity, inflammatory consequences of bacterial dissemination from the gut and sepsis, and immunity to common enteropathogens.

The outputs of the present project will be deeper knowledge of the mechanisms and agents driving increased severity in these infections: For instance, we know that interferons (IFNs), a family of hormone-like messenger substances with antiviral effects

leading to protection, can sometimes promote severe disease. However, we don't know how and when the damaging effect prevails over thebeneficial effect, and we plan to study the mechanisms leading to either tissue damage or to antiviral protection. As in vivo testing often involves blockade of the relevant pathogenic pathway to show improvement, we will produce data that directly paves the way towards human treatment. No IFN inhibitors are known, but through our mechanistic studies, upstream or downstream modules in the IFNpathway may be revealed as promising drug targets. Understanding how different subclasses of IFNs work will also allow us to identify more specific pathogenic drug targets to inhibit. In addition, our studies will show which dynamic pattern of IFN induction is linked to protection and which one to pathology, findings that might lead in the future to blockade of the pathway specifically early or late during infection.
Also, we know that genetic differences between inbred mouse strains change IFN levels from protective to excessive, but we don't know which is the causative genetic difference. As oneof our efforts is to identify the upstream IFN regulator that differs between these mouse strains, we will be able to identify genes that may define at-risk groups in the human population, due to their tendency towards excessive IFN responses. We are already studying a mouse model of a human genetic syndrome that may fall into this category. Therefore, all outcomes of this project represent direct and indirect benefits for alleviating respiratory and gut disease in humans. Similarly, for COVID-19, blockingthe excessive inflammatory processes that characterise the late phases of severe disease would have a dramatic and immediate beneficial impact in the clinic. All results generated in our studies will be made available to the scientific, clinical and lay communities, by publications with open access, presentations at scientific and clinical conferences, and outreach to the lay public which is a priority in our organisation. Reagents will be made freely available to the scientific community upon request.

Interactions with clinical groups will be sought to confirm or refute our findings in existing humandatasets or to set up trials to test this. These interactions will be developed as soon as we have certainty of our findings in our in vivo models, so depending on the individual programme this will be between the coming months and several years from now. Where applicable, IP support will be sought within the institute to determine whether patent applications are possible. Should our work lead to the identification of novel targets, then the medium- and long-term beneficiaries would be the general public (having access to novel immunotherapies against severe infections), and industrial partners whodevelop the products that we have tested or who develop products against the novel targets that we have identified. As far as research with pollutants is concerned, showing clear molecular links betweencomponents present in pollutants and changes in immune responses or in epithelial differentiation will inform and support policies towards improving air quality, the use of diesel exhausts and the reduction in smoking.

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

Maximal dissemination of our results on all levels, including scientists, clinicians, health policy makers,patient groups, the general public will be sought using all available channels, e.g. scientific publications, seminars, conferences, press releases, radio and TV interviews, blogs, social media, outreach activities such as school visits or the pint of science, and others. Wherever collaborations allow us to improve or accelerate outputs, we will use them, we will share reagents and make data relevant for ongoing epidemics and pandemics available as pre-prints prior to peer review.

Unsuccessful experiments will be published as far as possible, to minimise unnecessary repetition by colleagues. The translation team in our institute will be involved as early as promising targets are foundto expedite bench – to bedside development.

Species and numbers of animals expected to be used

- Mice: 50,000

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.

To understand the complex, multi-organ processes that characterise immune responses to infection, invivo models are indispensable, as no in vitro system is able to reproduce the complexity of the immuneresponse. Among the species available, mice are the species of choice, given the unparalleled range of tools for analysis and manipulation. Many immunological therapies in use today were first discovered and studied in mice. As the physiology and pathology of the immune system is similar between humans and mice, mice are very good predictors of mechanisms in action in human disease. Data obtained in our mouse experiments are complementary to other studies in our lab using representative in vitro systems and activities to compare our data to available patient data. As respiratory infections hit humans at all stages, and at-risk groups are the very young, the very old and obese individuals, we may use mouse stages covering the whole life span and conditions such as obesity. For enteric infections we will focus on adult mice initially, to understand disease processes.

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

Typically, mock-treated versus animals with immune-modulating treatments such as blocking antibodies or inhibitors (mostly administered intraperitoneally) or genetically wild-type and gene- modified mice will be compared. Animals will be infected intranasally or by oral gavage with a defined dose of a known pathogen strain, and either killed at a pre-determined time point to assess lung or gutdamage, as well as specific immune parameters that we consider relevant for severity, or mice will be monitored over the whole course of infection for clinical signs and weight loss to determine severity of the infection. All infections we study are self-limited and are resolved within maximally three weeks, unless animals reach humane end points before. Where pre-determined time points are chosen, we always aim to maintain duration of experiments to the minimum required to address the scientific needand obtain meaningful scientific data. In some protocols, two infections will be applied in short succession or with longer delays, to mimic better the human condition of sequential or overlapping infections.

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

As the disease models we study are respiratory and gut infections, mice suffer flu-like and enterocolitissymptoms respectively, including weight loss, hypothermia, laboured breathing, diarrhoea, hunched position, lack of movement, all to varying degrees. For all models, we have detailed knowledge of the days of highest severity (which, depending on the pathogen and the mouse strain, may last between one and four days) and will monitor mice daily on these days, using a detailed clinical score sheet and/or weight loss as the main parameters. Once these critical days are overcome, mice recover rapidly and are

undistinguishable from uninfected mice within a few days, similar to human influenza or Yersiniosis. Only a minority of mice will undergo a full-time course of infection to link interventions or genetic ablation to changes in severity, while the vast majority of mice will be killed at predetermined time points to study lung damage and immune parameters.

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 animaltype)?

We utilise a severe or a moderate protocol depending on the scientific questions we are addressing. Where severe influenza or COVID are modelled, we may need to use a severe protocol, but as outlined above, only a few mice will undergo the whole course of infection, while most will be killed at predetermined time points prior to the days of highest clinical scores. As the same immune parameter may be protective in mild to moderate disease but disease-enhancing in severe disease (similar to e.g.interferon or IL-6 in COVID-19), we often cannot study severe disease in moderate infection protocols.Initial comparisons will tell us whether or not this is the case, and the mildest possible protocol to model human disease will always be applied. This is further exemplified in moderate, self-limiting Yersinia infection models where the main study outcome is to investigate memory immunity, and as such the lowest infectious dose possible will be used that is still able to generate these memory immune cells while avoiding excessive pathology during primary infection. Also, when recovery from lung damage, either infection-induced or in other lung damage models, is studied, we need to have certainty of the lung damage occurring in the first place, which is more reliable and reproducible in severe protocols. More moderate protocols show a higher interindividual variability, which make interpretation of results difficult and require higher mouse numbers per group. Again, we will weigh upin each individual experimental setting which is the earliest time point possible to assess crucial immune parameters.

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

- Killed

A retrospective assessment of these predicted harms will be due by 16 August 2028

The PPL holder will be required to disclose:

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

As the host response to infection involves multiple immune and non-immune cell types, several organsand extensive cell recruitment, it cannot be fully modelled in vitro. For instance, the immune response to a respiratory infection normally involves the bone marrow, blood vessels, the lung, lung-draining lymph nodes and the spleen; while Yersinia

infections are characterised by dissemination from the gutto distal organs like the spleen and liver. Furthermore, resident immune populations (important targets of this work) are shaped by the complex interplay of tissue-specific signals and stromal cell types within different organs, that cannot be effectively recapitulated in vitro. However, whenever complementary in vitro experiments can be performed in organotypic models or on specific immune cells we will do this. We have an established primary lung epithelial cell culture system that we heavilyrely on to test a multitude of conditions, thus reducing mouse numbers drastically. Additionally, a recently funded grant in the group aims to model intestinal human memory immune cells in vitro by developing an organ-on-chip system for gut tissue, with great potential to reduce mouse numbers needed to study these populations in future.

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

We use cell culture systems in vitro that reproduce, at least in part, the complex composition of lung tissue. For these cultures, we use primary cells taken from lungs, instead of tumour cell lines as do most in vitro studies, to be able to study the behaviour of normal lung cells rather than cells that can grow indefinitely in cell culture. We closely follow the development of even more complex cell culture systems (called “lung-on-a-chip), but so far they are not able to mirror all the interactions between many different cell types in the living lung. Our existing models of primary lung epithelial cell or cultures of specific immune cells can be used to address specific questions in isolation, which allows us to replace some animal experiments by experiments in these cell culture systems. We are collaboratingwith clinicians and screen cell availability in biobanks to use infected patient samples for lung infections, to validate that our findings in vivo reflect clinical data, and to reduce the usage of mice.

Why were they not suitable?

Culture systems cannot reproduce the high complexity of immune responses that are dependent on cell movement from the bone marrow through the blood into infected organs, under the influence of systemic factors such as hormones, microbiota or the central nerve system. It is also impossible to fullyreproduce lung or gut tissue, composed of a great variety of different cells on a complex extracellular matrix. The possibility to replace animal experiments by in vitro experiments is so far limited to specific questions, and we actively pursue further development of in vitro systems in the lab to extend their potential. For example, we add immune cells to our lung tissue cultures with the aim to gain a more complete picture in cell cultures of lung immune responses.

A retrospective assessment of replacement will be due by 16 August 2028

The PPL holder will be required to disclose:

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

Reduction

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

studies, computer modelling, sharing of tissue and reuse.

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

This number is based on the volume of experiments we have performed in the last 13 years doing similar research, and on the current lab size and range of projects we are pursuing or plan to start,factoring in that we will be able to perform in vitro part of the experiments addressing our biologicalquestions.

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

For all infection experiments, we have long-standing experience for experimental design and optimal handling to reduce operator-dependent variance. For the design of mouse experiments, we follow therecommendations as outlined in the PREPARE guidelines (https://norecopa.no/PREPARE0) and the NC3R's Experimental Design Assistant. Some of the basic principles we apply are as follows:

We try to include several conditions (e.g. depletion or blockade of several cell types or cytokines)in separate treatment groups in the same experiment, so save on untreated controls and make full useof multifactorial design to enhance statistical power. Using the above online tools, power calculations will be performed to determine minimal treatment group size for maximal statistical information. In- house advice by statisticians in the bioinformatics facility is routinely sought.

We routinely perform experiments on both males and females, to make full use of the mousecolony and to avoid sex-bias in our results or discover sex-specific effects.

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

We participate in institute-wide animal tissue sharing from experiments to enable multiple studies-exvivo.

We store many organs from experimental mice in case that novel findings or future research directionsrequire analysis of these organs.

We constantly improve education and training for those working under this project license to ensure and improve animal welfare and minimise operator-dependent variability in results. This happens forinstance through the 3R newletters circulating in the institute, through conference attendance of lab members reporting back on new improved methods having an impact on the 3Rs, and through updating among colleagues using similar protocols in the institute.

Different treatment groups (e.g. mice treated with control or depleting antibodies) will be co-housed inthe same cage, to avoid artefacts driven by differences in microbiota composition. Where genetically modified mice are tested, litter mates will be used for similar microbiota and to avoid artefacts due to minor differences in the genetic background.

Where use of littermates is not possible, co-housing prior to experiments or exchange of cage beddingwill be ensured. This is easier for females but can be achieved for males if they are co-housed immediately after weaning, thus requiring early genotyping or

homozygous lines. Co-housing will be particularly challenging for ageing experiments with males, given that constant in-cage fighting often requires separation of males. We use guidance from the NC3Rs on monitoring aggression among males and when to take action when aggression is evident (https://www.nc3rs.org.uk/minimising- aggression-group-housed-male-mice). We also use enhanced environments in our mouse cages help reduce stress and in-cage fighting.

We mostly breed the genetically altered animals that we use ourselves to be able to respond to theexperimental needs by prompt and often transitory colony size adaption. This reduces waste from overbreeding.

To decide to archive lines by cryopreservation when not required over a longer period of time, we takelead from HO efficient breeding of GA animals https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/773 553/GAA_Framework_Oct_18.pdf).

Obtaining wildtype mice from in house facility-shared breeding allows better efficiency for largercolonies.

We often use small pilot studies to estimate the effect size and the directions for future experimentalsettings.

A retrospective assessment of reduction will be due by 16 August 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan 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 welfarecosts (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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use wild type and genetically altered mice of different inbred and potentially outbred genetic backgrounds in in vivo infection models, using human pathogens or the mouse equivalents of them, to identify determinants of disease severity und the mechanisms underlying severity. We are actively seeking to refine severity parameters to go beyond the present clinical scores and weight measurements. This includes the employment of lung function measurements where mice undergo using non-invasive techniques, such as unrestrained whole-body plethysmography that is used to analyse breathing behaviour in mice. This physiological technique allows the analysis of lung and airway function and has several advantages: data obtained can be directly compared to patient data asclinical plethysmography measures similar parameters, we measure directly lung function rather than indirect disease measures, and we may be able to detect severe lung injury earlier by

these measurements than by the clinical scores we presently use. Similarly, labelled GFP- or luciferase expressing or -encoding bacteria and viruses can be potentially used for non-invasive imaging of pathogen spread during infection. We are already using fluorescent bacteria for ex-vivo measurementsof bacterial load, but the ultimate refinement will be to measure this in vivo in a non-invasive manner, atechnology that we are actively developing. This would not only represent reduction (as fewer mice areneeded in repetitive measurements) but also refinement (as mice are not killed for measuring bacterial load but monitored with a non-invasive method). Such newer and more sophisticated parameters needto be linked firmly to the above-mentioned morbidity measures which still represent the gold-standard indicators of disease severity, before we can replace the latter to achieve refinement.

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

Less sentient animal species do not provide the physiological similarity to humans and the wide availability of tools for analysis and manipulation. More immature mice are not an option as embryos and newborns have only an immature if any immune system. We need a physiology in our in vivo models which is as close as possible to humans. Only mammalian organisms show the same degree of complexity as humans in their immune response, lung or gut structure, and other basic physiologicalparameters like metabolism.

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

We always aim to refine our procedures. For instance, we have started to replace intraperitoneal tamoxifen administration by oral gavage, and for serial blood collection we currently explore the use ofsaphenous vein rather than tail vein puncture. As mentioned above, we are attempting to pioneer imaging and lung function analysis techniques to follow pathogen spread during infection by non- invasive means, to reduce harm to the animals. Furthermore, tunnel handling will be rolled out across the institute within the next years, which causes less anxiety than traditional tail handling. We are guided by and seek advice from local NVS policy on non-invasive procedures, as well as improved pain management, to minimise harm.

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

Unless otherwise specified, the work in this project will be designed using the principle outlined in PREPARE guidelines for planning animal research and testing (2017) and in the LASA Guiding Principles on good practice for Animal Welfare and Ethical Review Bodies. We will also follow LASAGuidelines administration of substances.

With regards to infection models we take advice from the "Considerations for Infectious Disease Research Studies Using Animal " (Lesley A Colby, Lauriane E Quenee, Lois A Zitzow. Comp Med.2017 Jun; 67(3): 222–231; PMID: 28662751) as well as from “Refining procedures for the Administration of substances” (https://doi.org/10.1258/0023677011911345) and from the “guiding principles aseptic surgery” (https://www.lasa.co.uk/PDF/LASA_Guiding_Principles_Aseptic_Surgery_2010.2.pdf).

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

We are regularly updated within the institute on advances in the 3Rs from NC3Rs (https://www.nc3rs.org.uk/nc3rs-newsletters) and NORECOPA, and we actively seek information onpossible improvements in discussions with colleagues and collaborators at in-house meetings and external conferences. Whenever we are able to refine techniques without impacting the scientific validity of our work, we aim to implement advances, for example enriched environments for mice to reduce stress levels and avoid overgrooming.

A retrospective assessment of refinement will be due by 16 August 2028

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 forfuture work of this kind? During the project, how did you minimise harm to the animals?

7. Mechanisms and targets for chronic pain

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, ortheir effects, in man, animals or plants

Key words

neuropathic pain, immune system, neurones, targets, inflammatory pain

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is 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?

Pain accompanies a variety of clinical conditions and the management of pain remains a difficult task. Currently chronic pain is poorly treated by opiates and is resistant to alleviation from the use of non- steroidal anti-inflammatory drugs (NSAIDs). This lack of appropriate and effective treatments is due inlarge part to the incomplete understanding of basic neurology. More work needs to be undertaken to better understand the 'mechanisms' of chronic pain, so that we can develop new treatments that directly target the pain itself and provide pain relief in the absence of overt side-effects.

A retrospective assessment of these aims will be due by 07 August 2028
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 advancedor 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?

Given this shortage of suitable therapies for both neuropathic and inflammatory pain our research aimsto identify new mechanisms underlying chronic pain in order to find new targets for analgesic therapies(pain relief). Neuropathic pain is associated with trauma to a nerve or chemotherapy for cancer treatment. Inflammatory pain is associated with conditions such as rheumatoid arthritis.

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

This project will result in publications and data that will enable applications for more funding and generate new knowledge that can guide the identification of new targets for pain relief. Our pre-clinicalresearch aims at developing a mechanism based approach to the identification of new therapies whichreduce chronic pain in the absence of overt side-effects.

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

This project will identify new mechanisms which are responsible for chronic pain in animals and humans. The identification of key players in such mechanisms will provide new therapeutic targets for the relief of chronic pain in diseases like arthritis. The usefulness of our models lies in them replicatingboth the symptoms and the disease progression seen in patients. Thus, the findings of our pre-clinicalresearch will have direct relevance to the clinical problem and will provide important evidence for the therapeutic potential of new targets for the treatment of chronic pain. In the short-term we will identify new targets for drugs which reduce chronic pain. In the long-term, our findings will result in the development of new analgesics for chronic pain patients and the availability of new analgesics will improve their quality of life.

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

We will present our data at national and international meetings, and publish in scientific journals. We will keep active collaborations with industry so that we remain in contact with translational approach toscience.

Species and numbers of animals expected to be used

- Mice: 25,500
- Rats: 5,700

Predicted harms

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

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

The use of adult rodent models is crucial to our understanding of pain pathophysiology and the development of novel analgesics. Rodents will be employed in these studies as they are the lowest vertebrate group on which these types of experiment can be conducted to provide unique systems responding to drugs used in the clinic. In addition, the extensive use of rodents in biological researchhas already provided much information on pain processes.

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

We will induce conditions that resemble chronic pain in humans by surgical procedure or administrationof specific agents. We expect that our animals will walk less and lose some of their explorative behaviour. The duration of the experiments will be kept to the minimum time required to address specific aims: 1. To elucidate new mechanisms and mediators involved in chronic pain with a special focus on neuro-immune interactions in the spinal cord and in peripheral nerves including the dorsal root ganglia. 2. To determine the effectiveness of compounds as analgesic and anti-hyperalgesic agents in models of inflammatory and neuropathic pain with acute (hours) or chronic duration (several weeks).

We are interested in acute and chronic pain mechanisms. Some studies of sensory phenomena are possible in humans. However, some mechanistic questions require more invasive techniques so we undertake our work in rodents for this. We are interested in using animal models of human disease orpathology. Some of these models have short onset and duration (hours) and can be studied acutely. For example, the injection of a chemical agent like zymosan in the pad of hind paw is associated withpain-like behaviour that is observed and recorded for up to 24 hours.

The hind paw pad is innervated by the sciatic nerve and the cell bodies of these sciatic afferents are located in the lumbar dorsal rootganglia and can be studied as representing the treated afferents. Other models develop and change over time and experiments may last weeks. For example, the injection of collagen as a model of arthritis is associated with pain-like behaviour that is observed and recorded for several weeks. The prolonged time course of some experiments and the assessment of animal behaviour require the useof recovery protocols. Furthermore, the treatment of chronic disorders usually requires persistent or chronic treatments. Pain associated with the procedures in this licence will be controlled by analgesiaduring procedures (for example surgery), except where the principal aim is to study chronic pain mechanisms and in these models alternative pain relief will be used as directed by the NVS.

At the start, in models of pain (neuropathy, arthritis) animals will either receive administration of vehiclecontrol (no treatment), neuroactive substances and/or undergo alteration of gene expression and/or depletion of immune cells (Treatment).

In behavioural tests, animals will be trained and then assessed before and after treatment (Assessment). Functional assessment may be associated with pathway tracing and neuroimaging. Atthe end of assessment animals will be killed by schedule 1 and tissue may be collected for analysis (End).

Start:
We start with normal or genetically altered rodents, initially purchased from licensed breeders, or transferred from other academic institutions. Some of the genetically altered animals will be bred at ourestablishment and may be used where appropriate; these are

particularly useful for defining the role ofa particular target in the pain process, or for providing the means for examining the activity of a particular compound at a human target protein. We don't expected our genetically altered animals to show a harmful phenotype. Safety and efficacy of neuroactive agents (commercially available compounds or compounds generated by discovery programmes with satisfactory biological activity in vitro and/or known pharmacokinetic profile) will be determined in normal or genetically modified animals before use in disease models.

Model:

In a typical study, after having spent at least five days in the animal facility, animals are used in one ofthe pain models. Animals may undergo induction of a neuropathy or arthritis. The models are used to mimic some aspects of the clinical pathologies and understand pain mechanisms. In these models wetest the effect of compounds acting at specific targets and assess the role of a specific mechanism in the induction and maintenance of pain.

The models are as follows:

Peripheral neuropathies: Chronic pain associated with nerve trauma is poorly treated with current drugs. We use models of peripheral nerve injury with the final aim to elucidate new targets for innovative pain therapy. The nerve injury models consist of surgical lesions created under anaesthesia,such as cutting or crushing a particular peripheral nerve in one of the back legs of the animal. The cell bodies of sciatic nerves are located in the lumbar dorsal root ganglia and account for some 70% of the cells that can be studied as representing the treated nerves. Animals recover well from surgery and show increased sensitivity to painful or unpleasant stimuli in the hind-paw of the leg that the surgery was for several weeks and mild impairment of locomotor function for up to 1 week. Systemic drug- induced neuropathies closely model some neuropathic lesions frequently precipitating clinical problemssuch as chronic pain during cancer therapy in patients. In cancer treatments a dose-limiting side-effect of chemotherapeutic agents is the development of neuropathic pain, which is poorly managed by available drugs. We mimic clinical protocols with the aim of finding new mechanisms and targets for pain. For example, 5 day cycles of daily injections of vincristine is associated with significant mechanical hypersensitivity and a moderate cold hypersensitivity.

Induction of inflammation:. Inflammatory (nociceptive) pain is partially controlled by nonsteroidal anti-inflammatory drugs (NSAIDs) and the development of new analgesics would improve pain treatment.The study of the mechanisms of clinically relevant, inflammatory pain requires the use of animal models that induce some tissue injury. We will use models of inflammation that are restricted to one body part but also models of systemic inflammation. We will also use models that are relatively shortlasting (measured in hours to days) or longer lasting (several weeks). Control animals will be either untreated animals or sham treated. The several models listed are necessary as they underlie diverse mechanisms of activation of the pain pathways. However, most of them share comparable severity which will be addressed by keeping their duration short.
The models of inflammatory (nociceptive) pain facilitate the study of pain transmission and the characterization of novel analgesic compounds. In these models acute (seconds to hours) nociceptivepain is measured by spontaneous and evoked behaviour. Longer lasting pain is measured by evoked stimulation.
Induction of collagen-induced arthritis (CIA) and inflammatory arthritis. Chronic diseases such as rheumatoid arthritis display ongoing inflammatory disease associated with pain that is poorly managedclinically, especially when pain is present despite joint swelling

being controlled by disease-modifying drugs.

To mimic this type of disease, we need recourse to chronic models of active or passive immunization.These models are potentially more distressing for the animals and we therefore use them only for crucial studies and then only maintain them for the minimum time required to answer the experimentalquestion.

All compounds will not be tested in all animal models but rather they will be initially examined in the model and species most appropriate for that class of compound in which activity could be anticipated.

Thorough experimental procedures requires that the effects of a surgical procedure, whether on biochemical, morphological or behavioural end-points, be compared to the same endpoint in animalswhich have undergone a sham operation. This ensures that the effects observed are a result of the intended injury rather than a non-specific effect of surgery. This would require an extra group of animals in every experiment. However, the majority of the surgical techniques with recovery in this project are well established and we know from extensive experience that there is no sham effect, particularly with relatively simple procedures such as sciatic nerve injury. Therefore, in these cases we will not include a sham group but rather refer to historical control data to minimise the animals used under this project. For all techniques the effect of sham surgery will be reviewed periodically, and whena new experimenter is using the technique. This will ensure that their data are consistent with previouscontrol data before they can proceed with routine testing.

Treatment:

Our proposed experiments involve the treatment of animals with neuroactive compounds. The range ofcompounds to be tested requires the use of a variety of delivery techniques (including slow delivery directly to the CNS), sites and frequencies which will vary according to the behavioural test to be used.We may also investigate the neuromodulation potential of altering gene expression. The cellular inactivation of depletion of immune cells will be used to investigate the role of these cells and derived mediators in the development and maintenance of chronic pain states.

The animal may then be administered with a test substance either before or immediately after assessment of behavioural responses. In some behavioural tests there may be a period of trainingprior to entering the animal into a study model.

Assessment:

One way of achieving our objective of assessing the performance of the somatosensory system is to measure how manipulations affect the ability of the animal to detect and respond to its environment and to applied stimuli. Some of this information can be obtained simply by observing the animals in their cage or for instance in an open field. For assessment of nociceptive capacity, it is necessary to challenge the animal with painful or unpleasant stimuli that can be thermal, mechanical or chemical. The standard response of the animal to such stimuli is a co-ordinated withdrawal response or a periodof licking of the skin area that was affected by the stimuli. Most of the stimuli we apply are threshold stimuli: stimulus intensity is increased until the animal detects a minimal (threshold) amount of pain, atwhich point it is free to generate a withdrawal reflex. The time taken to achieve this, or the level of the stimulus at which this occurs, is a measure of the sensitivity of the nociceptive system, and varies withanalgesic drugs. Such tests do not damage (or

produce only minimal damage) to tissue (and are terminated by cut-off times in any case). We may therefore apply them repeatedly. Typically, experiments are either short term, when 3 or 4 testing sessions will take place in one day and the experiment then terminated; or long-term - perhaps lasting 3 weeks, when animals might be tested on alternate days.

For many of these tests, the animals need to be habituated and pre-trained on the relevant apparatus.This can sometimes require a few weeks of exposure to the tasks before a treatment or intervention isstarted. On many occasions results from these tests then can be used as a baseline to compare backto once the treatment or intervention has started.

For tests of chemical sensitivity, it is not usually possible to use threshold testing. Here it is more common to provide a dose of chemical (from which the animal cannot escape) and measure the amount of pain-related behaviour induced. A robust stimulus we use is the capsaicin test that featuresa brief period of paw flinching and guarding of the treated paw, which subsides after 30 minutes. We quantify the degree of nocifensive behaviour produced by the stimulus, and then kill the animal. We willnot combine more than two tests of chemical sensitivity in any given animal.

Another way in which we assess changes in sensory systems is an analysis of anatomical connections. To do so, we use anterograde and retrograde labelling techniques. The essence is that atracer compound is injected or applied to the neurons of interest that internalise and transport the tracer to other parts of the neuron. One example is the injection of tracers into a joint, where they are transported to the cell bodies of sensory neurons in the dorsal root ganglia and reveal for instance thesize of the cells that innervate the joint. The transport of tracers throughout the treated neurons typically takes a few days, and for some tracers can take up to 2 weeks. It is therefore necessary to apply the tracers in one operation (which might be before or after putative therapeutic treatments), allow the animal to recover for one or more days before taking tissue for post-mortem analysis, under terminal anaesthesia.

Functional imaging of brain structures is performed to evaluate whether microglia and neurones are activated in supra spinal areas. When testing the effect of a neuroactive substance on brain imaging, itis necessary to perform prolonged imaging sessions within a 24-hour period. This is in order to performanalysis before and after acute treatments. We may need to use neuromuscular blockers to reduce animal movements that would interfere with imaging measurements. Animals will be anaesthetised throughout the period of neuromuscular blockade.

A final method of assessment is to examine the biological properties of tissues taken from the animalsstudied under this licence. In some cases, isolated tissues will be studied in tissue culture. In others, tissues preserved with fixatives for (immuno)histochemical studies.

End:

At the end of the assessment animals are killed by methods deemed ethical by the law and tissue istaken for further experiments in the laboratory.

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

During most of the protocols in this project we expect that our animals will feel pain as this

is what we are trying to assess in the experiments however from our experience the animals normally do not showany pain behaviour in their natural environment unless evoked during one of our pain assessment behaviour tests. As well as this it may also have an impact on the animals walking ability and they maylose some of their explorative behaviour. The duration of these impacts can vary between the pain models used, for example animals that underwent nerve injury surgery may experience less mobility for the first few days after surgery and then gain it back and animals that have inflammatory induced may experience this impact for the duration of the swelling of the paw. Because of these impacts animals may gain less weight than than controls and in the case of the collagen-induced arthritis model, rats may lose weight. To help prevent weight loss animals are given diet on the floor for easy access and sometimes wet diet which is more appealing to help them keep weight. We make sure thatthe severity of our model is "moderate" whenever possible by limiting the time for which the animals are kept following the induction of the pain model. Furthermore, the use of neuromuscular blockers willbe limited to specific cases when we need to image activity of neurons and animal movements may cause interference. Animals will be anaesthetised throughout the period that neuromuscular blockers are used. The KBxN model of arthritis causes all paws to transiently swell (peak swelling at approximately day 5 followed by a gradual recovery until complete recovery by approximately day 17). Pain persists beyond 17 days but this can only be clearly obnserved when pain responses are evoked.This model causes a moderate level of suffering in our experience. The paw swelling associated with the collagen-induced arthritis model starts at approximately 11 days and peaks at approximately 18 days after induction of the model and the animals experience swelling of all paws. Again, pain persists persists beyond the swelling. In our experience, the degree of suffering lasts for longer than the KBxN model and the animals have a slow recovery. Animals with collagen-induced arthritis are killed up to 28days (rats) or 42 days (mice) after model induction.

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 animaltype)?

We have included eight protocols. Severity will be moderate in 6 protocols for about 60% of all animalsin each protocol and the remaining 40% control animals will experience mild severity. Severity will be mild in one protocol for few animals. Severity is considered to be severe for one protocol which includes the KBxN arthritis model in mice and the collagen-induced arthritis model in mice and rats. In this model, 100% of mice and rats that receive collagen to induce arthritis will experience severe severity (approximately 20% of all animals used in this protcol). However, control animals that do not receive collagen will experience moderate severity, as will animals that are part of the KBxN model (approximately 80% of animals in this protocol in total).

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

- Killed

A retrospective assessment of these predicted harms will be due by 07 August 2028

The PPL holder will be required to disclose:

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

animalswere affected?

Replacement

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

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

The use of animal models is crucial to our understanding of pain pathophysiology and the developmentof novel pain killers. The translational animal models which are going to be used in this project provide unique systems responding to drugs used in the clinic. We are principally interested in pain mechanisms. Some studies of sensory phenomena are possible in humans. However, some mechanistic questions require more invasive techniques that are not possible or feasible at present in humans. In vitro techniques are also not sufficiently advanced so they can model the integrated actionsof the nervous system. Thus, we will need to undertake some of our work in animals. Mostly we are interested in animal models of human disease or pathology, and so some of our experiments will makeuse of such models. Some of these models are short onset and short duration (hours) and can therefore be studied acutely in animals. For example we inject a chemical agent in the hindpaw and then measure the amount of pain-related behaviour that is induced. A given chemical agent produces abrief but strong period of pain, which subsides over a few minutes. In animals one sees a brief but intense period of flinching of the treated paw and guarding behaviour which are quantified. Flinches a defined as the number of times the animal raise hind paw; the spontaneous guarding behaviour is measured as the time of animal held the hind paw while stationary.

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

As well as the animal models, we use in vitro techniques to dissect mechanisms and look for alternatives, such as translatable markers. We also use cell lines when appropriate and primary humancells to look at mechanisms however these may not be suitable or all models and mechanisms we lookfor. For instance, we use sensory neurons in culture and immune cells in culture. We routinely search for non-animal alternatives outside of our lab, for example in the literature or through collaborations.
These techniques are always used prior to/alongside our work in animals.

Why were they not suitable?

The non-animal alternatives are not sufficiently advanced so they can model the integrated actions ofthe nervous system so we cannot asses the aims in these methods alone.

A retrospective assessment of replacement will be due by 07 August 2028

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is thereanything 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 routinely seek to reduce the number of animals studied by careful experimental design, the adoption of sensitive outcome measures with small variation and the study of only the most relevant time points. Where possible each animal is used as its own control. Where this is not possible groups of animals will be utilised. In these cases the numbers in each group will be the minimum required to allow valid statistical analysis. In all in vivo tests the number of animals in each group will be the minimum required to allow valid statistical analysis. The number of animals used will vary from procedure to procedure depending on the degree of variability in the experimental measures, but our extensive experience with these models has shown that group sizes of 6 – 8 animals are generally appropriate. For those procedures involving surgery it is scientifically more rigorous to include sham operated control animals in an experiment. However, for techniques which are well established and forwhich we know from experience that there is no sham effect we will not include such animals in every experiment, but refer to historical control data. The effect of sham surgery will be reviewed periodicallyand when a new experimenter is using the technique. It is possible in some instances to use in vitro cell systems to analyse, for example, the effect of inflammatory mediators on receptor and we plan to use such studies extensively. However, it is not yet possible to make stable cell lines of sensory neurones, our main cell type of interest, or to mimic the long term effects of neuronal damage or inflammation that occur in vivo. In these instances the use of animals is unavoidable. Also, by cryopreserving embryos we will reduce the number of animals culled in breeding programmes. Indeed,we have recently cryopreserved embryos from a number of our colonies, which are not going to be used until more research funding is available.

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

One of the methods used to help reduce the number of animals used was using the NC3Rs design assistant online tool to help calculate the most efficient number of animals for the experiments. As wellas this we have optimised some of our protocols so that we can use previous data from experiments rather than repeating and when possible each animal can be used as its own control. For some protocols this is not possible so groups of animals will be used and calculations will be made so we only use the minimum required to allow valid statistical analysis. We also utilise multiple behaviour testing when possible for the protocols to get more robust results leading to less repeating of the experiments and have optimised the behaviour tests used.

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

We have several years of experience doing these protocols and most techniques are well established in our laboratory. Therefore, we often can reduce the number of control animals to the very minimum and use historical data. For examples, to reduce numbers of animals may be retested with more than one compounds and we always make sure that retesting won’t cause more harm. As well as this we have had many experience in breeding animals and planning experiments so we can produce efficientbreeding to only

get the animals we need for the experiments and animals that can't be used for initial experiments will be used to tissue and in vitro work. We will use the NC3Rs design assistant where possible to help optimise the number of animals required and also perform pilot studies for any new compound/substance or experiment we use.

A retrospective assessment of reduction will be due by 07 August 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan 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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rodents will be employed in these studies as they are the lowest vertebrate group on which these types of experiment can be conducted to get reasonable results and their extensive use in biologicalresearch has already provided much information on pain processes. This is why we will continue touse mice and rats for our pain models. We use more mice than rats as mice can be genetically modified. We use rats in models for which they are the species of choice such as collagen-induced arthritis.

The models we use are designed to study pain and at this time these are the only models available to accurately study this. The severity of most models is moderate but severity is mild for control groups ofanimals in each model. Because we cannot change the models we have refined them to cause the least amount of extra pain and suffering than what we need to explore. This means that in some of ourmodels the pain we use is normally evoked pain so the animals should not feel pain during times where we are not testing them or at least they do not show pain or distress in their behaviour during times of not testing. As well as this we have maximised the methods of these tests and procedures used to create and study this model. We clarify the type of model we are using such as neuropathic pain or inflammatory pain and have limited the methods needed to study each as well as the limiting the time animals are needed to be kept following the induction of each pain model. An example is that one model induces swelling of the paw so to prevent any unnecessary distress and suffering we keep this model for a short time and monitor the animals daily and measure swelling.

We also have refined our procedures so with invasive procedures like surgery all animals will receive post-operative intensive care to ensure high standards of welfare are maintained as well as analgesicsto reduce any extra pain from the procedures. This care will include cages remaining on heated mats, administration of saline, provision of soft, easily digestible food. On some occasions we may not provide analgesia as for instance, neuro-active agents under examination are likely to exert analgesic effects themselves.

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

Behavioural tests require the animal to be vigilant and less sentient animals would not be able toperform our tests.

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

To minimise welfare costs to the animals we adopt several measures, all animals will receive environmental enrichment and when possible we always adopt group housing. As well as this we minimise stress in the procedures we do, such as giving animals training prior to behavioural testing toreduce stress from the test and giving post-operative care after surgeries or invasive procedures. All animals will receive post-operative intensive care to ensure high standards of welfare are maintained. This will include cages remaining on heated mats, administration of saline, provision of soft, easily digestible food.

We will provide analgesia where possible and the most appropriate method will be selected to minimise any welfare costs to the animals (this could also include the route and dose of administration or choice of pain model/assay) and that the duration of any suffering will be minimised. When analgesia may not be provided as it would interfere with our studies of pain mechanisms, for animals inpain because of pain induction we make the animal comfortable and provide group housing and intensive care.

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

We follow the International Association of Pain (IASP) guidance on the use of animals in pain researchand the NC3Rs ARRIVE guidelines which provide recommendations to improve the reporting of animalexperiments and for improving the reliability of published research.

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

We are regularly updated and informed by our animal facility colleagues, who organise meeting andsessions that I regularly attend.The NC3Rs regional program manager communicates any new initiatives and incentives.

A retrospective assessment of refinement will be due by 07 August 2028

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 forfuture work of this kind? During the project, how did you minimise harm to the animals?

8. Mechanisms underlying abnormal heart rhythm and function

Project duration

5 years 0 months

Project purpose

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

Key words

Heart disease, Arrhythmia, Cardiomyopathy

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

Disorders of the rhythm and function of the heart are an important cause of death in clinical medicine.Our proposal focuses on how disturbances in heart cells lead to abnormal cardiac rhythm and contraction and how the nervous system, infections or hormones in the body might regulate this pathology in genetically modified rodents.

A retrospective assessment of these aims will be due by 13 July 2028

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 advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We hope our studies will lead to increased understanding of the pathobiology of heart rhythm disorders, animal models of human arrhythmic syndromes and cardiomyopathies and potentially newtherapies for those diseases.

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

We will communicate our advances through presentation at international meetings and publication inwell recognised peer review journals. Our work may also result in new research tools or medicines.

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

In the short term new ideas will be circulated in the cardiovascular research community. In the longterm we hope this feeds into the development of new therapeutic strategies for patients.

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

We have national and international collaborations where we have made our mice available to researchgroups in the UK, Europe and USA. We will continue these collaborations but also forge new ones as new ideas emerge. We are also regularly approached for collaboration and training as we have a unique set of technologies we implement in the study of the cardiac electrophysiology of rodents.

Species and numbers of animals expected to be used

- Mice: 2000 mice per year

Predicted harms

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

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

We typically use young adult mice in most of our protocols. We use mice as there are a range of wellcharacterised genetic tools and resources. Furthermore, our technical interventions can be implemented reliably with scientific credibility in this species and in these age groups.

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

A stepwise approach will be used so that initial studies will be carried out using tissues from animals bred under the licence or will be done under a general anaesthetic from which the animals will not recover. As the lines of inquiry progress studies may involve surgery to, for example, implant devices that allow blood pressure and other measurements to be made. Some of these transmit radio waves to allow recordings to be taken from the animal following its recovery from the surgery without the animal being aware that the measurements are being made. In some studies the animals use wheels or treadmills so that the effects of exercise can be examined. Finally we use surgical procedures to mimic common cardiovascular pathologies. The protocols which cause the most deleterious effect for the animals involve an operation to place a thread around a coronary artery so that the blood supply topart of the heart is restricted to mimic a heart attack or to apply controlled pressure around a blood vessel in the abdomen to mimic high blood pressure. All surgery is conducted using general anaesthetics and the same types of aseptic measures to prevent infection as are used in human operating theatres. The animals will receive pain killers following the surgery. The animals are also monitored very closely and will be euthanised to prevent unnecessary suffering if they develop signs set out in the licence.

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

The most likely impact is expected to be pain from the surgical procedures. All surgery is conducted using general anaesthetics and the same types of aseptic measures to prevent infection as are used inhuman operating theatres. The animals will receive pain killers following the surgery. The animals are also monitored very closely and will be euthanised to prevent unnecessary suffering if they develop signs set out in the licence. A number of the protocols may lead to impaired cardiac function and heart failure. For example, after ligation of a coronary artery and recovery as a model of myocardial infarction and heart failure the animal may suffer pain and distress from the surgery. We have optimised the surgery so that the vast number of animals now recover (>95%) an do not suffer from sudden cardiac cardiac rupture. These animals may develop heart failure indicated by reduced activity,piloerection, increased respiratory rate, weight loss and pedal oedema. We monitor these animals carefully for these side-effects and keep the animals no longer than is necessary to achieve the scientific objectives of the study (typically less than a month in this example).

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 animaltype)?

The majority of procedures are mild in severity (70% often related to breeding mice for ex-vivo studiesafter schedule 1 killing) with only 25% being moderate in severity. A remaining 5% of mice are subjected to a severe protocol.

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

- Killed

A retrospective assessment of these predicted harms will be due by 13 July 2028

The PPL holder will be required to disclose:

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

Replacement

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

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

The analysis of cardiac rhythm and function and associated pathophysiological situations often requires the use of intact animals. Complex physiological processes involving the function of a numberof interacting body systems are being examined. The normal working of the heart requires the function of several distinct organ systems including a functioning nervous system, vascular and renal function and respiratory function. As such these cannot be reconstituted fully using in vitro experiments. Thus such analyses require an in vivo analysis of the integration of the function of different organs and for the results to be extrapolated to human physiology these analyses need to be performed in intact animals.

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

We use various cell based approaches including cell lines and human induced pluripotent stem cellsdifferentiated into heart and other cells to investigate cardiac arrhythmia and function. We have extensive links with clinicians looking after patients with these diseases. We are also performing genome wide association studies in am for ECG traits and these yield new molecules for study.

Why were they not suitable?

It is impossible to fully understand the interaction of different organ systems without the use of animalsand the function key molecules needs to be observed in their cognate tissue. There are limitations to the kind of interventional studies and tissues you can obtain from patients.

A retrospective assessment of replacement will be due by 13 July 2028

The PPL holder will be required to disclose:

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

Reduction

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

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

The numbers are based on the number required to deliver our scientific aims in our current fundedprojects. I anticipate maintaining this level of activity over the five years of the licence.

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

The numbers of animals used will be minimised by maximising tissue use from each animal and by designing experiments according to good statistical and scientific principles. Important experimental design features will ensure that the correct physiological conclusions are reached. Useful online tools and policy documents are available (e.g. ARRIVE guidelines and NC3R's Experimental Design Assistant). The structure of our study protocols also allows repetitive imaging and prolonged telemetrythat reduces the number of animals that to be used to address the scientific questions.

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

We have optimised the breeding stategies for many of our lines. We have ongoing collaborationswhere tissues are shared.

A retrospective assessment of reduction will be due by 13 July 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan 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 welfarecosts (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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We mainly study genetically manipulated rodents particularly mice. Using new genetic technologies wecan delete genes in only some organs and\or at only some times during the lifetime of the animal. In general this allows us to reduce the severity of overall impact to the animals' health. The most severe aspects of the proposal are the models of heart attack achieved by tying off the coronary artery and increased blood pressure achieved by constricting the main blood vessel leaving the heart. The ligationof the coronary artery mimics the process of thrombotic vascular occlusion in the large arteries of man during myocardial infarction and constricting the aorta that of hypertension and\or aortic stenosis.

There are no real alternatives to mimicking these important pathological processes in a controlledfashion. It should be borne in mind that cardiac arrhythmia is a significant health

problem in thesesettings and results in a significant numbers of patients feeling unwell or even dying.

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

The use of more immature life stages does not mimic the behaviour of organ systems in the adult animal. Other less sentient animals have a role but they do not reproduce the physiological responsesin mammals. For example, fish such as zebrafish don't have lungs and the development of the heart isdifferent. In many of experiments we need to observe the behaviour of key physiological process suchas heart rhythm, blood pressure in the awake animal.

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

We are developing and using cutting edge technologies that remove the need for surgery during datacollection. For example the “ECGenie” which avoids the need to implant telemetry devices and sequential imaging techniques reduce animal numbers as it is possible to watch the progression ofpathology in a single animal.

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

The NC3Rs website has a very useful library of resources and guidelines (https://www.nc3rs.org.uk/3rs-resources). Important advances are reported in the scientific literature.

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

Our local NVS and animal staff continuously provide helpful information. I receive regular notificationsfrom the NC3Rs and attend conferences showcasing advances in the 3Rs. I have been a committee member and reviewer for the NC3R Crack-IT initiatives and I am currently on the MRC Genetically Engineered Mouse Models committee. All these channels allow me to stay up-to-date with best practice and care.

A retrospective assessment of refinement will be due by 13 July 2028

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 forfuture work of this kind?

- During the project, how did you minimise harm to the animals?

9. Molecular control of neutrophils and endothelial cells in inflammation and repair

Project duration

5 years 0 months

Project purpose

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

Key words

endothelial cell, neutrophil, inflammation

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?

This project investigates molecular processes that underpin the generation, propagation and resolutionof inflammation by analysing (i) the molecular control of neutrophils, abundant circulating immune cellsand endothelial cells, the building blocks of blood vessels as well as (ii) cross-talk between cell types, in particular the key players mentioned above, in inflammation.

A retrospective assessment of these aims will be due by 07 August 2028

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 advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Inflammation is an important part of any immune defence against infections. It is tightly regulated to ensure that infectious agents are killed while the body's own tissues are preserved, and any bystander damage is promptly repaired once the threat has been removed. As part of the 'healthy' inflammatory response, vessel walls become a little leaky, allowing plasma fluid and proteins to enter the surrounding tissue. Moreover, immune cells, notably neutrophils, breach vessel walls to reach inflamedsites and fight infections. However, breakdown of the tight regulation of immune cells and their interplay with blood vessels can result in important tissue damage. This is the case in chronic inflammation, where the immune system is in overdrive and harms the body's own tissues. Similarly, inacute situations, an overblown inflammatory response is not helpful. This is illustrated for example by COVID-19 or 'flu, where severe disease, e.g. in the elderly, is the result of an excessive inflammatory response rather than the viral infection per se. The interplay of immune cells with blood vessels is particularly obvious here, and can result in accumulation of plasma fluid in the lung, which in turn can interfere with breathing.

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

This work will result in an improved understanding of molecular events in both neutrophils and endothelial cells that occur during inflammation and its resolution. We aim to identify why things go awry, and how neutrophils contribute not only to generating inflammation but also to healing of bystander damage in healthy organisms. By making use of disease models in conjunction with analysing patient samples, we aim to understand how these mechanisms go awry in disease with a view to identifying better treatment for patients in the long run. In the short term, this work will result innew knowledge which will be published in specialist journals.

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

In its different guises inflammation is one of the biggest killers, but unfortunately at present, we only have access to a small number of blunt treatments, that often target the symptoms but not the cause ofinflammation. In the long term our work will contribute to improved therapies for those suffering from excessive inflammation. Our contribution will be an indirect one, because we aim to understand how inflammation works in so-called pre-clinical work. In other words we prepare the foundations for future clinical work that will use the knowledge we and others generate to identify the best drugs for particular inflammatory processes.

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

Prior to embarking on the study, pilot experiments will be used for calculations called 'power analyses' that work out how many repeats of any experiments will be required to reach statistically sound conclusions. Once completed, this work will be published in

specialist journals and discussed with specialist audiences at conferences. To ensure that the work will reach its full potential, publications willinclude detailed study protocols and information on statistical analyses that were performed.
Furthermore, publications will not only report those approaches that were successful, but any pitfallsencountered along the way will also be written up. The aim of this is that ultimately unsuccessful experimental approaches will not be repeated by others.

Species and numbers of animals expected to be used

- Mice: 4600

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.

Inflammation is very complicated and cannot easily be modelled. Mice will be used to interrogate neutrophil and endothelial cell function and neutrophil-endothelial interactions in inflammation. Using genetically altered mice can allow us to interrogate the function of a particular gene in both endothelialcells and neutrophils. Mice are the model of choice since their immune system is very well understood
and serves as a model of the human immune system. The models we use are well established modelsfor human disease and they are performed using adult mice .

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

Most of our models involve the induction of inflammation. These models involve administration of substances that cause inflammation in a specific place, e.g. the lung. We then analyse signs of inflammation, e.g. by weighing the animals and analysing their tissue (e.g. blood, lungs). In some cases we administer drugs to test whether doing this will reduce the inflammation. The actual experiments last from several hours to a couple of weeks to analyse early events during the inductionof inflammation or induction and resolution of inflammation.

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

Most of our experimental models are of very short duration and do not result in very noticeable adversesymptoms. An exception is the viral infection model, where mice will display weight loss and have a reduced activity level prior to making their recovery following infection with influenza virus. In most models used here, preparatory steps are likely to be associated with more adverse effects than the inflammatory models themselves. In some cases mice are reconstituted with stem or progenitor cells after having received a dose of irradiation that ablates the stem cells which form the blood. Unless newblood forming stem cells are administered (i.e. the mice are being 'reconstituted'), this is lethal. The lethal dose of irradiation is required here to avoid rejection of the graft by the original immune cells (which would make the mice very unwell). If, however, reconstitution with haematopoietic stem cells is successful, the irradiated mice will recover and be absolutely fine. This is a well established tool in the laboratory (and the wider field); in many instances it can avoid the use of a much larger

number of mice. In our experience deaths following irradiation and reconstitution is extremely rare (far less than 1%).

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

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

Of the total of 4600 mice projected to be used over the course of 5 years, it is expected that the severity experienced by 41.5% (1900) will be sub-threshold, by 15% (680) mild, and by 40.5% (1870)moderate, while the severity of 3% (150 mice) is expected to be severe.

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 07 August 2028

The PPL holder will be required to disclose:

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

Replacement

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

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

Using genetically altered mice allows us to interrogate the function of a particular gene in both endothelial cell and neutrophil. Moreover, to analyse inflammation we need the entire body. For thisreason we perform experiments in mice.

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

Isolated (human) neutrophils and cultured endothelial cells alone and in combination.

Why were they not suitable?

Human neutrophils are not amenable to genetic manipulation, and specific inhibitors do not exist for allprocesses of interest. Co-culture models allow us to study an interaction between isolated endothelial cells and neutrophils. While instructive this is unfortunately not adequate to fully model inflammation that occurs in an organism, which is much more complex and includes involvement of additional factors in the body.

A retrospective assessment of replacement will be due by 07 August 2028

The PPL holder will be required to disclose:

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

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen 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?

Publications and previously conducted studies offer an insight into mouse numbers typically requiredfor studies. The estimation provided above is based on such estimates.

Since neutrophils are extremely short lived they cannot be cultured and genetically modified. Thereforelarge numbers of animals (hundreds per year per project) are used as a source of genetically modified neutrophils for analysis in vitro. Moreover, the generation of a new genetically modified mouse line requires a lot of breeding, in particular where more than one genetic modification is required, e.g. for a conditional knock-out, or even just for re-derivation into a unit followed by breeding to homozygosity or where back-crossing to a particular genetic background is required. This results in the use of a large number of mice (>100 per line even with modern approaches)

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

For those experiments that analyse inflammation in vivo, we perform pilot studies prior to designing definitive experiments involving larger cohorts; experiments will be planned, conducted and reportedaccording to NC3R and ARRIVE guidelines.

One of our projects analyses conditionally HoxB8-transformed haematopoietic progenitor cells from mice. We and others already showed that these can be used to derive HoxB8 neutrophils, which behave like neutrophils in many ways. We currently have a project underway in which we aim to genetically manipulate HoxB8 neutrophils to avoid the generation of new mouse lines as reservoirs ofneutrophils for analysis in vitro. We also trial whether HoxB8-transformed progenitors can be administered to mice for in vivo experimentation, potentially further reducing the requirement for breeding genetically altered animals for experimental purposes with a view to solely analysing neutrophil behaviour.

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

The majority of mice we use are bred in-house. Wherever possible we plan experiments very carefully so that we can answer several questions at once by using more than one read-out for the study, e.g. byanalysing more than one tissue. Breeding all animals in-house means careful planning to ensure that not too few nor too many mice are born. However at times there will be some mice that we cannot use in experimental cohorts. We

use tissue from such mice to optimise new in vitro experiments, and for pilot studies.

A retrospective assessment of reduction will be due by 07 August 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan 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 welfarecosts (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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Inflammation is a highly complex process that cannot be modelled based on cells in culture alone. We use mice and have carefully chosen experimental models, employing models of very short duration foranalysis of early events in inflammation. This reduces the time in which suffering can occur. Where weanalyse also the resolution of inflammation, we carefully titrate the stimulus again with a view to induceminimal suffering required while achieving our scientific objectives.

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

Although different to humans, mice represent at present the best available animal model that is representative of inflammation in human beings. Not only do mice have comparable immune system and inflammatory responses to humans, there is also a wealth of genetic mutants that allow us to decipher the importance of individual regulators. This is important since some of these may representgood drug targets, which is what we hope to find.

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

Most of our inflammatory models are very short with a duration often only of hours. These models werecarefully selected because they will reduce the length of any suffering. In our previous experience with these models we have not observed clinical signs in mice on these models.

The pulmonary infection model (protocol 7) is the exception to this. This model involves administrationof virus (e.g. a mouse adapted influenza A) to cause lung inflammation. Dose finding experiments areperformed where viruses are titrated carefully to ensure that the lowest titre commensurate with the aim of the experiment is being used. While animals typically make a full recovery from this model, the protocol has been classed severe in case some of our GA animals may be more susceptible to the lung inflammation caused by the viral infection.

Animals on this model are weighed daily and are carefully monitored and scored for clinical signs (posture, activity level, responsiveness, temperature, breathing, piloerection), with weighing and scoring performed twice daily while the mice are acutely unwell. Clearly defined endpoints are applied for any animals exceeding severity levels (no spontaneous activity, no response to touch or a clinical score >4 for more than 24 hours; loss of >25% body weight compared to original body weight, or >20%for >5 days).

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

We stay up to date on best practice guidelines and updates by making use of the NC3Rs website(www.nc3rs.org.uk)

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

We make use of the online resources provided by NC3R and follow developments in the literature. We moreover keep informed on new developments with the help of informational seminars and events heldlocally, and where applicable change our practice accordingly.

A retrospective assessment of refinement will be due by 07 August 2028

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 forfuture work of this kind?
- During the project, how did you minimise harm to the animals?

10. Rodent regulatory genotoxicity

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, ortheir 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 aimsmentioned in paragraph (b)
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Genotoxicity, Pharmaceuticals, Agrochemicals, Industrial Chemicals

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 orscientific needs it's addressing.

What's the aim of this project?

The aim of this rodent regulatory genotoxicity project is to evaluate the potential of pharmaceutical (human or veterinary/animal health) or non-pharmaceutical (agrochemicals, food additives and industrial chemicals) compounds to cause genetic damage in rodents, principally the rat and mouse.

Genotoxicity is a term used to describe the ability of a compound to damage the genetic information within the cell causing mutations which may lead to cancer in the future. Thus, genotoxicity studies arepart of an overall work package as part of the safety evaluation

process.

A retrospective assessment of these aims will be due by 10 August 2028

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 advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

A key benefit of this programme of work is the provision of safety data to facilitate sound regulatory decisions when assessing the risks to humans when the test substances are produced, transported orused.

This work is vital to the development of safe substances such as pharmaceuticals (human or veterinary/animal health) or non-pharmaceuticals (agrochemicals, food additives and industrialchemicals) that people will come into contact with.

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

The output of this project will be the provision of safety data to facilitate sound regulatory decisionswhen assessing the risks to which humans, animals, plants or the environment are exposed when substances are produced, transported or used.

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

The public and animals will benefit from these outputs.

The data produced in this project allows the support of ongoing clinical trial programs and aids in the ability obtain a product licence to market a substance. The development of new medicines, veterinary/animal health products, food additives, agrochemicals and industrial chemicals is necessaryfor the continued success of efforts to combat disease, maintain food supplies and achieve improvements in the quality of life.

It is a fundamental expectation that such substances should not pose an unacceptable risk to the health and well-being of the human population or target animal populations, or to the environment. This project contributes directly to that expectation, and facilitates the development of products that willhave minimal adverse impact.

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

Development and validation of new tests or modifications to existing assays will lead to an improved battery of tests for hazard and risk assessment. In addition, many new tests or modifications may allowmore thorough assessment of genetic hazard in one step, thus eliminating the need for extensive further testing and reducing overall animal usage.

Wherever possible, data from multiple end points will be obtained from the same animal.

Species and numbers of animals expected to be used

- Mice: 5500
- Rats: 10000

Predicted harms

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

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

Adult rats and mice are the species specified in the regulatory test guidelines. Some studies also useadult genetically modified mice.

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

Animals will be exposed to test substances (as detailed earlier) via routes including inhalation, application to the skin, orally, in food or water and injection either into the skin, muscle or bloodstream.This may require anaesthesia or periods of restraint on a number of occasions.

Administration/infusions of substances can be performed using delivery devices such as catheters in blood vessels. Animals may be blood sampled to confirm exposure, or restrained to enable exposure.Animals will be humanely killed at the end of the study and tissues harvested for examination.

Most of the dosing techniques, manipulations or investigations do not cause any lasting adverse effects, but a small number of animals may show temporary moderate distress due to, for example,withdrawal of blood.

The vast majority of studies will last for days rather than weeks.

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

When dosing an animal by injection or taking blood, the amount of pain an animal feels is similar to what a patient would feel having an injection performed by a doctor. If we have to repeatedly inject animals using a needle and syringe, we would choose different sites to do this where possible. If wecan take blood samples when an animal is deeply unconscious then we do. If we need to take repeated blood samples or need to dose repeatedly then we try and use different sites. Of course, everyone who performs these procedures is trained to a high standard.

The genetically modified animals we use are specifically required by global regulators for a specifictest.

Following test material administration, adverse effects may be observed in some animals. This may include weight-loss, ruffled fur, subdued behaviour and breathing abnormalities for example. We do observe our animals at least twice a day, and the people who do this are trained to recognise the signswhen an animal is ill. If an animal is ill, we would check it

more frequently, and get more senior staff involved in its care for advice, including vets. Humane endpoints will be applied where required.

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 animaltype)?

Administration of test substances may result in mild to moderate signs of toxicity, usually at the highestdose level. The rats and mice used in these studies may show effects at all dose levels, but they are expected to be transient. Experience shows that under the last licence (~80%) showed transient subtlemild clinical signs such as reduced weight gain or weight loss, subdued behaviour and fur ruffling.
Moderate signs (hunched posture and abnormal breathing) of adverse effects were seen in someanimals (~15%), usually in the higher dose groups.

Despite the close monitoring some animals (<5%) sometimes inadvertently experienced severe toxicological adverse effects such as repeated convulsions, persistent laboured breathing or indeed were found dead. Lethality and/or severe effects are not the desired outcome and animals will be closely monitored and promptly humanely killed at predetermined humane endpoints to minimise the likelihood of unexpected death as far as possible. Animals which exceed the project license severitylimits are reported to the Home Office.

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

- Killed

A retrospective assessment of these predicted harms will be due by 10 August 2028

The PPL holder will be required to disclose:

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

Replacement

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

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

Regulatory guidelines require the use of animals to investigate genotoxicity. The in vivo tests (in animals) are conducted because some agents are mutagenic in vivo but not in vitro (in non animal alternative tests). The in vivo tests also include additional relevant parameters such as absorption, distribution, metabolism and excretion, which may modulate the genotoxic effects of a test substance.
You cannot fully model the complex interactions as seen in animals solely in non animal alternativetests.

We will however remain vigilant to the possibility of the development and emerging use of

any nonanimal regulatory acceptable alternatives should they become available in future.

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

The ECVAM database and other literature searches were conducted to determine if any non-animal alternatives were available. However, the animal study is preceded by an in vitro assay, the results ofwhich are used to optimise the design of the animal study.

All studies will be assessed to verify that there is a need to conduct the study and that there is no otherdata or approach that could avoid in vivo tests.

Why were they not suitable?

No non-animal alternatives are accepted alone by Regulatory authorities. However, if there is data orprevious test results available that mean in vivo tests are not required, procedures will not be conducted for that purpose.

A retrospective assessment of replacement will be due by 10 August 2028

The PPL holder will be required to disclose:

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

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen 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 are based on the numbers used during the last five years and the expected demand for thisservice.

The Regulatory Guidelines usually indicates the design and number of animals included in a study therefore, there is lesser scope in genetic toxicology for reduction than in other fields of work. Attentionis paid to good study design to use the minimum number of animals in the most refined way to achievethe aims of the study.

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

Some study design decisions may have to be made based on data from bacteria or cell based assaysor from preliminary data in rodents, from which a progressive approach to the accumulation of information is adopted. This orderly sequence of data collection reduces the number of animals used and restricts the procedures to which they are subjected.

For studies that are being performed at a later stage, where studies in the rodent (and

other species)may have already been performed, decisions on study design can usually be made with a higher degree of confidence leading to lower animal use. Scientists and Statisticians will be consulted at anearly stage as required, so that advice can be given on implementation of the 3Rs and study plans developed which minimise severity of procedures applied as far as possible.

ICH guidelines promote the assessment of genotoxic effects by including the relevant end-points into other toxicity studies that are required for regulatory submission. This has clear advantages in terms ofanimal reduction, however, the study designs must meet specific requirements, so that they are acceptable to regulators and that further animal studies can be avoided.

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

Regulatory guidelines define the minimum testing requirements for adequate data/statistical analysesfor the majority of assays and the study plans used generally adhere to these guidelines/recommendations. Where no guideline/ recommendations exist, animal numbers are selected on the basis of published literature and/or internal validation data that identify the minimum number of animals required for adequate statistical power.

We will always seek to minimise the use of control groups and multiple dose levels, where this isappropriate.

Where possible we will use the same animal to get as many of the required experimental outputs wecan.

A retrospective assessment of reduction will be due by 10 August 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan 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 welfarecosts (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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rodents (either rats or mice) are used because their use is mandated by regulatory bodies who carry out the relevant risk assessments/safety evaluations for the studies in this project. There is considerable experience and background data for the species and studies in this project and the mostrefined methods will be used.

In a few studies we use a transgenic mouse (Muta™Mouse). This is for a specific test (looking atpossible gene mutations) and is to satisfy a global regulatory testing guideline.

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

The species used is generally the same as the rodent species used for the general toxicology studies.The toxicology and/or toxicokinetics from the general toxicology studies can then be used to inform dose selection, sample times etc for the genotoxicity tests, thus reducing animal usage. Species selection may also be driven by known absorption, distribution, metabolism or excretion (ADME) differences between rodent species.

The Regulatory test guidelines require the use of young adult animals.

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

Studies are performed in a stepwise manner, starting with preliminary studies using small numbers ofanimals where there is limited information, a so called 'pilot study'. This gives the highest prospect ofrefining and optimising the programme e.g. by optimising specific doses of substances given to achieve the desired scientific endpoints in the main study and also in consequence minimising the pain, suffering, distress or lasting harm for the animals used on study.

All animals are regularly monitored for signs of any adverse effects on their health or wellbeing, and to prevent unnecessary suffering, early pre-determined humane end-points are applied under appropriateveterinary guidance (e.g. modification/withdrawal of treatment with the test item, or humane killing of affected animals).

Veterinary surgeons are on hand at all times to give welfare advice on any concerns seen by technicalstaff looking after the animals.

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

ICH (2011) EMA/CHMP/ICH/126642/2008. Guideline S2(R1): Guidance on Genotoxicity Testing andData Interpretation for Pharmaceuticals Intended for Human Use.

ICH (2018) EMA/CHMP/ICH/83812/2013. Guideline M7(R1). Assessment and Control of DNA Reactive(Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk.

OECD Guideline For The Testing Of Chemicals (2016): In Vivo Mammalian Alkaline Comet Assay, TestGuideline 489

EC Commission Regulation No. 2017/735. Method B.62: Mutagenicity – In Vivo Mammalian AlkalineComet Assay. OJ L 112/180.

OECD Guideline for the Testing of Chemicals. (2016) Genetic Toxicology: Mammalian ErythrocyteMicronucleus Test, Guideline 474.
EC Commission Regulation No. 2017/735. Method B.12: Mutagenicity - In vivo Mammalian Erythrocyte Micronucleus Test. OJ L 112/54

US EPA (1998) Health Effects Test Guidelines; OPPTS 870.5395 Mammalian Erythrocyte

Micro-nucleus Test. EPA 712-C-98-226.

US FDA (Redbook 2000), Toxicological Principles for the Safety Assessment of Food Ingredients.
IV.C.1.d. Mammalian Erythrocyte Micronucleus Test.

Japanese Ministry of Agriculture, Forestry and Fisheries. Test Data for Registration of Agricultural Chemicals, 12 Nohsan No. 8147, Guideline 2-1-19-3, Agricultural Production Bureau, November 24,
2000.

Japanese Ministry of Health and Welfare. Evaluation and Licensing Division, Pharmaceutical andMedical Safety Bureau, Notification No. 1604, 1 November 1999.

OECD Test No. 475: Mammalian Bone Marrow Chromosome Aberration Test (2016).
OECD Test 488 Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays (2022).
For blood sampling and dosing then the following guidelines/literature will be used:
First report of the BVA/FRAME/RSPCA/UFAW joint working group on refinement, Laboratory Animals,27, 1-22 (1993).

A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routesand Volumes, Journal of Applied Toxicology, 21, 15-23 (2001).

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

Through the regular review of non-animal alternative developments / resources, attendance ofscientific conferences and animal welfare forums and reviews of scientific literature.

A retrospective assessment of refinement will be due by 10 August 2028

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 forfuture work of this kind? During the project, how did you minimise harm to the animals?

11. Safety Testing of Chemicals, Plant Protection Products, Biocides and Substances added to Food or Feed Products Using Small Animal Species

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Improvement of the welfare of animals or of the production conditions for animalsreared for agricultural purposes
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aimsmentioned in paragraph (b)

Key words

Toxicology, Non-Pharmaceuticals, Regulatory, Safety Assessment, Small animals

Animal types	Life stages
Mice	adult
Rats	adult

Animal types	Life stages
Rabbits	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 orscientific needs it's addressing.

What's the aim of this project?

This licence authorises the conduct of studies in small laboratory animal species (rats, mice and rabbits) with the aim of evaluating the toxicity, their ability to cause skin and eye irritation, skin sensitisation and tumorigenicity (ability to cause cancer) of non-pharmaceuticals (agrochemicals, biocides, food additives /foodstuffs, ingredients of house-

hold chemicals (where legislation allows) andindustrial chemicals). This is to aid in the development of new chemicals, and to provide mandatory information to regulatory authorities to allow marketing approval (i.e. to show that they are safe when they come into contact with humans).

A retrospective assessment of these aims will be due by 03 September 2028

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 advancedor 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 (and the public expects) that substances we are exposed to are safe or that theirpotential hazards are well understood and documented.

The data generated from the studies performed under this project will be used to inform decision- making processes on substances under development and, where appropriate, to satisfy governmentalregulatory requirements necessary to gain marketing authorisation or product registration.

This safety assessment is of immense importance along with other non-rodent and non-animal studiesin demonstrating to governments and the public the safety of these substances or highlighting their known hazards and safe handling.

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

This project licence authorises the conduct of in vivo safety studies in laboratory small animal speciesto evaluate candidate molecules and novel and currently-registered substances in terms of systemic toxicity, toxicokinetics, irritation /sensitisation or the potential to cause or influence development of tumours.

The overall benefit of this project is that it generates high quality data that is acceptable to regulatory authorities and enables internal decision making within our clients' organisations. This project will alsoensure that chemicals and pesticides that the general population are exposed to are safe.

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

Our customers will benefit, as the data we generate will allow them to progress their substances underdevelopment and, where appropriate, to satisfy governmental regulatory requirements necessary to gain marketing authorisation.

The studies ensure that non-pharmaceuticals such as food additives, agrochemicals and industrial chemicals that the human population are exposed to during their lives are safe or that their hazards areknown as that they can be handled safely.

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

Where confidentiality permits, data, study design and best practice will be openly shared at conferences, workshops, webinars, blogs and publications.

As 3R's benefits are also realised under this project licence, these will be shared more widely withother establishments.

Species and numbers of animals expected to be used

- Mice: 19900
- Rats: 42900
- Rabbits: 4560

Predicted harms

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

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

Rodents (Rats and mice) will be predominantly used in this project along with Rabbits. Only adultanimals will be used.

Species choice and use of specific animal models is determined by the need to generate regulatory acceptable data. Where a choice of species is possible, care is taken to select the most biologically appropriate species, and the species which most closely relates to man. Studies to assess the types ofmaterial covered by this licence are usually performed on small animal species.

Generally the rat is the rodent species of choice in safety assessment. Rats are large enough to provide repeated blood samples, thus requiring significantly fewer rats than mice to achieve the sameobjective. Mice may be used when considered a more appropriate species, for example, if they more readily absorb the test material, are more relevant biologically or improved tolerance depending uponobjective of the study.

Rabbits may be used when considered a more appropriate species, for example non-pregnant range finding studies prior to conducting reproductive toxicology studies in pregnant rabbits or local tolerancetesting.

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

Animals will be given the "test material" under investigation in a way which mimics possible human exposure. As the most likely route of exposure is orally the majority of animals will receive the test material either mixed in their food or directly by insertion of a flexible rubber catheter/semi rigid plasticor metal cannula in to the oesophagus. For some test materials the oral route of administration may not be appropriate for example the material is more likely to come in to contact with skin or other bodymembranes (e.g. the cornea of the eye for example). Most animals are treated daily; occasionally studies may require several doses within 24 hours or exposure to the test material for a number of hours each day for example by placing the material on the skin and covering it with a gauze dressing. The length of study depends on the likelihood of repeated human exposure and ranges

from a single administration for example to assess accidental contact through to daily administration for 2 years to explore possible long term effects (when looking for the potential for a chemical to cause cancer).

Blood and urine samples may be taken to measure the level of test material or its metabolites with ananimal's circulatory system. These may also be analysed to detect any effects on body systems and organs for example liver or kidney function.

Study animals are closely observed at least twice daily by highly trained technologists who monitor forany signs of discomfort. Other measures such as food consumption and bodyweight may be used to closely monitor for treatment related effects. Veterinary surgeons are employed on a full- time basis and are available 24/7 to provide clinical treatment, guidance on animal welfare and the conduct ofprocedures including appropriate surgical technique, anaesthesia and analgesia.

The majority of animals are expected to have mild adverse effects of treatment such as reduced weightgain or changes in appearance or behaviour. A small number of animals (usually limited to the highest doses evaluated in early studies) may show more moderate adverse effects. The nature and type of effect varies dependant on the biological systems affected, however, these usually result in findings such as reduced food consumption, weight loss and changes in behaviour such as decreased activity. Humane endpoints will be adopted or dose levels reduced if animals show excessive effects. Longer term studies are expected to have progressively less adverse effects.

Many toxicological effects of the test material are not evident during the in-life phase of a study and donot impact the animals' wellbeing. Only through macroscopic and microscopic examination of the tissues from each animal, can evidence of all toxicological changes be fully assessed and the scientificvalue of each animal maximised. In order to undertake these evaluations the animals must be killed humanely at the end of a study.

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

When dosing an animal by injection or taking blood, the amount of pain an animal feels is similar to what a patient would feel having an injection done by a doctor. If we have to repeatedly inject animals using a needle and syringe, we would choose different sites to do this where possible. On some occasions (eg jugular sampling in mice) we can take blood samples when an animal is deeply unconscious. If we need to take repeated blood samples or need to dose repeatedly then we try and use different sites. Of course everyone who performs these procedures are trained to a high standard.

Routinely we need to take a urine sample for analysis, so we would then put an animal into a special cage which is smaller than their normal cage. The animal can still move around. Virtually every animalwill get used to their new cage within about 15 minutes and are fine.

Dosing with chemicals may cause adverse effects in some studies. Experience shows that the majority of animals are not expected to show any clinical signs of suffering (either no clinical signs or normal background signs expected of the rodent strain). A small percentage may show transient subtle to mild clinical signs. Moderate signs of adverse effects may be seen in some animals, usually in the higher dose groups. Lethality and/or severe effects are not study objectives in any of the protocols within this licence, but for preliminary studies that may be the first animal studies with limiteddata available, a very small percentage of animals may inadvertently show severe findings before theyare

immediately and humanely killed.

We do observe our animals at least twice a day, and the people who do this know the signs when an animal is ill. If an animal is ill, we would check it more frequently, and get more senior staff involved inits care for advice, including vets.

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 animaltype)?

On the last project, about 70% of animals displayed mild severity, around 25% of animals were classified as having displayed moderate severity and less than 5% were in the severe severity category. This is because these studies can last between a few days and weeks to up to a year, and although the individual procedures are usually mild in nature on their own, the cumulative effects makethem moderate overall

It's impossible to predict the proportion of severities expected on a service licence like this, as this willbe dependent on what study types we are asked to perform.

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

- Killed
- Used in other projects
- Rehomed
- Kept alive

A retrospective assessment of these predicted harms will be due by 03 September 2028

The PPL holder will be required to disclose:

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

Replacement

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

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

At present there are no scientific and legally acceptable evaluations of systemic toxicity which will satisfy regulatory requirements and provide sufficient safety data other than use of animals. Validatedin vitro tests for specific organs and biological pathways are available and used to replace or refine procedures wherever possible. If new in vitro methods become available and achieve regulatory acceptance during the course of this project they will be validated and used to replace in vivo procedures. Where available, review of scientific articles, non-animal methods and other animal data such as metabolism information will be utilised to reduce animal use.

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

There are no other non-animal alternatives for the work being undertaken on this project. The regulations we are following will not allow safety decisions to be made on non-animal systems alone.

In vitro and in silico methods (test tube or computer work not using animals) are used in combination with animal studies to inform study designs and assist in understanding of potential toxicity but cannotyet replace in vivo (animal) studies.

Why were they not suitable?

Although there are in vitro tests that can model some parts of how chemicals get into our bodies, and how our body deals with them, and can identify undesirable effects, for example, there is no series of invitro tests that brings all these complex happenings together, like we see in animals and humans.

That is why we need to test chemicals in animals, as they have similar physiology and processes as humans, and that testing gives us a good idea what may happen if they were ever exposed to humans.

A retrospective assessment of replacement will be due by 03 September 2028

The PPL holder will be required to disclose:

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

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen 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 we have used are based on figures of previous usage from previous projects, or a projection thereof (based on estimated incidence) based on requests received from customers in the past. It is, however, impossible to accurately predict the number of studies that may be performed, inthe circumstances.

The regulatory guidelines we follow for each study usually indicate the number of animals in a study;otherwise, the number used is the minimum to achieve the aims of the study.

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

Studies are designed to provide maximal scientific value from the minimum number of animals, whilstusing sufficient animals to meet scientific objectives, and regulatory guidelines. Statistical input is sought, where appropriate, to strengthen the overall scientific

quality and relevance of studies.

Where available, sensitive analytical techniques may be used to reduce animal numbers.

Wherever practicable, and by looking across studies, the combination of endpoints eg general toxicity,reproduction and developmental toxicity, mutagenicity etc in studies is considered, to reduce overall animal usage.

As most studies involve the examination of tissues following treatment opportunities for re-use are verylimited. Tissues are collected to support drug and in vivo developments from any surplus stock animals.

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

We will try and get as many outputs as we can from a single animal where possible, without adverselyaffecting its welfare. So if we need to get several different samples, for example, we will often do that in the same animal, rather than use separate ones, when possible.

Before our main studies, we use smaller groups of animals to get an idea of the doses we need to usefor the main studies. These studies are important as it gives us confidence that the doses we are using are correct prior to testing them in bigger groups of animals required by global regulators.

A retrospective assessment of reduction will be due by 03 September 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan 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 welfarecosts (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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Most of our models involve dosing animals with test substances, and sampling them, with many outputs taken after the animals have been humanely killed. This is generally the least invasive set ofprocedures that can be done to give meaningful outputs to make scientific decisions about further tests, or to determine the safety of a test substance.

Throughout our studies, our animals are checked at least twice a day. This allows us to see over a period of time, whether dosing each individual animal is causing any adverse clinical signs. if this isthe case, we can take action: get veterinary advice, add food

supplements and extra bedding if needing, and even reduce dose levels or stop dosing completely.

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

Rodents (rats and mice) and rabbits will be used in all of the studies conducted under this licence.Rodents are considered to be of the lowest neurophysiological sensitivity (their brain function and physiology) that will allow us to achieve the study aims and are considered suitable for predicting what's likely to happen in humans. Rabbits will be used for specific studies where rodents are not aphysiological or regulatory option.

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

Many of the procedures performed on our rodents and rabbits like blood and urine sampling, cause only transient distress to the animals. Blood sampling procedures are similar to and about as painfulas having a blood sample taken by a doctor or a nurse. Blood volumes are kept to a minimum.

Confining animals in special cages to allow us to take urine samples is similarly of little distress to theanimals.

Where animals are planned to be restrained in tubes for longer periods (eg 30 minutes or greater), training of the animals occurs for increasing periods prior to treatment commencing to accustom the animals. Dosing and sampling procedures 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 and will be the minimum consistent with the scientific objectives of our studies. In addition, suffering willbe further minimised by implementing clearly defined humane endpoints.

For mice, sham dosing prior to the start of test article administration has been shown to allow the animal to be accustomed to the restraint and dosing procedure without actual liquid administration andwill be conducted for all oral gavage studies. Also handling in mice can be stressful if not conducted ina suitable manner. Therefore, mice will be cupped when handled where possible to reduce the stress,lifting via the tail should be kept to a minimum.

In addition, care is taken to provide as much environmental enrichment as possible. This includes, butis not limited to, plastic shelters in their cages, wood blocks and balls (short studies up to 13 weeks duration) to gnaw on and push around; mice are occasionally given swings, mice and hamsters are generally given extra bedding for warmth and food supplements are given as appropriate.

In some tests we may use animals that are genetically altered, for example, transgenic mice becauseof their susceptibility to tumours. These animals are specially bred and don't display any harmful clinical signs due to their conditions.

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

Regulatory Guidelines

OECD Guidelines

OECD 405 – Acute Eye irritation/corrosion

OECD 407 – Repeated Dose 28-day Oral Toxicity Study in Rodents

OECD 408 – Repeated Dose 90-Day Oral Toxicity Study in Rodents

OECD 410 – Repeated Dose Dermal Toxicity: 21/28-day Study

OECD 411 – Sub-chronic Dermal Toxicity: 90-day Study

OECD 417 – Toxicokinetics

OECD 420 – Acute Oral Toxicity – Fixed Dose Procedure

OECD 424 – Neurotoxicity Study in Rodents

OECD 429 – Local Lymph Node Assay

OECD 451 – Carcinogenicity Studies

OECD 452 – Chronic Toxicity Studies

OECD 453 – Combined Chronic Toxicity/Carcinogenicity Studies

Summary of Considerations in the Report from the OECD Expert Groups on Short Term and LongTerm Toxicology

Notes for guidance on repeated dose toxicity. Committee for Proprietary Medicinal Products (CPMP),2010. CPMP/SWP/1042/99 Rev 1

Guideline on the evaluation of control samples in non-clinical safety studies: checking for contamination with a test substance. Committee for Medicinal Products for Human Use (CHMP), 2005.CHMP/SWP/1094/04

Note for guidance on carcinogenic potential. CPMP, 2002. CPMP/SWP/2877/00

EU Directive 91/414/EEC – evaluation, authorisation, approval of active substances at EU-level and national authorisations of plant protection products (PPPs); EU Feed Hygiene Regulation (183/2005);EU Regulation 882/2004 on official controls for feed and food law (and animal health and animal welfare); EU Biocides Regulation 528/2012 and EC 1907/2006 REACH Regulations

Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008and Directive 2000/13/EC.

Dosing and sampling and other documents

Diehl et al. A good practice guide to the administration of substances and removal of blood, includingroutes and volumes. Journal of Applied Toxicology: 21, 15-23 (2001)

Gad et al. Tolerable levels of nonclinical vehicles and formulations used in studies by multiple routes inmultiple species with notes on methods to improve utility. International Journal of Toxicology: 1-84 (2016)

NC3Rs: Recommendations from a global cross-company data sharing initiative on the incorporation ofrecovery phase animals in safety assessment studies to support first-in-human clinical trials (Regulatory Toxicology & Pharmacology, 2014).

LASA/NC3Rs: Guidance on dose selection for regulatory general toxicology studies for pharmaceuticals.

Guidance on the conduct of regulatory toxicology and safety evaluation studies. UK Home Office 2005

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

This will be achieved by consultations with our Named Information Officer, colleagues in Animals Technology, and by attending appropriate training courses and conferences, or getting feedback fromsuch events.

A retrospective assessment of refinement will be due by 03 September 2028

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 forfuture work of this kind? During the project, how did you minimise harm to the animals?

12. Safety testing of medicinal products using dogs and minipigs

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

Regulatory, Safety Assessment, Dogs, Minipigs

Animal types	Life stages
Beagles	adult
Minipigs	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.

- 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?

The project aims to test pharmaceuticals (for human use) to determine the scientific and/or regulatory endpoints in non-rodent (dog or minipig) toxicity, pharmacokinetics and metabolism for submission to regulatory authorities, to satisfy governmental regulatory requirements and for safety assessment purposes.

These studies are run to satisfy the requirements of UK/EU (and sometimes international regulatory authorities) who are independent of governments) which require the testing of pharmaceuticals in a non-rodent species. Study designs are based on OECD and ICH guidelines for Pharmaceutical and medical device testing

A retrospective assessment of these aims will be due by 09 September 2028

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?

New medicines have the potential to be of benefit in new or improved disease treatments. Before potential new medicines are administered to humans or animals their safety must be evaluated. This testing is a mandatory legal requirement and provides information on risks to people taking new medicines. Often, the new pharmaceuticals we test in this programme will be designed to be better than existing treatments, possibly with fewer or less severe side effects.

At present there are no alternatives that don’t use animals that are scientifically, ethically or legally acceptable as replacements for systemic toxicity or safety assessment. Most new medicines are tested in rodents (mainly rats and mice) before being tested in a second, non-rodent species like the minipig or dog.

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

The overall benefit of this project is that it supports the development of safe, new medicines to improve the health and quality of life of human patients by generating high quality data that is acceptable to regulatory authorities and enables internal decision making within our client's organisations.
Achievement of the objectives of this licence will enable safe development candidates to progress and will also help to remove unsuitable candidates from the development pipeline at an early stage, thus saving animals and resources.

Study reports will be included in regulatory submissions to allow regulatory authorities to make judgements on whether to permit clinical studies or to licence a drug. Global guidelines recognise that the justification for animal-based regulatory toxicology and safety testing is the need for regulatory authorities to have sufficient information to assess the risks to which humans or animals are exposed by the use of new drugs. Supporting studies, including preliminary studies, will enable appropriate dose selection and appropriately focussed observations and investigations in the definitive regulatory studies.

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

Patients will benefit from these studies as this work will contribute to the development of new drugs that help alleviate human conditions. These new drugs may work better in the clinic, relieve or cure diseases and have better side effect profiles. We may, by our work, also contribute to better knowledge and understanding of these types of drugs, and that knowledge may be used to develop further new drugs.

One of the key benefits is the production of data that is required by regulatory authorities, to ensure medicines can be dosed safely to humans. These drugs that will be tested are

for a variety of conditions, in some cases where there is an unmet clinical need to treat such conditions.

In addition, the models on this project may be used to assess the safety or other in life properties of a new drug and find a dose that causes no effect. This is important when planning future trials in humans or animals, to make sure any starting dose in a clinical trial is safe for the patients taking it.

Our customers will also benefit, as the data we generate will allow them to progress their new drugs into clinical trial, or otherwise if they are found to have adverse side effects.

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

The work will be shared with customers who will use it to determine their future strategy, or for submission in documents required by regulatory authorities. Whilst we have no direct control over what happens to the data after we have shared it, we trust from information given to us that it is used for regulatory purposes or to support regulatory purposes (e.g. to support drugs progressing to clinical trials). Where appropriate, we collaborate with our customers to share data we have produced in the form of scientific publications that are in the public domain.

We are able to advise our customers on which studies are required in their development programme and on suitable study designs, based on our experience and on knowledge gained from previous post-registration feedback from customers and/or regulators, leading to focussed and effective studies.

It is difficult to predict how the benefits of any work done on this project will be seen in the future due to confidentiality issues. However, this work will contribute to the safety of pharmaceuticals that can be administered to humans, (either by informing on safety and allowing to progress to clinical trials, or preventing pharmaceuticals reaching the market due to safety issues), which in itself reduces the overall number of animals used (by preventing further testing).

Species and numbers of animals expected to be used

- Beagles: 4800
- Minipigs: 3000

Predicted harms

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

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

The dog or minipig are used in these studies, as they are well characterised species with a lot of background scientific data available over many years. They also satisfy the requirements of global regulatory authorities for safety evaluation in non-rodent species, which is required by law prior to testing in humans.

As stated earlier, most pharmaceuticals are tested in a rodent species prior to testing in a non-rodent species, as covered by this project.

It is a legal requirement in the UK that dogs (or cats or equidae) may only be used in a programme of work involving regulated procedures when the objectives of the work cannot be achieved by using another species. In this project, the dog will only be used when use of the minipig would not achieve the aims of the experiment, or satisfy regulatory authorities. All requests for studies using dogs are assessed by means of an internal review process; the review panel, including scientists, project licence holders and responsible persons under ASPA, consider the information presented to reach a consensus decision, and will only approve the use of dogs where there is robust justification that the study could not be successfully performed using minipigs instead.

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

Animals are dosed by the intended/likely route of human or animal exposure (for example oral administration, injection or infusion), and observed regularly to monitor appearance, behaviour and clinical health. Routinely, animals can expect to be blood sampled and undergo ophthalmoscopy.

Some animals may undergo a surgical procedure under general anaesthesia, eg placement of a deep vein catheter for intravenous infusion, or implantation of a monitoring device or minipump. Investigative

procedures carried out in these studies are similar to diagnostic procedures that might be used medically to monitor progress of a human patient and include, for example, collection of blood and urine samples for laboratory investigations, or ECG monitoring to assess heart rate/function, or examination of the eyes using an instrument similar to those used by opticians. Animals undergoing surgery receive the same sort of care as a patient would in hospital. We discuss their pain relief and use of antibiotics with a veterinary surgeon before we start. We administer drugs as necessary and give them plenty of time to recover from surgery before we use them in experiments. These surgical procedures are carried out only for essential purposes.

If we need to take a urine sample for analysis, we may put an animal into a special collection cage which is smaller than their normal cage. The animal can still move around.

Other more unusual tests might include assessment of neural function, taking small samples of tissue under general anaesthesia, collection/examination of body fluids, collection under general anaesthesia and examination of lung washings or spinal fluid, body temperature by rectal thermometer. A minimal degree of restraint or confinement may be required for some procedures. Where appropriate, positive reinforcement training (using treat rewards) is used to encourage co-operation in (and minimise any stress of) handling/procedures.

Some animals may be used on procedure on more than one occasion (re-use); such re-use is limited and strict criteria are applied, eg veterinary examination indicates that it is appropriate to do so. Some animals (dogs only) may be re-homed via the establishment's rehoming scheme if it is in their best interests, but most animals are humanely killed at the end of the study to allow detailed examination of the organs.

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

When dosing an animal by injection or taking blood, the degree of pain or discomfort an animal feels is similar to what a patient would feel having an injection done, or blood taken by a doctor.

Animals undergoing surgery receive the same sort of care as a patient would in hospital. We discuss their pain relief and use of antibiotics with a vet before we start and administer drugs as necessary.

Dosing with drugs may cause adverse effects in some studies. Experience from the last licence shows that roughly half (43%) of animals display only mild severity with the remaining 57% displaying moderate severity. Lethality and/or severe effects are not expected to occur, in any of the protocols in this licence.

We observe our animals at least twice a day, and the people who do this know the signs when an animal is ill. If an animal is ill, we would check it more frequently, and consult vets and other senior animal care staff for advice and guidance in its care.

Most animals are expected to experience no, or only mild, adverse effects during the course of the study such as slight weight loss. A small percentage of animals may show more significant adverse effects, such as more marked weight loss, reduced activity, vomiting or tremors. No animals would be expected to die or to suffer prolonged adverse effects as a result of the procedures, and where necessary early humane end-points are applied, under veterinary guidance as necessary, to prevent this; such end-points might include interventions to discontinue dosing, or to provide supportive treatments, or if necessary to humanely kill the animal.

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

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

On the last project, about 50% of animals were classified as having experienced mild severity, the rest were classified as moderate. The moderate severities in the last project would have been due to treatment-related signs of moderate severity (mostly in prelims) or because a surgical procedure e.g. cannulation was involved. It's impossible to predict the proportion of severities expected on a service licence like this, as this will be dependent on what study types we are asked to perform. However, a distribution between ‘mild’ and ‘moderate’ severities similar to those in the last project are anticipated.

All protocols on this licence are classified Mild or Moderate only, there is no intention to perform any procedures that are Severe in nature.

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

- Killed
- Kept alive
- Rehomed
- Used in other projects

A retrospective assessment of these predicted harms will be due by 09 September 2028

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?

Pharmaceutical testing in animals is a mandatory legal and regulatory requirement and provides information on risks to people and animals taking new medicines. At present there are no alternatives that don't use animals that are scientifically, ethically or legally acceptable as replacements for systemic toxicity assessment.

We maintain a constant awareness of regulatory guidance and ensure that where non-animal methods exist which fulfil the regulatory requirement, they are used in preference to animal studies.

The regulatory requirements are mainly for UK/EU regulators, but occasionally other regulators in other countries like the US for example. If the requirements for these non-UK/EU tests are over and above the requirements for a UK/EU regulators, or the test required is more severe, then we consult the Home Office to ask for prospective authority to run such tests.

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

There are no other non-animal alternatives for the work being undertaken on this project. The regulations we are following will not allow safety decisions to be made on non-animal systems alone.

In vitro and in silico methods (test tube or computer models work not using animals) are used in combination with animal studies to inform study designs and assist in understanding of potential toxicity but cannot yet replace in vivo (animal) studies.

Why were they not suitable?

Although there are in vitro tests that can model some parts of how drugs get into our bodies, and how our body deals with them, and can identify undesirable effects, for example, there is no series of in vitro tests that brings all these complex events together, as in the whole (animal or human) organism.

That is why we need to test new drugs in animals, as they have similar physiology and processes as humans, and that testing gives us a good idea what may happen if they are subsequently used in humans.

A retrospective assessment of replacement will be due by 09 September 2028

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 we have used are based on figures of previous usage from previous projects, or a projection thereof (based on estimated incidence) based on requests received from customers in the past. It is, however, impossible to accurately predict the number of studies that may be performed, in the circumstances.

The numbers of animals used in each study are in some cases specified in the regulatory guidelines; where not specified, numbers are based on established minimum regulatory expectation, or on scientific estimates of the minimum numbers required to meet study objectives.

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

Studies are designed to provide maximal data and statistical power (where appropriate) from the minimum number of animals considering that it is better to increase the number of animals used to achieve the objective than to use too few animals and risk having to repeat the study.

For regulatory studies, guidelines require the number of groups and animals per group to be adequate to clearly demonstrate the presence or absence of an effect of the test substance; core study designs are based on international guidelines where these exist. Otherwise reference is made to standard study designs with input from statisticians, where appropriate, to identify the optimum number balancing the need to achieve study objectives while avoiding excessive animal use. These internal designs are reviewed and updated in line with changing external guidelines and internal refinements that either minimise numbers or reduce severity.

Whenever possible, common species of animals are used such that a large amount of control background data is available. This reduces the need for large control groups.

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

We will try to get as many outputs as we can from a single animal where possible, without adversely affecting its welfare. So if we need to take several different samples, for example, we will often do that in the same animal, rather than using separate ones, when possible.

Before our main studies, we use smaller groups of animals to get an idea of the doses we need to use for the main studies. These preliminary studies are important as they give us

confidence that the doses we are using are correct prior to testing them in bigger groups of animals as required by global regulators.

A retrospective assessment of reduction will be due by 09 September 2028

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.

This project will use juvenile and adult dogs and minipigs. We only use dogs when minipigs are unsuitable for scientific reasons.

The models we use are the least invasive procedures, for the least amount of time necessary to get the information we need. They are carried out using standard and recognised techniques by fully trained staff. We also have veterinary clinicians on hand for advice and on the occasions we have to anaesthetise the animals and for general advice on animal welfare.

If we have to repeatedly inject animals or withdraw blood using a needle and syringe, we would choose different sites to do this where possible to minimise local adverse effects.Where appropriate we place temporary cannulas in blood vessels to reduce the number of needle punctures necessary. If we can take blood samples when an animal is deeply unconscious, then we do so.

For all surgical procedures pain relief will always be provided. Surgical procedures will be carried out aseptically and to at least the Home Office minimum standards for aseptic surgery, and in accordance with the principles set out in the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017) (LASA is the Laboratory Animal Science Association). Any animals that undergo surgery will get the same standard of care as a patient who needed surgery in hospital.

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

There is a scientific and regulatory requirement for safety/toxicity data in non-rodent species such as dogs or minipigs to supplement rodent data and enable a complete risk assessment. We use minipigs in preference to dogs wherever possible; (a legal requirement in the UK), and dogs are only used where necessary to achieve the study objectives, ie when the minipig is unsuitable (for example due to species-specific differences from humans, confounding pharmacology or toxicological responses, or practical limitations due to anatomy or physiology).

Most of these studies require repeat dosing for days, weeks or months, to assess potential adverse effects in man, so it is not practical to perform them under terminal anaesthesia.

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

Animal welfare is of utmost importance and Good Surgical Practice will be observed for any animal undergoing surgical procedures. Surgery will be conducted using aseptic techniques (to prevent infection) which meet at least the standards set out in the Home Office Minimum Standards for Aseptic Surgery. Before we start surgery, we agree with a Vet what pain killers or antibiotics the animals need both before and after the surgery. When animals are recovering from surgery, we give them extra heat and monitor them closely until they are fully recovered and showing normal behaviour. We then check them at least twice daily before they go on study.

During dosing and restraint, animals are constantly and closely watched for signs of distress. If equipment is used to enable us to achieve the scientific aims of the study (e.g. confinement in a metabolism cage for urine collection), then we would habituate animals to this equipment prior to dosing. Most animals habituate well to this equipment, but if they don't (rare) we remove them from the study

If we have to repeatedly inject animals or withdraw blood using a needle and syringe, we would choose different sites to do this where possible to minimise local adverse effects.Where appropriate we place temporary cannulas in blood vessels to reduce the number of needle punctures necessary. If we can take blood samples when an animal is deeply unconscious, then we do so. All personnel performing these procedures are trained to a high standard to minimise adverse effects.

All procedures are subject to ongoing assessment and technique improvement and we participate in cross-company working parties on best practice. Animals are regularly reviewed for general health and veterinary staff are on call at all times to assess any adverse events and provide supportive care and treatment as appropriate.

Animal welfare costs are minimised by the careful selection of dose levels to reduce the likelihood of unexpected toxicity, and the application of rigorous and comprehensive humane endpoints.

Socially compatible species are routinely group housed with environmental enrichment which encourages species specific behaviours without adversely impacting study outcomes.

Individual studies are designed to cause the least possible suffering by frequent review of practices, provision of highly skilled technical staff and veterinary support, purpose built facilities and a clear focus on animal welfare. Any confinement or restraint is restricted to the minimum required to achieve the scientific objectives of the study and all study plans/protocols are reviewed for adherence to welfare guidelines and best practices by the site’s Animal Welfare and Ethical Review Body (AWERB).

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

ICH Safety Guidelines–see www.ich.org/products/guidelines/safety/article/safety-guidelines.html
Note for Guidance on Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies S3A (1994)
Pharmacokinetics: Guidance for Repeated Dose Tissue Distribution Studies (S3B) (1994)

Duration of Chronic Toxicity Testing in Animals (Rodent and Non Rodent Toxicity Testing) S4 (1998) Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals S6(R1) (2011)
Safety Pharmacology Studies For Human Pharmaceuticals S7A (2000) Immunotoxicity Studies for Human Pharmaceuticals S8 (2005) Nonclinical Evaluation for Anticancer Pharmaceuticals S9 (2009)

Guidance on Non-clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorisation for Pharmaceuticals M3(R2) (2009)

OECD Guidelines – see www.oecd.org or www.oecd-ilibrary.org

OECD 409 – Repeated Dose 90-Day Oral Toxicity in Non-Rodents (1998) OECD 417 – Toxicokinetics (2010)
OECD 452 – Chronic Toxicity Studies (2009)

Summary of Considerations in the Report from the OECD Expert Groups on Short Term and Long Term Toxicology (2006)

Notes for guidance on repeated dose toxicity. Committee for Proprietary Medicinal Products (CPMP), 2010. CPMP/SWP/1042/99

Guideline on the evaluation of control samples in non-clinical safety studies: checking for contamination with a test substance. Committee for Medicinal Products for Human Use (CHMP), 2005. CPMP/SWP/1094/04

LASA/NC3Rs: Guidance on dose level selection for regulatory general toxicology studies for pharmaceuticals. http://www.lasa.co.uk/pdf/lasa-nc3rsdoselevelselection.pdf

Notes for guidance on non-clinical local tolerance testing of medicinal products. CPMP, 2001. CPMP/SWP/2145/00

LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017) http://www.lasa.co.uk/wp-content/uploads/2017/04/Aseptic-surgery-final.pdf

Diehl et al. A good practice guide to the administration of substances and removal of blood, including routes and volumes. Journal of Applied Toxicology: 21, 15-23 (2001)

Gad et al. Tolerable levels of nonclinical vehicles and formulations used in studies by multiple routes in multiple species with notes on methods to improve utility. International Journal of Toxicology: 1-84 (2016)

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 Named Information Officer, colleagues in Animal Technology, and by attending appropriate training courses and conferences, or getting feedback from such events.

A retrospective assessment of refinement will be due by 09 September 2028

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. Regulation of the mucosal immune response during infection with pathogens or pathobionts and inflammation in rodents

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Infection, Immune response, tuberculosis, peritonitis, Inflammatory bowel disease

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 orscientific needs it's addressing.

What's the aim of this project?

The over-arching aims of this Project Licence are to study the regulation of the mucosal immuneresponse to infection. Specifically, the aims are to:

Identify molecular pathways regulating resistance and susceptibility to Mycobacterium tuberculosisinfection, the causative agent of the disease tuberculosis.

Identify molecular pathways regulating mucosal immune responses in the intestine and the peritonealcavity.

A retrospective assessment of these aims will be due by 13 September 2028

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 advancedor 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?

An individual responds to infectious agents and other foreign substances to control and block diseasefrom progressing. An understanding of the immune response is thus critical for control of infectious disease and conversely to block the immune response from over-responding to microbes or foreign substances and causing damage to the individual.

Mycobacterium (M) tuberculosis studies

Tuberculosis (TB), caused by infection with the bacterium Mycobacterium (M) tuberculosis, remains a major cause of death from infectious disease, with 1.5 million deaths in 2020 1.6 millions deaths estimated in 2021 and a major need for new drugs. In addition to new antibiotics, therapies to enhance the individuals' immune response are badly needed to help eradicate this disease. It is knownthat a quarter of the world's population are estimated to have been infected by the pathogen, M. tuberculosis, but only approximately 10% of those individuals go on to develop TB disease. The individuals that remain healthy appear to control and eliminate the infection and so are resistant to the disease. These individuals only show evidence of exposure to M. tuberculosis, by a specific skin test or blood test, but remain healthy. The mechanisms underlying progression to active TB or control of the infection are unclear. An understanding of the early immune response in the airways which determineswhether an individual controls the infection or progresses to disease is critical to identify mechanisms of disease outcome. We are currently studying this in the bronchoalveolar lavage (BAL) of human TB patients and their contacts funded by a large grant and will compare and test findings from these studies in our experimental mouse models. It is necessary to test molecules, cells or pathways that areidentified during our studies of human TB, in experimental in vivo models to help to identify the mechanisms determining the outcome to M. tuberculosis infection. There is a need to understand why in some cases the infection is controlled whilst in other cases active TB disease develops. The aim is to identify the early immune events which determine disease progression or control, and thus to identify new host-directed therapies to enhance the effects of antibiotics in human TB. Currently, a combination of antibiotics is required, many of which can cause severe side effects, and they have to be administered for many months, leading to people not taking the drugs and to drug resistance.
Hence other therapies to enhance the protective immune response are badly needed.

It is essential to study the immune response to M. tuberculosis in vivo in experimental mouse models,since there is evidence from new highly-sensitive radiography of TB patients and their contacts, that the immune response starts in the immune organs and only later is evident in the infected lung tissuewhere immune cells migrate in to kill the pathogen. Thus, testing of molecules, cells and pathways in mouse models of TB resistance or susceptibility in vivo is essential to define targets for immune intervention and for the development of new host-directed therapies.

The experimental model that most resembles the human infection resulting in a controlled

immune response or the development of active TB disease, that we can use in the UK and with which we can perform advanced immunological analyses, is the mouse model. Importantly, there are TB resistant and susceptible mouse strains, which when infected with M. tuberculosis, show similar lung pathologyand blood gene signatures of the immune response to human TB.

Intestinal immunity studies

Infection of the intestine via the oral route can be controlled or lead to intestinal inflammation, in somecases inflammatory bowel disease (IBD), and if there is dissemination it can cause systemic disease, such as peritonitis, which is a disease that can occur if microorganisms escape the gut and cause inflammation in the inner lining of the abdomen or other conditions. It is essential to study the intact mouse model since immune cells migrate into the tissue from immune organs during infectious challenge. In addition, we complement and replace some of the work in animals with epithelial organoids, which can be cultured with some of the immune cells represented in intestinal infections, which could be isolated from humans or the experimental model, for in-depth mechanistic studies reducing the number of mice used.

There is a high global burden of intestinal infection. The pathways of protection or pathogenesis, wherethe disease progresses or is controlled, are not clearly understood. Moreover, the burden of IBD (Crohn's Disease and Ulcerative Colitis) is rising globally, with substantial variation in levels and trendsof disease. Why some individuals develop IBD, whereas the majority of the world do not, is crucial for formulating effective strategies for preventing and treating IBD and other associated intestinal disorders resulting from infection. Understanding how individuals make an inflammatory and immune response to microbes in the diet or environment, pathobionts (microbes which invariably infect immunocompromised individuals) or pathogens which can infect and cause disease in otherwise healthy individuals, is crucial. This will enable the formulation of effective strategies for preventing andtreating intestinal infections and associated pathologies, such as IBD, to block inflammation and disease development and promote control of the pathogen and also understand how in some cases some infections can disseminate to the peritoneal cavity causing peritonitis.

Mice have a comparable physiology to humans and the inflammatory and immune response in the gut triggers a similar cascade of events as in humans. In particular, the human immune-system's reactionsto gut microbes and diet, and pathogens which infect otherwise healthy individuals or pathobionts which are microbes which invariably infect immunocompromised individuals, are highly recapitulated inmice. An understanding of the molecules and pathways of control and disease are an essential focus of our work, which will identify targets for therapeutic intervention.

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

The expected benefits of the work will be mechanistic understanding in the immediate future and therapeutic benefit in the longer period. This project could answer key questions about the immune response to infection or foreign substances that lead to control/resistance or development of disease.

They can be summarized as follows:

We expect the mouse models used in this project to generate new insights regarding the development of the immune response to infection in the lung with the bacterium M.

tuberculosis in models of resistance or susceptibility, which resemble active TB disease or asymptomatic controlledinfection.

The TB infection models will assist in defining infection response stages for early intervention duringthe development of TB disease. The models will also assist in the design of future anti-TB therapies, since we aim to use these models to test the effects of molecules on the immune response, that we identify in human TB. It is important to study lung microenvironment and the draining lymph nodes in the mouse since the lymph nodes have been shown in human TB to show an early immune response to the pathogen. The long-term aim would be to identify molecules/pathways tested in the experimentalmodels which can efficiently be targeted to control M. tuberculosis infection in humans and stop the progression to active TB disease, and to define molecules during early stages of infection which determine disease outcome and guide earlier therapeutic intervention than is currently possible.

We expect mouse models of intestinal and/or peritoneal infection used in this project to generate new insights regarding the development of the immune response in the gut to infection with different microorganisms, which would lead to control of infection, or lack of control of the infection, intestinal inflammation, or IBD, and additionally if there is dissemination which could result in systemic diseaseand/or peritonitis.

By studying the involvement of certain inflammatory components and systemic signals in models ofperitoneal and intestinal infection and IBD, we aim to clarify the mechanism of controlled responses inthe peritoneal cavity and in the intestine or those that contribute to disease and so help to identify targets as potential host-directed therapies to control disease.

Finally, this work will benefit the basic research community by increasing our knowledge of theimmune response in health and disease.

At the end of the studies supported by this licence, the outputs will include publications and seminars at national and international institutions, as well as presentations at national and internationalconferences. In some cases, the results of the work will be presented to the public at public engagement events.

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

These outputs will play a major role in informing other researchers nationally and internationally of key findings defining the molecules and pathways of the immune response that: (i) control disease or contribute to disease outcome in experimental models of TB resistance and susceptibility to resemble the spectrum of human TB; and (ii) experimental models of intestinal and peritoneal infection leading tointestinal damage and/or peritonitis due to infection, or in more extreme cases of intestinal diseases such as IBD. In the long term these findings may lead to the development of biological therapies to treat TB disease, where there is a great need due to a shortage of effective antibiotics particularly due to drug resistance, with around 1.6 million deaths per year world-wide. By defining molecules during early stages of infection with M. tuberculosis, which determine disease outcome, these findings may guide earlier therapeutic intervention than is currently possible due to difficulties in diagnosing TB. We may also define new targets for potential host-directed therapies during intestinal inflammation and/or peritonitis and in IBD.

The output of this work will initially be mechanistic information on the immune response in

infection (TB) and intestinal infection, including IBD. In the mid and long-term the benefits of defining potential host-directed therapies, will reach the pharmaceutical industry and clinicians, resulting in improvement of care for patients, to guide future potential treatments, by identifying new potential drug targets for either disease. The work will also provide valuable information as to the early immune events determining disease outcome to guide doctors in the clinic for the management of TB or intestinal infections/peritonitis and IBD with disease, that may have spread to other sites in the body and in somecases cause systemic disease.

Within the duration of this licence, we expect short-term benefits such as the publication of discoveriesin scientific journals and at conferences. We also expect that the identification of novel molecules, cellsand pathways of protection or disease progression, together with large databases of gene expression in TB, or in intestinal and/or peritoneal infection and resulting intestinal diseases such as IBD that are generated will be of use for the wider scientific community.

Where we make large datasets, defining the immune response in the different models, these will bemade accessible to researchers and industry.

As all the studies supported by this licence fall within the category of basic research, we expect thatthere mostly will be longer-term benefits. The knowledge gained will be used in further studies to investigate new pathways of resistance and susceptibility in either TB or intestinal or peritoneal infection and IBD.

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

We will look to maximise the outputs of any significant findings through interactions with the scientificcommunity through presenting at seminars and conferences. We collaborate with researchers in Portugal, Spain and other parts of the UK and share data with our collaborators at regular meetings and with our in-house colleagues at institute-wide seminars and lab meetings.

New findings resulting from mucosal infection of genetically altered (GA) animals and mouse models ofdisease will be made available to other researchers, multiplying the impact of our work. Most importantly all big data regarding gene expression underlying the immune response during the differentimmune responses obtained from these studies will be made available to other researchers and the public, using accessible tools and thus contributing to reduction, refinement and replacement of animals by minimising any unnecessary repetition in their future studies. For example, publication of large datasets arising from our studies, of gene expression changes in tissue or blood during early stages of mucosal infection with different doses of the infecting pathogen, will help refine our own continuing studies to limit discomfort to animals, and help others in refining their studies to understand the outcome to infection with reduced numbers and reduced suffering to animals.

Species and numbers of animals expected to be used

- Mice: 30,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures,

includingduration of the experiment and number of procedures.

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

Our area of research is the immune response to infection of the lung, modelling TB disease, and toinfection of the intestine/gut and peritoneal cavity. We also need a model organism to model humanoral infection, intestinal inflammation such as inflammatory bowel diseases (IBD) and if there is dissemination systemic disease and/or peritonitis.

The reasons why mice are the best choice as experimental models, for TB and inflammatory boweldiseases, can be summarized as follows:

the physiology of the immune response in TB, intestinal inflammation, IBD and peritonitis in mice isconsistent with the human diseases.

The genome of a mouse is easy to modify allowing the study of particular genes in the immuneresponse process. Mice can also be modified to be genetically prone to infection (genetically engineered mouse models) to mimic these diseases.

In mice, there are many models that are either commercially available or available from academic depositories, as well as well-defined techniques for production. There are also many scientific tools likeblocking antibodies etc that are largely available, minimizing the need for use and breeding of genetically altered mice. Adult mice will be used.

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

For TB, mice will typically be infected by aerosol with different strains of M. tuberculosis on the first day. They may be treated with drugs or antibodies by injection (weekly, or more regularly, as required)to test immune pathways and targets of resistance or susceptibility to the infection and progression of TB disease. To alter gut bacteria, diet may be modified or antibiotic treatments may be administered. We are interested as to how the changes by drugs, antibodies, diet, or any immune modulators (thesecould be antibodies against immune molecules/targets that we define and wish to test) will impact the host response and the outcome to infection with M. tuberculosis, and its response to therapy.

The animals will have their disease monitored over time using symptoms, for example weight loss, andpossibly non-invasive imaging techniques, since they will have internal disease. Extra care will be taken when infection is with highly virulent strains of M. tuberculosis which can result in more severe disease at an earlier stage, and/or if M. tuberculosis infection is accompanied by administration of immune modulators or is performed on genetically altered mice or strains of mice e.g. C3HeB/FeJ, which are TB-susceptible. The duration of the experiments will vary depending on the mouse model and M. tuberculosis strain, or if immune modulators are co-administered, and will consider many features of the disease specific to the experiment. We always aim to maintain duration of experiments to the minimum amount of time required and the minimum symptoms and extent of disease required toaddress the scientific need, particularly resembling human TB.

For diseases of the intestine or peritoneal cavity eg IBD or peritonitis, mice will be orally infectedby oral gavage with the infectious agent (some infections on the first and second days), or by intra- peritoneal infection. They may be treated with drugs or antibodies by injection (weekly, or more regularly, as required) to test immune pathways and targets of resistance or susceptibility to the infection and progression of disease

(inflammatory bowel disease/peritonitis/wasting disease). To altergut bacteria, diet may be modified or antibiotic treatments may be administered. We are interested as to how the changes by drugs, antibodies, diet, immune modulators, genetic alterations in the mice, willimpact the host response to infection of the gut or the peritoneal cavity, and its response to therapy.

The animals will have their disease state monitored over time using symptoms, for example weight loss, diarrhoea; and possibly non-invasive imaging techniques, since they will have internal disease. Extra care will be taken when infection is accompanied by administration of immune modulators (thesecould be antibodies against immune molecules/targets that we define and wish to test) or in genetic altered mice. We always aim to maintain duration of experiments to the minimum amount of time required and the minimum symptoms and extent of disease required to address the scientific need, particularly resembling human intestinal inflammation after infection, peritonitis and IBD. The duration of the experiments will vary depending on the model and will consider many features of the disease specific to the experiment, including the nature of the infection. We always aim to maintain the durationof experiments to the minimum required to address the scientific need.

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

In most cases animals used in this license will either develop symptoms of the lung disease TB; or inthe gut symptoms of intestinal inflammation such as inflammatory bowel disease; or in the peritoneal cavity symptoms of peritonitis.

Lung infection: The models of TB will vary with respect to possible adverse effects in the lung, and potentially weight loss, however the majority of mice (TB-resistant C57Bl/6) will develop only very mild disease, while others (up to 5%) (TB-susceptible C3HeB/FeJ) may develop severe disease, which is essential to be able to model human TB disease. The symptoms may be affected by the strain and dose of M. tuberculosis and/or by co-administration of immune modulators and/or if performed in genetically altered mice, and the duration of each experiment which can range from 14 days to a maximum of 100 days. Infected mice kept for the longer time periods (over 26 days) will be those infected with very low dose of a low virulence strain of M. tuberculosis, where the duration is needed to obtain developed lung pathology resembling the different stages and heterogeneity of human TB. In allcases mice are monitored daily and mice will be humanely killed if reaching or before reaching the humane endpoint.

Intestinal infections: The gut symptoms of IBD; or in the peritoneal cavity symptoms of peritonitis; willalso vary according to the dose and strain of the infectious agent (pathogen eg. Escherichia [E.] coli, Citrobacter [C.] rodentium, Enterococcus [E.] faecalis, or the pathobiont Helicobacter [H.] hepaticus).
Experiments will be conducted usually over a few days to 2 weeks, but in some cases may need to bekept for up to a month to resemble human disease. If immune modulators and/or genetic altered mice are used, this may accelerate or delay the time course of disease, but all mice are monitored daily andmice will be humanely killed before if reaching or before reaching the humane endpoint.

We have much knowledge and experience of all the models that we use, both for lung infection and forthe gut and peritoneal cavity infections (for the latter we also seek additional expert advice if needed from our collaborators and experts on experimental mouse models of IBD in new experiments). We aremostly able to predict well the time

frame over which the animals will not show adverse effects as we discuss in more detail above for either lung or the intestine or peritoneal cavity. The use of certain interventions with molecules of the immune response, or antibodies directed against them, can make this slightly less predictable, but the animals are monitored closely in this case to minimise any suffering and we aim to terminate experiments before the onset of potential adverse effects and humanely kill mice before they reach the humane endpoint. Undesirable affects could be limited to the site of the infection (for example, lung – pneumonia or TB; or gut – ulceration or IBD) or by general signs of poor health, such as weight loss, piloerection, hunching, increase breathing rate (especially when infection has spread), but in cases may become systemic resembling human disease. Again, mice developing such symptoms will be monitored carefully and humanely killed if reaching or before reaching the humane endpoint.

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 animaltype)?

The expected severities predicted by this proposal are mainly mild and moderate, with one severe protocol. Only up to 400 mice are expected to approach this severe level, in order to reflect the extreme of the lung pathology in human TB disease. Mice will be monitored carefully and humanelykilled if reaching or before reaching the humane endpoint.

For phenotyping we anticipate that mice will if anything only reach mild symptoms since although theycarry genetic alterations in genes involved in immune pathways, the mice are housed in a facility with pathogen-controlled measures.

We anticipate that only 10-20% of mice may reach moderate severity for the immunomodulation Protocol, the rest being mild, since although the mice may be administered immune modulators or blockers of the immune response, the mice are housed in a facility with pathogen-controlled measures.

We anticipate that mice infected with the bacterial strains, E. coli, C. rodentium and E. faecalis, will only show mild to no effects in wild type mice. However up to 50% of the mice may reach moderate severity upon infection with these pathogens, if they are either genetically altered mice with changes inan immune molecules, or if they are wild type mice administered with immune modulators or blockers of the immune pathways during infection.

Wild type mice infected with H. hepaticus show no symptoms, however, up to 50% of the mice may reach moderate severity upon infection with this pathogen, if they are either genetically altered mice with changes in immune molecules, or if they are wild type mice administered with immune modulatorsor blockers of the immune pathways during infection.

During infection with M. tuberculosis of wild type mice or genetically altered mice, or mice co- administered with immune modulators or blockers of immune pathways, it is expected that most will only reach mild to moderate severity, with only up to 200 400 mice approaching severe symptoms. Since this is a chronic infection, where although immuno-modulation may manifest adverse effects, careful monitoring of mice will allow clinical predictions to facilitate humanely killing of the mice beforethe humane endpoint is reached.

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 13 September 2028

The PPL holder will be required to disclose:

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

Replacement

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

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

The experimental model that most resembles a human controlled immune response to M. tuberculosis, or the development of active TB disease, that we can use in the UK, and with which we can perform advanced immunological analyses, is the mouse model, where there are TB resistant and susceptible mouse strains, which when infected with M. tuberculosis, show similar lung pathology and blood gene signatures of the immune response to human TB, supporting our need to work in in vivo TB mouse models. It is essential to study the immune response to M. tuberculosis in vivo in experimental adult mouse models, where the response is detectable in immune organs as well as infected tissue, since highly-sensitive radiography of TB patients and their contacts has shown that theimmune response starts in the immune organs and only later is evident in the infected lung tissue where immune cells migrate in to kill the pathogen. Thus, testing of molecules, cells and pathways in mouse models of TB resistance or susceptibility in vivo, and dissecting the early airway and immune organ response to M. tuberculosis is essential to define targets for immune intervention and for the development of new host-directed therapies.

We are using adult mice which are necessary for dissecting and testing mechanisms underpinning the immune response to M. tuberculosis and to infections of the gut or peritoneal cavity with other infections. Lower organisms and cell culture do not capture the complexity of the immune response that determines outcome of infection, specifically protection or progression to disease. Therefore, thesestudies must mainly be performed within the animal as the complexity of these changes and the number of players involved cannot be modelled in the laboratory. Experimental models of TB resistance and susceptibility have been defined which most closely resemble human disease, at the level of pathology, gene expression and the immune response which may help in identifying targets to therapies in human disease and also identifying immune mediators of disease outcome which could help in guidance for earlier therapeutic intervention.

Intestinal and peritoneal cavity infections: In the past we had used cellular assays of immune cells to define genes, molecules and pathways of the immune response that had potential to help to maintain stability or homeostasis in the gut and provide protection

against inflammation during system perturbations such as infection. However, these cellular assays are not able to model migration of cellsfrom other immune organs which are likely to play an important part in these mechanisms. It is therefore essential to study the intact mouse model since immune cells migrate into the intestinal tissue from immune organs during infectious challenge and can contribute to host damage or regulation of the response. Thus, to test cells, molecules and pathways that we have defined as potentially important in regulating or controlling damage to the host, it is essential to use the intact mouse models of gut and peritoneal infection and inflammation, to support the development of new host-directed therapies.

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

Lung infection and TB: In the past in research leading up to the current programme, we had used cellular assays of immune cells to define molecules and pathways of protection or disease progressionwith M. tuberculosis and other infections and control of the infection by immune cells. Our research of the past years has identified the cells, genes and pathways that potentially regulate the molecules of the immune response to protect against TB or result in progression to the disease using mouse modelsand analysis of human TB disease. It now is necessary to test these molecules, cells and pathways as targets for immunotherapy using experimental mouse models.

We have studied the literature and have gained experience from our own studies and found no non- animal alternatives which could address the questions of the immune response to M. tuberculosis in cell culture or organ culture. Although lung-on-a-chip models of M. tuberculosis infection have been published, these models will not capture the immune response to the pathogen which is initiated in theimmune organs with immune cells which once activated migrate back to the infected tissue to kill the pathogen. The experimental model that most resembles a human controlled immune response to M. tuberculosis, or the development of active TB disease, that we can use in the UK, and with which we can perform advanced immunological analyses, is the mouse model, where there are TB resistant andsusceptible mouse strains, which when infected with M. tuberculosis, show similar lung pathology and blood gene signatures of the immune response to human TB.

Intestinal infection: We will additionally use long-term expansion of epithelial organoids, which contain some of the immune cells represented in intestinal infections as well as epithelial cells of the gut. Cells used for organoids could be isolated from humans or the experimental mouse models and could help to replace as well as reduce the number of mice used. However, these can only be used forin-depth local mechanistic studies as they do not reflect the involvement of immune organs where immune cells are activated and then migrate to the mucosal/intestinal tissue.

In both cases, given the limitations of these in vitro approaches, it is essential to use a whole mousemodel of lung or gut disease to test these immune molecules or their antagonists since the immune cells once taken from animals and used in in vitro in culture cannot recapitulate the whole immune response in the body, which is complex, and cell culture is no longer useful.

Why were they not suitable?

In vitro cell culture cannot capture the complexities of the physiological immune response since afterinfection, for example of the lung by aerosol, cells in the lung become activated and then in turn activate immune cells that traffic to the lymph nodes become further

activated and multiply and then return to the lung to kill the infectious agent. It is not possible to model these complex multi-organ events in the in vitro assays. The same is true for infections of the gut through the oral route, or invasive infection such as through the blood or peritoneal cavity.

Even the most sophisticated lab-based model systems cannot convey the complexity of the microenvironment of the immune response to infection, or the impact that this infection can have tocause disease either locally or systemically on an animal. The complexity of the whole animal is required to investigate the impact of infiltrating immune cell types alongside both the local and systemic response of the infection.

Although lung-on-a-chip models of M. tuberculosis infection have been published, these models willnot capture the immune response to the pathogen which is initiated in the specialized lymphatic immune organs where immune cells become activated and migrate back to the infected tissue to killthe pathogen.

For study of intestinal infections, we will additionally use long-term expansion of epithelial organoids, which contain some of the immune cells represented in intestinal infections as well as epithelial cells ofthe gut. Cells used in these systems can be isolated from humans or the experimental mouse models, which could help to replace the number of mice used. However, these can only be used for in-depth local mechanistic studies. It is still essential to study the intact mouse model since immune cells migrate into the intestinal tissue from immune organs during infectious challenge and can contribute tohost damage or regulation of the response.

We have considered multiple options of non-animal approaches, however, given the limitations of invitro isolated organ/cell culture approaches, in both lung and intestinal/peritoneal infections, non- animal alternatives are not useful for our current and future studies.

A retrospective assessment of replacement will be due by 13 September 2028

The PPL holder will be required to disclose:

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

Reduction

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

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

The number of animals to be used has been estimated on the basis of the previous 20 years of work ofmy lab and the current landscape of projects of the lab going forward, staffing in my lab and the funding I have for the next 5 years.

We use statistics and past experiments to define the minimum number of mice required for

statistical significance to obtain robust reproducible data to inform the immune response during infection of micethat determines outcome.

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

We employ several strategies to try to limit the number of mice in the study:

We will always aim to maximise the amount of data (pathology information and gene expression) weget from each mouse, for example by obtaining multiple tissues or experimental read-outs from the same mouse.

Lung: We always maximise the data from the animals we use. For example, when taking lung tissue for a histological estimate of pathology, one lung lobe is taken for RNA/DNA genomics analysis or flowcytometry. Similarly, when performing bronchoalveolar lavage on euthanised mice (to compare to human TB bronchoalveolar lavage samples), we also obtain the paired lung tissue from the samemouse for parallel analysis.

Intestine: We always maximise the data from the animals we use by taking tissue from the gut of eachmouse for histological estimate of pathology, for RNA/DNA genomics analysis, and for flow cytometry or other analyses.

Also, we will limit the use of genetic models (that often require many generations breeding) by treating the mice with chemical agents or antibodies to either to block immune-system components or enhancethem.

We use(d) statistics and past experiments, including pilot experiments, to define the minimum numberof mice required for statistical significance to obtain robust reproducible data to inform the immune response during infection of mice that determines outcome. We can always rely on our in-house statistician for any additional advice whenever we need.

For most of the experiments, quantitation is required and we will use the minimum number of animals to provide an adequate description, generally on the basis of previous experience (our own or from the literature). Pilot experiments will use approximately 5 mice per group, which should be sufficient if a significant result is obtained. The experiment will be repeated to obtain further significance if: (a) there are only small differences; in this case it may have to be repeated with larger numbers of mice and/or modifications; (b) does not work; in this case it would be repeated in the presence of candidate molecules/modulators; (c) to obtain statistically reproducible results which are set using power analysis, generally using a significance level of 5%, a power of 80% and at least practicable difference between groups of 20%. Once a desired effect has been obtained it may be necessary to use a greaternumber of mice per group in order to facilitate obtaining rare immune cells involved in the response and to analyse their function (e.g. phenotype by flow cytometry, cytokine production and other immune parameters). For example, numbers of cells like dendritic cells, or small populations of T cells are limiting, so these experiments may need to use between 10-50 mice per group in order to be able to purify enough cells for subsequent genomics and transcriptomics analyses. Cryopreservation of gametes, embryos, tissues and cells is routine and will ensure that the minimum number of mice is bred.

Each experiment (including the pilot experiments) will include a small group of animals that are susceptible to infection with the respective pathogen or pathobiont and develop a well-defined course of disease in response to a given dose of pathogen. This group will serve

as an internal control for theexperiments involving infections, allowing comparison of results from different experiments (performedat different times, with different doses and/or batches of pathogens etc.), in wild type mice, mice of a different genetic background, mice genetically altered in a molecule under test, or mice administered an immune modulator. This group will also provide the quality assurance for the virulence of the innocula, which is necessary for comparison of results across experiments.

We will use the PREPARE guidelines: https://norecopa.no/PREPAREand the Experimental Design Assistant: https://eda.nc3rs.org.uk/.

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

Pilot studies have been performed for all experiments in the past and will be executed when neededfor future experiments, however, with care to still ensure robust and reproducible data is obtained.

The majority of experimental mouse numbers per group are decided based on years of experience, statistics and our publications (or those of others when investigating a new experiment regimen). Our aim is to use to the minimum number of mice per group to produce reproducible results within a group(eg. this can depend on the dose of infecting pathogen) which shows a statistical difference to control animals. In the past we have used power calculations when required and will do so again if needed.

We obtain all tissues possible from the same infected/challenged mouse, for procedures including immune assays, histology to inform the level of inflammation, and gene expression changes to inform the immune response determining outcome. This enables the maximum information to be gained fromthe minimum number of mice. Whenever possible, we will share animal tissue from experiments to enable multiple studies ex vivo. We are committed to improving education and training for those working under this project licence.

We also use gene expression data from our own published studies and studies from others to understand the patterns of the immune response, which will help inform the necessary experimental timepoints for the study, thus reducing mouse numbers by avoiding inclusion of unnecessary timepoints. These data are available to the research public and so help to reduce mouse numbers needed for experiments at large by providing an immense database and resource of the immune response in infection and diseases. In our publications we have made the data highly accessible via acomputer app that we constructed.

Regarding breeding of mice, we share mouse colonies with other researchers, which are obtained bycentralised breeding, minimising numbers used and avoiding duplication.

We breed many of the genetically altered animals ourselves in order to promptly adapt colony sizes torespond to the experimental need and reduce wastage from overbreeding.

We also take lead from HO Assessment Framework of Efficient Breeding of Genetically Altered Animals (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/773 553/GAA_Framework_Oct_18.pdf) and take decisions to archive lines by cryopreservation when not required over a period of time.

The NC3Rs have also recently published a guidance document about the sharing and archiving of GAmice which is of use: https://www.nc3rs.org.uk/3rs-resources/breeding-and-colony- management/sharing-and-archiving-ga-mice

Obtaining wild type mice from in house facility-shared breeding allows better efficiency for largercolonies.

A retrospective assessment of reduction will be due by 13 September 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan 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 welfarecosts (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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use wild type mice and genetically altered mice lacking components of the immune response tostudy the immune response to infection with: 1) M. tuberculosis, via aerosol) to model the spectrum of human TB (from asymptomatic, resistant to progression to TB) and; 2) the pathogen C. rodentium, andthe pathobionts H. hepaticus, E. coli and E. faecalis (via intraperitoneal or oral gavage) to model intestinal infection, peritonitis and human IBD.

Lung infection: We will use wild type mice and genetically altered mice lacking components of the immune response to study the early immune response in the airways to infection with the pathogen M. tuberculosis, via aerosol to model the spectrum of human TB (from asymptomatic, resistant to progression to active TB disease) using models which we have previously refined and published. Our studies of these early immune events in the airways (and sometimes blood) will mainly concern early time-points which we have carefully determined from the literature and our own previous studies wheremost mice will show only mild disease.

Only TB-susceptible mice at later time-points after infection may start to show symptoms which could lead up to severe disease. This is essential to present a model which most resembles human TB disease, at the level of lung pathology including granuloma and cavity formation, but will only occur in asmall percentage/number of mice. In these experiments, mice are monitored daily and mice will be humanely killed if reaching or before reaching the humane endpoint.

The models of TB will vary with respect to possible adverse effects in the lung, and potentially weight loss, however the majority of mice (TB-resistant C57BL/6J) will develop only very mild disease, while others (up to 5%) (TB-susceptible C3HeB/FeJ) may develop severe disease, which is essential to be able to model human TB disease. The symptoms

may be affected by the strain and dose of M. tuberculosis and/or by co-administration of immune modulators and/or if performed in genetically altered mice, and the duration of each experiment which can range from 14 days to a maximum of 100 days. We have a very clear knowledge from our past experiments and our publications, of the strain and dose of M. tuberculosis in the different genetic strains of TB resistant and susceptible mice which results in the different extents of disease. These generally range from mild to moderate, with only up to5% approaching severe symptoms. Infected mice will only be kept for the longer time periods (over 26 days) when infected with very low doses of a low virulence strain of M. tuberculosis, where the durationis needed to obtain lung pathology resembling the different stages and heterogeneity of human TB. Mice are monitored daily and mice will be humanely killed before reaching the humane endpoint to minimise suffering.

Intestinal infections: The gut symptoms of IBD; or in the peritoneal cavity, symptoms of peritonitis, will also vary according to the dose and strain of the infectious agent (pathogen e.g. C. rodentium, or pathobiont e.g. H. hepaticus, E. coli, E. faecalis). Experiments will be conducted usually over a few days to 2 weeks, but in some cases may need to be kept for up to a month to resemble human diseaseif the invading pathogen or pathobiont only leads to mild disease as can be the case with H. hepaticus.Mice are monitored daily, and mice will be humanely killed if reaching or before reaching the humane endpoint. If immune modulators and/or genetic altered mice are used, this may accelerate or delay the time course of disease, but mice are monitored daily and mice will be humanely killed if reaching or before reaching the humane endpoint, to minimise suffering. We monitor the mice carefully within eachexperiment to ensure reduction of the suffering experienced by the animals.

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

Other animal models have been published studying the immune response to mycobacterial infection ofthe lung, or intestinal infection, but these lack the organ and physiological complexity needed for translation to humans. This complexity needs to be recapitulated in vivo in adult mice to make the investigation of infection to the microenvironment of the lung, peritoneal cavity and intestine possible and to resemble the major burden of human TB disease and intestinal/peritoneal disease, which is in adults. The requirement here is to have a physiology which is as close as possible to adult humans, and only mammalian organisms such as mice have the same complex immune system as humans, including at the immune organ level e.g. lymph nodes and immune cell activation and migration to tissue, which is only fully developed in adult life.

Modelling human TB disease: It is necessary to test molecules, cells and pathways that we have discovered from research on human TB and experimental mouse models as targets for immunotherapyor to define early immune determinants of disease outcome, using experimental mouse models where outcome can be determined and linked to the immune response. Therefore, terminally anaesthetised mice will not capture the immune events related to outcome. Models of TB have been published with infection of zebrafish with Mycobacterium marinum, but these models again do not exhibit a physiologywhich is close to that observed in humans. This model does not capture the innate and adaptive immune responses in the immune organs which resemble human TB disease and model the mechanisms behind why some individuals infected with M. tuberculosis will control the infection while others will progress to active TB disease. Mouse models of TB resistance and susceptibility have been published however, which do recapitulate the spectrum of TB in humans and thus offer the most accurate model of human TB disease.

Intestinal and peritoneal cavity infections: In the past we had used cellular assays of immune cells isolated from animals that have been humanely killed, to define genes, molecules and pathways of the immune response. This allowed us to discover immune molecules and cells that could potentially offer protection against infections, which but on the other hand could potentially cause damage to an individual, or regulate the immune response to limit host damage. However, these molecules and pathways need to be tested in intact adult experimental mouse models of intestinal infection to define new targets for the design of host-directed therapies to block inflammatory diseases of the intestine and the peritoneal cavity. Infection of the intestine via the oral route can be controlled or can lead to intestinal inflammation, and in some cases IBD. Where there is dissemination, systemic disease such as peritonitis may occur, which is a disease that can occur if microorganisms escape the gut and causeinflammation of the inner lining of the abdomen, or other conditions. In both of these instances, immune cells migrate into the intestinal tissue or peritoneal cavity from immune organs during infectious challenge and can contribute to host damage or regulation of the response. We will additionally use long-term expansion of epithelial organoids, which could help to reduce the number ofmice used, since these organoids are isolated from either animals which have terminally anaethetised or from human biopsies. These organoids can be cultured with some of the immune cells representedin intestinal infections and can only be used for in-depth local mechanistic studies. These organoid systems are not able to model dissemination or cell migration. Hence, it is still essential to study the intact adult mouse model since immune cells migrate into the intestinal tissue from immune organs during infectious challenge and can contribute to host damage or regulation of the response. Thus, to test cells, molecules and pathways that we have defined as potentially important in regulating or controlling damage to the host, it is essential to use the intact adult mouse models of gut and peritoneal infection and inflammation, to support the development of new host-directed therapies.

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

We have access to cutting edge techniques and experts within various fields of medical research. We actively share refinement and improvements in techniques and seek to constantly improve our modelsto ensure that we are minimising any harms to the animals, as this also helps to improve the accuracyof our study and reduce artefacts caused by stress. For example; we are vigilant to ensure careful monitoring of experimental mice within each experiment and use our previous experience and experiments to guide us, as well as advice from other experts when needed.

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

Unless otherwise specified, the work in this project will be designed using the principle outlined in thePREPARE guidelines for planning animal research and testing (2017) and from the following links:

https://view.pagetiger.com/RSPCAAvoidingMortalityResearchReport/RSPCA
https://www.dropbox.com/s/wls05epsbykinhh/administration_substances.pdf

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

We regularly receive updates on advances in the 3Rs from within our establishment from NC3Rs. Where we are placed to refine techniques without impacting the scientific validity

of our work we aim toimplement advances. The NC3R Regional Project Manager keeps our institute informed on key advances and knowledge. As experts in the field we are up-to-date with all the latest publications and are up to date with the latest research from conferences and collaborations, thus we are fully aware of any refinements published, that could help us in refining our experimental mouse models further. The fact that we ourselves are researching the cellular changes and immune signatures during the early airway response in TB patients and their contacts – most of whom control the infection whilst 10% progress to active TB disease, allows us to model closely the experimental mouse models and only test genes and molecules that show a potential role in human TB disease or control. We use the literature of human IBD and other intestinal infections and inflammation to again identify potential genes and pathways of protection or disease, and always refine our experimental mouse models totest potential targets relevant to human disease.

A retrospective assessment of refinement will be due by 13 September 2028

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 forfuture work of this kind?
- During the project, how did you minimise harm to the animals?

14. Identification and characterisation of therapeutic targets for cardiac disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Heart, Heart Failure, Cardiovascular, Therapy, Cardiac remodelling

Animal types	Life stages
Mice	adult, neonate, embryo, juvenile, pregnant
Rats	adult, neonate, embryo, juvenile, pregnant
Rabbits	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 orscientific needs it's addressing.

What's the aim of this project?

The project aims to define the functions of selected components of mechanisms/processes involved incardiac remodelling across a broad range of animal models of heart disease.

A retrospective assessment of these aims will be due by 16 September 2028

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 disease causes 160,000 deaths each year in the UK which equates to 435 people perday. There are around 7 million people living with cardiovascular disease in the UK with a healthcare cost of ~£9 billion and a cost to the UK economy of £19 billion a year.

A common end point of many cardiovascular diseases is heart failure (HF); a complex clinical syndrome caused by structural and/or functional cardiac abnormality. Over 500,000 people in the UK have been diagnosed with HF and despite optimised medical therapy mortality rates remain unacceptably high (5-year mortality rates are ~50%). HF has a major adverse effect on quality of life commonly leading to fluid retention in the lungs causing undue breathlessness even at rest, swelling ofthe legs/ankles and depression.

There are multiple cardiac diseases that lead to HF including; heart attacks (myocardial infarction; MI), high blood pressure (hypertension) and structural changes to the heart (cardiomyopathy). These diseases result in cardiac remodelling; a complex array of molecular and cellular changes within the heart that clinically manifest as altered heart architecture and contractile function. Initially, patients withcardiac remodelling may demonstrate compensated contractile function. However, a significant proportion of these patients will undergo progressive deterioration of cardiac remodelling leading to HF.

Novel therapeutic strategies to preserve heart function and limit cardiac remodelling are therefore urgently required to treat patients with cardiac disease and limit progression to HF thereby improvingsurvival rates and quality of life. Identification of new therapeutic targets can be achieved by using animal models to enhance our understanding of the mechanisms/processes involved in adverse cardiac remodelling and HF.

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

Through increasing our understanding of the science underlying the functional and structural changesto the heart that occur after a range of cardiac diseases we can inform the development of therapeuticstrategies to prevent heart failure. Such treatments would reduce the socioeconomic burden of heart failure in the UK and improve the quality of life of patients living with heart disease.

Short Term Outputs:

The primary expected benefit is the generation of **new knowledge, defining and characterising themechanisms/processes important for cardiac remodelling** in a broad range of cardiac diseases. This work will lead to publications in scientific journals and presentation of research findings at local, national and international conferences.

Medium Term Outputs:

Components of identified mechanisms/processes (which can be at any level e.g. molecular, organelle, cellular, extracellular, organ etc.) will then be manipulated to **generate new data on the importance of selected components for cardiac function in the whole animal**. Importantly, this new data will inform preclinical studies aimed at determining the therapeutic potential of using these components as targets to limit progression of HF in patients with cardiac disease, for which there remains no cure. The

data generated will be used to support future grant applications.

Long Term Outputs:

In the longer term, the potential benefits of this study are that data generated may have far reaching implications for treatment of a range of cardiac diseases benefitting patients and clinicians by contributing to the **development of effective therapy** which will ultimately reduce the economic andhealth burden of HF. Knowledge gained on cardiac remodelling may also **contribute to clinical guidelines** for the treatment of patients with HF.

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

The expected benefits arising from this project are multi-fold:

Short Term Benefits: The main beneficiaries will be scientists in the field through are advancementsin knowledge and understanding of heart failure.

Medium to Longer Term Benefits: Again, scientists in the field will be the main beneficiaries but this aspect of the project will also be beneficial to pharmaceutical companies looking to prioritise targets fordrug development; a huge unmet clinical need. Securing industrial interest promotes two-way flow of knowledge and guidance to help expedite and focus not only the mechanistic research itself but also the future potential for translational development. The ultimate aim is for any positive findings to lead tochanges in clinical practice and in turn enhance the quality of life of patients with cardiovascular disease.

An additional benefit over the duration of the programme of work will be staff and students working on this programme becoming independent scientists trained in the implementation of 3Rs. This new cadreof skills trained people strengthen the research communities required to meet future scientific challenges.

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

All of the approaches we intend to use have been described in the literature and we have built up considerable expertise in assessing cardiac function (phenotyping) and complex microsurgical procedures over the last 10 years. We have also published in high profile publications the use of studies using gene therapy to treat hearts with MI and genetically modified animals. I have establishedcollaborations to learn their methodology for induction of heart disease due to increased blood pressure. Moreover, my close collaboration with international colleagues who are actively developing new small molecule compounds means we can be the first to test these compounds in our rodent models, addressing our key objectives and achieving many of the above benefits quicker (e.g., development of a new gene therapy and drugs for cardiac disease).

The data will be presented at national and international conferences, public engagement outreachactivities, academic journals and press releases by those involved in the programme of work. We publish our studies in peer reviewed scientific journals and will also aim to use journals that permitnegative studies where appropriate.

Species and numbers of animals expected to be used

- Mice: 27500
- Rats: 15000

- Rabbits: 1800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration 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 will be used because of our ability to mimic clinically-relevant cardiovascular disease in these species. Furthermore, they provide an effective way of enabling genetic modification thereby enhancing our understanding of the role of genes and development of cardiac disease. Adult rabbits will be used for some experiments as they provide an intermediate sized heart to mimic humandisease but also because the heart muscle cell from this species more closely resembles some aspects of human heart muscle cell function compared to rodents. We will also utilise heart tissue from rodent neonates where we require to culture cardiac cells for prolonged periods of time; a difficult technique achieved with adult cardiac cells. The most appropriate models will be chosen based on ourongoing studies or from the published scientific literature.

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

During this project we will typically induce cardiac disease in animals purchased in from a recognisedbreeder or animals bred in-house that are genetically manipulated. These models of cardiac disease can be induced surgically or non-surgically. In separate experiments, we take mice without cardiac disease and assess cardiac function and can test interventions.

We will then assess the characteristics of the cardiovascular system in our models at baseline and post-induction of disease. This typically includes measurement of the electrical activity of the heart (ECG), cardiac contractile function, blood pressure, cannulation of blood vessels for monitoring, blood sampling, administration of substances and microsampling. Some animals will undergo acute/chronic administration of substances/components to modulate physiological function, induce cardiac remodelling and/or required for imaging (all with appropriate controls). These substances/components include potential therapeutics. Additionally, some animals will undergo delivery of components that canalter gene function.

Upon terminal anaesthesia some animals will undergo more in-depth assessment of the cardiovascularsystem. Animals will then be humanely killed and tissue taken for examination. Where minimally compromised tissue is required this will be done under anaesthesia.

The duration of model can be as little as one day or as long as 52 weeks. A typical animal would undergo one surgical/nonsurgical procedure to induce cardiac dysfunction, cardiac imaging (echocardiography), blood pressure measurements at weekly intervals, and more in-depth assessmentof the cardiovascular system under terminal anaesthesia and be on procedure for between 1 day and 8weeks.

The duration that the animals will be exposed to cardiac disease will be the minimum required to obtainsufficient data about the acute and chronic changes in cardiac structure and function. At the end of the procedures the animals will be killed humanely.

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

The expected impact/adverse effects for the animals on the project are dependent on the model of cardiovascular disease. Any animal that develops clinical signs of heart failure arising from cardiovascular disease will be humanely killed with an option to assess cardiac function under terminalanaesthesia beforehand, with discussion if necessary, with the NVS and named animal care and welfare officer (NACWO).

Surgical induced cardiac disease: e.g. myocardial infarction and transverse aortic constriction.

Deaths from animals undergoing complex microsurgery predominately occur during the operationunder anaesthesia and therefore pose limited cost to welfare. From this point, we have a post- operative mortality rate of up to approximately 25% on a rolling (typically quartely) basis.

Non-surgical induced cardiac disease: e.g. myocarditis

Although strain specific the mortality is approximately 10%. Infected animals (e.g. with Coxsackievirus B3) are closely monitored during the acute phase of the infection, looking for signs indicative of heart failure. An intestinal inflammatory reaction could develop with this model and in certain mouse strains. Mice that show this response (e.g. weight loss, hunching) rather than cardiac specific, will be assisted with placement of gelatinous food or treats inside the cage and will also be monitored closely. The non-cardiac signs are expected to be transient and improve over time but if any animal develops “severe” signs such as marked and persistent (>48 hours) it will be humanely killed.

Non-surgical induced cardiac disease: e.g. heart failure with preserved ejection fraction (HFpEF).

Most of the manipulations to induce HFpEF by themselves are not expected to produce significant adverse effects. However, some manipulations that induce high blood pressure can sometimes lead tofatalities, usually associated with dramatic cardiovascular events such as ruptured blood vessels (aneurysms), abnormal heart electrical activity (arrhythmias), sudden cardiac failure or massive stroke.Animals used in studies involving such manipulations will be monitored at regular and frequent intervals and any whose welfare gives rise to concern at any point will receive prompt veterinary attention or will be humanely killed. Except in the case of small group sizes (e.g. <10 animals) where e.g. 1 death out of 6 animals may represent a >15% mortality rate, we do not typically expect overallmortality on a rolling basis (typically measured quaterly) to increase above 15%.

The mouse models detailed do not show any changes to their behaviour or health as a result of anychange to their genes.

Pain as a result of surgical procedures: all animals undergoing surgery may experience some pain. Pain relief (analgesics) will be given in consultation with the named veterinary surgeon (NVS) and foras long as necessary.

Models on procedure and phenotyping:

Mortality risks due to anaesthetic for imaging/ECG - risks are minimal and are the same for anaesthesia (<1%). The NVS will be consulted should this increase above 10%. In all cases, the number of anaesthetic/imaging sessions will be kept to the minimum possible and will never exceed 32sessions. The imaging sessions represent a non-invasive way to monitor progression of disease and inform determination of humane endpoints. Gaps between sessions will be as long as possible and typically no less than 24 hours.

More in-depth cardiac phenotyping (e.g. pressure-volume catheter measurements) - transient discomfort from induction of anaesthesia. Intra-operative mortality rates due to these measurementsduring terminal anaesthesia are <15%. Pressure drops will be monitored in order to mitigate animal deaths intra-operatively. Administration of substances are not expected to have adverse effects but ifthese occur they will be discussed with NVS.

Blood loss is minimal during cannulation - risk of major blood loss is rare, but if it occurs, the animalwill be killed by a Schedule 1 method.

Injections & blood sampling - Intravenous and subcutaneous injections carry the risk of blood clots, bruising and blood loss. These adverse effects will be minimised by good technique. Blood sampling will not exceed 15% of total blood volume in any 28 day period and hence should not result in anaemiaor (reduced blood volume) hypovolaemia.

For implantation of substance delivery devices (e.g. mini-pumps) or devices enabling continuous phenotyping (e.g. telemetry devices) - Post-operative pain will be controlled by analgesic drugs givenon the advice of the NVS.

The substances which are administered to modulate physiological function/cardiac remodelling. Most of the substances which are administered to modulate physiological function are not expected to produce significant adverse effects. However, some substances do cause adverse effects. Although they are used according to published literature, they can sometimes lead to fatalities usually associated with dramatic cardiovascular events such as ruptured blood vessels (aneurysms), abnormalelectrical activity (arrhythmias), sudden cardiac failure or massive stroke. Animals used in studies involving such drugs will be monitored at regular and frequent intervals and any whose welfare gives rise to concern at any point will receive prompt veterinary attention or will be humanely killed. When some substances are applied to animals models in which there is already organ/tissue pathology a mortality rate of between 20-25% has been reported. Except in the case of small group sizes (e.g. <10 animals) where e.g. 2 deaths out of 8 animals may represent a 25% mortality rate, we do not typically expect overall mortality on a rolling basis (typically quaterly) to increase above 15%.

Any animal that develops clinical signs of heart failure will be humanely killed with an option to assess cardiac function under terminal anaesthesia beforehand, with discussion if necessary, with theNVS and named animal care and welfare officer (NACWO). For slightly longer periods of weight loss
e.g. >20% of pre-operative body weight over a 3-4 day period, advice will be sought from the NVS andacted on promptly or animal humanely killed.

Any animals kept singly-housed will be handled with due care and empathy and will be provided withsuitable enrichment.

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 animaltype)?

All animals experiencing surgical and non-surgical procedures to induce cardiac disease are denotedas severe (~100% severe) (e.g. myocardial infarction, transverse aortic constriction, myocarditis and heart failure with preserved ejection fraction (HFpEF)). All animals undergoing these procedures are given analgesia.

All breeding and maintenance of genetically modified animals is mild (~100% mild) as is obtainingminimally compromised organ/tissue/blood.

The cumulative effect of those animals not undergoing induction of cardiac disease but who will experience repeated monitoring measures, ECG, echocardiography, drug, gene manipulation, is notexpected to exceed moderate severity (~100% moderate) as the least harmful route and lowest volume of administration will be chosen for delivery of substances, the nature of the monitoring methods are broadly non-invasive and animals will be acclimatised or familiarised with these within thestudy design.

In our previous experience of similar studies involving multiple assessment/intervention steps in combination with genetic modifications, we have identified no lasting harm as a result of cumulativeadverse effects. Animals on these studies maintain weight, show normal behaviour.

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

- Killed

A retrospective assessment of these predicted harms will be due by 16 September 2028

The PPL holder will be required to disclose:

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

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered 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 difficult to obtain viable human heart muscle including suitable non-disease human heart muscle.There is considerable variation in age, medication and underlying pathology of any obtainable humantissue and there is the likelihood of progressive disease being present. It is also not possible to investigate the processes at well-defined time points after a single incidence of damage. Substantial prior and continuing organ/tissue/cell experiments will inform and limit the number of animal experiments required and where possible as much information from one animal will be obtained.

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

Access to human tissue to study cardiovascular diseases is limited due to various factors including: (a) the inability of heart muscle cells to replicate to any great extent which limits acquirement of human heart tissue for experimentation and (b) the plethora of drugs which patients with cardiac disease are on which can confound data. Both (a) and (b) limit access to appropriate control cardiac tissue from healthy patients. These factors lead to the crucial need for the development and characterisation of animal models of cardiac disease which mimic human heart disease for which no alternatives exist. Weare aware of the limitations of alternative preparations in cardiac research but where possible we will utilise data collected from organs and tissue experiments (including stem cells, cardiac slices) to replace the requirement for animals. However, when positive data are generated in cell systems, the next step is to progress to animal models, before final translation to clinical trials in humans.

Why were they not suitable?

For some of the early studies, the cell culture models are suitable. But there reaches a point where there are limitations associated with interpretation of results in these models; cells are of immature status and therefore results may not be predictive of the cellular response in the adult heart. The immature status of stem cells and use of culture for cardiac slices precludes these preparations beingof sufficient similarity to adult cardiac tissue to inform accurately their translational potential relative tolive animal models. Furthermore, the complexity of the 3D beating heart and its inter-relationship withthe circulation and other organs cannot be replicated yet in sufficient detail using alternative models.

A retrospective assessment of replacement will be due by 16 September 2028

The PPL holder will be required to disclose:

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

Reduction

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

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

These numbers are for the full 5 years duration of the project licence and are based on ongoing projects within the group and with collaborators. We have also been awarded a 5-year programme grant and additional project grants and so the animal numbers also incorporate the experiments outlined in these proposals. Pilot studies performed on previous licence provide information on the lowest group sizes required for each experiment and we have a good understanding of the degree of variation in our models from extensive studies and the literature. The estimated numbers are based onthese group sizes for the appropriate comparison of intervention and control groups.

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

We have considerable expertise in minimising the number of animals required whilst ensuring generation of robust data as evidence by publication track record. We will use statistician advice whererequired. Sample sizes will be set from our knowledge of the literature, pilot experiments, previously performed experiments. For the majority of measurements this leads to a typical group size of 8-15 animals. We will use serial measures where possible to gain as much information as possible without the need to increase animal numbers. Where appropriate, we will randomly assign animals to experimental groups and blind studies and utilise PREPARE and NC3Rs' ARRIVE guidelines to guide best practice. In terms of genetically altered mice, where suitable lines already exist, animals will beobtained from the relevant supplier. Otherwise, we will make/obtain the required lines with help fromBiological Services and collaborators. We will measure production and breeding performance and ensure the minimum numbers of animals are used in the programme. We will aim to minimise shamsurgeries as neccesary but cannot remove their need due to the requirement of control data in publications.

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

Pilot studies will be used to optimise dose and efficiency where appropriate. We will share tissue withinour group across experiments and with collaborators and other groups within our institution where it does not compromise the experiment. Efficient breeding will be employed where possible including efficient breeding strategies, replacing breeders before reproductive performance declines, and replacing non-productive breeders as soon as possible.

The flexibility of being able to use mouse/rat/rabbits is aimed at reducing and refining the number of animals used rather than increasing them. Experiments will not be repeated in both species where unnecessary. The decision as to what species is to be used for a particular set of experiments will depend upon a clear decision at that time as to whether the use of the species tissue with the particular technique maximises the ability to detect a difference between experimental groups for eachmeasure and hence decreases the numbers of animals used.

A retrospective assessment of reduction will be due by 16 September 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan 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 welfarecosts (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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse, rat and rabbit animal models will be used as determined by the scientific question and the relevance of the species for human translation. The models used within the licence represent key cardiovascular diseases of humans and have been optimised to cause the least pain, suffering and distress to the animals. We have developed clear humane end points in order to limit potential sufferingand these will be implemented as necessary. Our protocols have been chosen based on the literature to provide maximum detailed information necessary to understand the process of adverse cardiac remodelling whilst at the same time ensure that the animals under investigation experience the least pain, suffering, distress or lasting harm. Phenotyping of our models (and respective controls) and administration of drugs/interventions are performed by the least severe/painful method available e.g.often administering via food/drink, use of non-invasive assessment of cardiac function e.g. echocardiography or under terminal anaesthesia.

Myocardial Infarction: This model typically involves surgically-induced permanent or temporary ligation of the coronary artery to cause myocardial heart damage. Animal can recover within hours to afew days due to compensatory heart function. This type of injury falls within the severe band. Appropriate analgesia is given pre- and post-operation to reduce pain.

Transverse Aortic Constriction: This model typically involves surgically-induced ligation of the aorta to induce and increase in cardiac muscle size. Animal can recover within hours to a few days dueto compensatory heart function. This type of injury falls within the severe band. Appropriate analgesia is given pre- and post-operation to reduce pain.

Myocarditis: This model typically involves injection of a pathogen to induce inflammation of the heart. This type of injury falls within the severe band. We have developed clear humane end points inorder to limit potential suffering and these will be implemented as necessary.

Heart Failure with Preserved Ejection Fraction: This model typically involves administration of substances to induce high blood pressure (hypertension)/metabolic challenge with or without a diet that leads to increased weight gain. This type of injury falls within the severe band. We have developedclear humane end points in order to limit potential suffering and these will be implemented as necessary.

We will utilise ARRIVE guidelines to report data and use blinding and randomisation where appropriateto reduce unconscious bias.

The ultimate benefits arising from the project licence will be to improve diagnosis and treatment ofcardiac disease.

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

There are no alternatives to using an animal model to examine the consequences of MI and other cardiac diseases on cardiac function at the level of the whole heart and single cell. Many international groups have established rat, mouse and rabbit models of cardiac disease and these represent the lowest mammalian vertebrate group in which you can fully characterise human relevant adverse cardiac remodelling. We will continue to utilise our current laboratory data to inform us whether severeprocedures are required or can be obtained using protocols of lower severity.

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

We will utilise in all models post-operative monitoring and care (including e.g. analgesia where appropriate, maintenance of body temperature, softened diet to encourage eating etc) to minimise anysuffering. Animals will be regularly checked to determine if any deterioration in their condition or the adverse effects of therapy. Should this occur we have defined limits and end points that determine ouraction which can include advice from the veterinary surgeon and humane killing.

Animals are regularly monitored and pain can be assessed by using scoring sheets and will inform theabove post-operative care provided to the animals. Acclimatisation and regular handling of animals reduces stress as does training in certain functional assessment procedures e.g. exercise testing and blood pressure measurement.

The decision as to what species to use for a particular set of experiments will depend upon: (a) obtaining the maximum signal to noise ratio, e.g. some antibodies work best in a particular species, (b)physiological mechanisms under investigation e.g. calcium handling function in rabbit tissue is more similar to humans and (c) use of transgenic breeding. The appropriate anaesthesia and pain relief measurements will be undertaken in all animal experiments as will aseptic techniques. We will constantly review the literature and N3CRs website for ways to refine the severe disease models and other routine techniques (e.g. serial blood microsampling).

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

Percie du Sert et al - The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research(2020).

PLOS Biology https://doi.org/10.1371/journal.pbio.3000410Local guidelines and NC3Rs website.
Zacchigna S, Paldino A, Falcão-Pires I, Daskalopoulos EP, Dal Ferro M, Vodret S, Lesizza P, Cannatà A, Miranda-Silva D, Lourenço AP, Pinamonti B, Sinagra G, Weinberger F, Eschenhagen T, Carrier L, Kehat I, Tocchetti CG, Russo M, Ghigo A, Cimino J, Hirsch E, Dawson D, Ciccarelli M, Oliveti M, LinkeWA, Cuijpers I, Heymans S, Hamdani N, de Boer M, Duncker DJ, Kuster D, van der Velden J, Beauloye C, Bertrand L, Mayr M, Giacca M, Leuschner F, Backs J, Thum T. Towards standardization ofechocardiography for the evaluation of left ventricular function in adult rodents: a position paper of the ESC Working Group on Myocardial Function. Cardiovasc Res. 2021 Jan 1;117(1):43-59. doi: 10.1093/cvr/cvaa110. PMID: 32365197.

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

We will continue to utilise the scientific literature for the latest refinement to our models and phenotyping to ensure the lowest possible level of suffering. Scientific conferences together with locallyrun seminars/workshops/training will be used. The NC3R webpage also will provide a forum for best practice and expertise in functional assessment.

A retrospective assessment of refinement will be due by 16 September 2028

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 forfuture work of this kind? During the project, how did you minimise harm to the animals?

15. Identification of New Therapeutic Targets and Investigation of Biological and Synthetic Therapies for Ischaemic 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, ortheir effects, in man, animals or plants

Key words

Cardiovascular Disease, Ischaemic Heart Disease, Lower Extremities Damage, Diabetes Mellitus andHypercholesterolemia, Therapeutic Angiogenesis

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 orscientific needs it's addressing.

What's the aim of this project?

This project is designed to better understand the causes of ischaemic disease, a reduction in blood flow caused by the occlusion of major arteries by “atherosclerotic plaques” (e.g., the build-up of fats, cholesterol and other substances) and the death or misfunction of small blood vessels (also called “microvessels”): capillaries and arterioles. These conditions are particularly frequent in people affectedby diabetes mellitus and/or high cholesterol level in their blood (a condition which is called “hypercholesterolemia”). The

ultimate goal of this research is to develop new therapies that are able tomeet the clinical needs of the patients who suffer from ischaemia in their heart or legs.

A retrospective assessment of these aims will be due by 21 September 2028

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 advancedor 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?

Arteries thickening or hardening by fat in the blood (this is called “atherosclerosis”) results in insufficient blood supply to tissue and organs. Current treatment for poor blood supply to the heart andlegs often consists of surgical interventions used to remove fat and material obstructing the vessel.

However, there are limitations because these treatments are not always possible and can often leave the patient with side effects and disabilities requiring additional interventions. Moreover, oxygen and blood deprivation to the heart and limb is not exclusively caused by an obstruction of one or more arteries. In fact, small vessel disease (called “INOCA”) can cause a type of oxygen and blood starvation in the heart that cannot be treated by surgeons or interventional cardiologist, and it can even cause a heart attack (this type of heart attack is called “MINOCA”). It is widely accepted that no other optimal cure is currently available for small vessel disease in the heart. In the lower leg, particularly in diabetic patients, small vessel disease causes ulcers which are difficult to heal and prone to infections and gangrene, often leading to the need for foot or leg amputation. These ulcers represent a very severe treat to the patients’ capacity to walk and overall wellbeing. Moreover, both the presence of leg ulcers and the foot or leg amputation are associated with increased mortality. It is estimated that almosthalf of the patients will die in the 5 years following a first-time leg, and the risk of death highly increases in patients with diabetes.

Treating insufficient blood supply in diabetic patients represents a big and unresolved challenge. Diabetes Mellitus represents one of the major threats to human health in the 21st century1, 2. DiabetesUK has recently reported that there are 3.9 million people living with diabetes in the UK. Around 700 people a day are diagnosed with diabetes. That's the equivalent of one person every two minutes.
According to the International Diabetes Federation, the number of patients with DM will further increase by 35% in the next 30 years. Cardiovascular disease is the leading cause of death in the diabetic population, where the prevalence of heart disease is responsible for two-thirds of deaths. The Framingham Heart Study, developed in the USA in the late ‘70s, was the first to show the increase of cardiovascular disease across all age groups for individuals with diabetes compared with those withoutdiabetes. Diabetes promotes oxygen and blood deprivation to the heart muscle and limb by worsening arteries thickening and promoting small vessel disease. Moreover, diabetes disrupts the mechanism normally taking place after sudden blood deprivation to a tissue (heart, limb) and which consist of the formation of new small vessels in the attempt to restore blood flow.

Unfortunately, currently available anti-diabetic drugs are not able to avoid cardiovascular complicationsin diabetic patients. It is also worrying that diabetic patients have more adverse events and complications after the surgical interventions used to unblock the arteries to re-establish the circulationin the heart or legs.

Our studies will increase the option to treat blood and oxygen deprivation in the heart and lowerextremities by enhancing the formation of new vessels around the affected area. We will also investigate therapies to cure poor blood/oxygen supply in patients with diabetes and high bloodcholesterol level.

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

This project licence will contribute to global efforts aiming to provide a definitive treatment of ischaemicdisease in the heart and limb muscles. It will identify new therapeutic targets and test therapeutic approaches.

Our work will allow to:

Improve the understanding of the process that causes tissue ischaemia (i.e., insufficient blood perfusion). This will allow identification of new “therapeutic targets” (i.e., disease-causing molecules,whose activity can be modified by a therapeutic intervention).

Identify new therapeutic strategies to protect the blood vessels and regenerate them once theyare lost to ischaemic disease.

The expected outputs include: 1) publications of scientific articles in highly respected journals; 2) presentations at national and international conferences and workshops); 3) Knowledge gained from thetherapeutic experiments on animals described in this project have the potential to lay foundations for the treatment of diabetic patients suffering from blood vessel disease in the heart and legs; 4) Use of new methods and techniques to promote the 3Rs principle (replacement, refinement, reduction) in various areas of this project. For example, where possible we will test potential therapeutic strategies using cultured cell lines in the laboratory instead of whole animals (replacement), we will employ a technique recently described to experimentally induce heart attacks which unlike traditional methods does not require traumatic tissue incisions with potential blood loss (refinement), we have powerful statistical methods and assistance at our disposal to determine the minimum number of animals necessary to obtain meaningful results (reduction).

Who or what will benefit from these outputs, and how?
Progress in the areas described above will not only be of significant interest to the wider cardiovascularbasic science research community but could also provide animal data to initiate a “clinical translational pathway” and underpin part of the regulatory requirements for setting up clinical studies or trials.
Longer term benefits would be to render therapies developed in the laboratory accessible to patients with diabetes and heart or vascular complications, to improve their health-related quality of life, and tohave positive impacts on their long-term morbidity and mortality.

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

Several factors will ensure the maximal output of this project:

Collaboration within our multidisciplinary team, comprising highly skilled staff working with expertisein animal models, molecular and cellular biology, data analyses and clinical studies.

Collaboration between Institutions. Our Institution is a leading organisation nationally and internationally in medical research. We have optimal support and access to top-class facilities andexpertise which attract multiple collaborators.

Our capacity to integrate animal work with analyses on clinical samples, cell models and bioinformatic analyses will maximise the value of the animal data.

Being part of national and international consortia, which favour the exchange of information and thecontinuous training of staff and exposure to new concepts and discoveries, will enhance the dissemination of our work.

Dissemination of our research results via publications of scientific articles in highly respectedjournals and presentations at national and international conferences and workshops.

Species and numbers of animals expected to be used

- Mice: 14625
- 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.

Preliminary studies of cell functions will be carried out before engaging with animal studies. We will beusing adult mice and rats for these studies to reflect the average age of the human population usually affected by ischaemic disease in their heart and legs. Multiple studies on rats and mice have reproduced key features of ischaemic disease, diabetes and high levels of cholesterol in the blood.
Mice are particularly valuable because of the range of genetically modified animals available for this project. However, rats are larger in size and hence are preferable for studies where therapeutic substances engineered in the laboratory are implanted in the animal’s heart. Moreover, an individual rat produces about 10 time more material, which we can use for cell and molecular biology studies tocomplement the in vivo investigation.

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

The animals in our project are typically used as models of the human conditions of poor circulation (ischaemia) in the leg or heart. Working with these ischaemia models, we will investigate the mechanisms underpinning the disease and test the therapeutic potential of substances, especially theircapacity to promote angiogenesis (the growth of new blood vessels) and improve the circulation.
Animals will develop ischaemia as consequence of diabetes and/or high blood cholesterol and/or willbe induced by a surgical intervention to occlude a major blood vessel which deliver the blood flow to the heart or a leg. We also have a separate protocol used to test

the proangiogenic activity of substances in healthy mice.

Typical scenario 1:

A mouse will be microchipped and will progessively develop diabetes and/or high cholesterol blood level (caused by GAA, high fat diet feeding, 5 IP injections of low dose streptozotocin or one single IV injection with a Pcsk9). Before and after the onset of diabetes and/or high blood cholesterol level, bodyweight will be measured once per week and blood will be taken from a tail vein for blood tests (with minimal rest intervals of 7 days). After at least 1 month from onset of diabetes and/or high blood cholesterol, the mouse will receive superficial skin wounds (one per leg) and, at the same occasion, it will also receive a therapeutic proangiogenic treatment (typically by gene therapy or exosome therapy),via injection in the limb adductor and gastrocnemius muscle and/or by topical application on the woundarea. Next, the mouse will be submitted to weekly non-invasive colour laser analyses of the blood flow in the limb muscles and wound area. At the same occasion, when the mouse is still anaesthetised, the wound area will be measure with a caliper. The mouse will be finally perfusion/fixed for histology.
During the whole protocol, the mouse will be regularly checked for immediately recognisable adverseevents.

Typical scenario 2:

A mouse will be microchipped. Next, it will receive (at the same occasion) unilateral limb ischaemia surgery, superficial skin wounding (one per leg), a therapeutic proangiogenic treatment (typically by gene therapy or exosome therapy), via injection in the limb adductor and gastrocnemius muscle and/orby topical application on the wound area. Next, the mouse will be submitted to weekly non-invasive colour laser analyses of the blood flow in the limb muscles and wound area. At the same occasion, when the mouse is still anaesthetised, the wound area will be measure with a caliper. The mouse will be finally perfusion/fixed for histology. During the whole protocol, the mouse will be regularly checked for immediately recognisable adverse events.

Typical scenario 3:

A mouse will be microchipped and will progessively develop diabetes and/or high cholesterol blood level (caused by GAA, high fat diet feeding, 5 IP injections of low dose streptozotocin or one single IV injection with a Pcsk9). Before and after the onset of diabetes and/or high blood cholesterol level, bodyweight will be measured once per week and blood will be taken from a tail vein for blood tests (with minimal rest intervals of 7 days). After at least 1 month from onset of diabetes and/or high blood cholesterol, the mouse will receive (at the same occasion) unilateral limb ischaemia surgery, superficialskin wounding (one per leg), a therapeutic proangiogenic treatment (typically by gene therapy or exosome therapy), via injection in the limb adductor and gastrocnemius muscle and/or by topical application on the wound area. Next, the mouse will be submitted to weekly non-invasive colour laser analyses of the blood flow in the limb muscles and wound area. At the same occasion, when the mouse is still anaesthetised, the wound area will be measure with a caliper. The mouse will be finally perfusion/fixed for histology. During the whole protocol, the mouse will be regularly checked for immediately recognisable adverse events.

Typical scenario 4:

The animal (either mouse or rat) will be microchipped and will progessively develop

diabetes and/or high cholesterol blood level (caused by GAA, high fat diet feeding, 5 IP injections of low dose streptozotocin or one single IV injection with a Pcsk9). Before and after the onset of diabetes and/or high blood cholesterol level, body weight will be measured once per week and blood will be taken froma tail vein for blood tests (with minimal rest intervals of 7 days). After at least 1 month from onset of diabetes and/or high blood cholesterol, the animal will receive a therapeutic proangiogenic treatment (typically by gene therapy or exosome therapy), via IV injection. Next, the animal will be submitted to bi-weekly non-invasive echocardiographic analyses. The animal will be finally perfusion/fixed for histology. During the whole protocol, the animal will be regularly checked for immediately recognisableadverse events.

Typical scenario 5:

The animal (either mouse or rat) will be microchipped. The animal will experience a heart attack (eithertype 1 or type 2 myocardial infarct) and at the same occasion, it will receive a therapeutic proangiogenic treatment (typically by gene therapy or exosome therapy), via cardiac delivery. Next, the animal will be submitted to bi-weekly non-invasive echocardiographic analyses. The animal will be finally perfusion/fixed for histology. During the whole protocol, the animal will be regularly checked for immediately recognisable adverse events.

Typical scenario 6:

The animal (either mouse or rat) will be microchipped and will progessively develop diabetes and/or high cholesterol blood level (caused by GAA, high fat die feeding, 5 IP injections of low dose streptozotocin or one single IV injection with a Pcsk9). Before and after the onset of diabetes and/or high blood cholesterol level, body weight will be measured once per week and blood will be taken froma tail vein for blood tests (with minimal rest intervals of 7 days). After at least 1 month from onset of diabetes and/or high blood cholesterol, at the same occasion, the animal will experience a heart attack(either type 1 or type 2 myocardial infarct) and receive a therapeutic proangiogenic treatment (typicallyby gene therapy or exosome therapy), via cardiac delivery. Next, the animal will be submitted to bi- weekly non-invasive echocardiographic analyses. The animal will be finally perfusion/fixed for histology. During the whole protocol, the animal will be regularly checked for immediately recognisableadverse events.

Typical scenario 7:

A mouse will be microchipped and receive an sc injection of (cooled liquid) Matrigel containing a testsubstance or placebo. The mouse will then be monitored for adverse effects, but otherwise left undisturbedfor the remainder of the protocol (up to 4 weeks), when it will be perfusion-fixed for histological analyses.

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

Adverse effects during the protocols to be used in the current programme may occur as consequenceof the procedures employed to induce diabetes, heart attacks or damage to blood vessels in the lowerextremities, similar to those observed in patients with ischaemic disease.

We expect the diabetic animals will experience adverse effects after surgically induced heart or limbischaemia more often than the non-diabetic animals. We do expect mice and

rats to have similar frequency and severity of adverse effects.

The most likely adverse effects to occur relate to changes in eating habits and body weight in diabeticanimals.

Animals will have poor circulation in the leg in the first week after induction of limb ischaemia (up to 80% animals) and temporary difficulty in ambulation in the first day thereafter. Some animals will have to be humanely killed in the first week after the operation to prevent development of foot necrosis following limb ischaemia surgery (up to 3% in non-diabetic mice and up to 10% in diabetic mice). Afterthis critical time window, the animals with limb ischaemia will progressively recover blood perfusion andmostly remain symptom-free until the end of the protocol.

Up to 30% of animals (both mice and rats) that will be induced with a severe heart attack (technically called “myocardial infarction” or “MI”) are expected to die during the duration of the protocols, due to the sum of the acute consequence of the injury and post-MI heart failure. The expected cumulative death is higher (up to 40%) when an heart attack is induced in animals with pre-existing diabetes and/or high cholesterol level. The animals can experience acute mortality in the first days after the MI operation. Moreover, symptoms of heart failure (difficulty breathing, reduced mobility, fatigue) might appear after 2 weeks, leading to prompt humane killing. Some animals will die when still anaesthetised and will therefore not experience any pain or stress. In our research program, the mortality rate after severe MI is high for mice and rats. The death rate in the animals is comparable to the human patients,where the severe heart attacks are frequent. In people, acute MI is associated with death in circa 1 outof 3 people and death often occur prior to arrival at the hospital. Approximately half of all patients are re-hospitalized within 1 year of their heart attack. Unfortunately, these numbers increase for the diabetic people.

Relevant foreseeable stresses also include the need for repeated anaesthesia maximum three times inthe week of the operation to induce heart or limb ischaemia and then at the maximal frequency of onceper week) for in vivo imaging and repeated intravenous blood drawing, up to the limits authorised and at weekly or longer intervals. Rarely, diabetic animals will develop severe forms of diabetes.

Unexpected adverse effects could also arise from the test substances and their delivery.

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 animaltype)?

Mild or subthreshold: 54% mice, 5% ratsNon-recovery: 0.5% mice, 1% rats
Moderate: 40% mice, 68.5% rats

Severe: 5.5% mice, 25.5% rats

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

- Killed

A retrospective assessment of these predicted harms will be due by 21 September 2028

The PPL holder will be required to disclose:

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

Our project integrates *in silico*, *in vitro* and *ex vivo* work with animal work.

The use of animals is an essential component for our research programme. Studying disease progression consecutive to heart attack or poor circulation of the lower extremities involves examining the intact heart and limbs and vasculature system in the whole animal, as both respond to the complexinterplay between mechanical stress, autonomic nervous system, vascular, endocrine and inflammatory systems.

Small Mammals are also essential to screen therapeutic candidates before proceeding to preclinicalwork in large animal models and clinical validation in first-in-human studies. Both the UK Medicines and Healthcare products Regulatory Agency (MHRA) and the USA Food and Drug Agency (FDA) require proof of efficacy in animal species for clinical trial approval.

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

In our work we use different type of experiments and replacement has been possible in part of our work using cells cultured in dishes or using human tissue samples derived from biopsies from volunteering patients undergoing surgery. We also make use of publicly available data deposited byother scientists, which we process using computational algorithms.

Why were they not suitable?

Ischaemic disease develops progressively and is influenced by the environment, where metabolisms, hormones, immune and blood cells, the nervous system and the gut bacterial to name just a few all communicate with the blood vessels of the heart and limbs. Therapeutic angiogenesis is also dependent of the environment. It is not yet possible to recreate the complexity of this environment in adish or *in silico*. It is possible that our research could contribute to develop new knowledge progressively supporting the advancement of *in vitro* and mathematical modelling alternatives to work on living animals.

A retrospective assessment of replacement will be due by 21 September 2028

The PPL holder will be required to disclose**:**

- What, if any, non-animal alternatives were used or explored after the project started, and is thereanything 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 projected number of animals reflects the number necessary to achieve the scientific objectives outlined in the programme of work described in this research programme and this has been informedby our previous experience and that of others.

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

We have made an effort in limiting the numbers of animals in each our research programmes by providing adequate statistical advice (i.e., power calculations to establish the minimal number of animals required per group and per experiment), and assistance in experimental design to all our personal licensees. We are continuously undertaking literature and web searches (Google, pubmed.com, nc3rs.org.uk) in an effort to find alternatives to any procedure that causes more thanmomentary pain or distress.

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

Use of pilot studies involving a small number of animals is an integral part of our experimental designand may be used for example to identify the safest, reasonable dosage of a novel compound (small scale dose/response study) to be used in subsequent larger studies. This approach will help reducingnumbers of animals used in this project.

Refining skills is a continuous process required to reduce variability from data collected in animals, thus skills precision help reducing number of animals in a study. With this aim we have actively engaged in several joint sessions with other experienced scientists from other groups in the department to optimise surgical techniques and imaging methods and share experience. Wheneverpossible, we will engage in visits to other Labs in the UK and overseas with excellent records on mouse/rat work.

Using the latest imaging technologies available to assess rodent cardiac and vasculature function (echocardiography, MRI techniques), by means of which multiple new key parameters can be measured, we have striven to maximize the information obtained from each animal during the same imaging study. These novel parameters show promise in the detection of subtle cardiovascular changes in rodents treated with new compounds, which would potentially be missed by conventionalimaging, this allows us to use fewer animals than before to identify differences between treated and control groups.
From each animal, we will typically obtain more than one type of quantitative data. As example capillary density (by histology) will be obtained on heart and limb specimens collected from animalswhich have already undergone in vivo imaging

At the end of a study, we seek to acquire as many organ samples as possible from

euthanisedanimals.

A retrospective assessment of reduction will be due by 21 September 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan 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 welfarecosts (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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Everything will be done to attenuate pain, discomfort, infections and stress. We will adopt refined microsurgical and aseptic techniques to minimise the adverse effects of surgery. Precautions include pain killer administration, supplementary heat and mashed food at floor level post-operatively, and if post-operative recovery is slower than expected supplemented oxygen is provided. Animals displayingany of the following clinical signs — breathlessness, discolouration of the skin, reduced mobility, eyes or nose discharge, diarrhoea, darkening and ulceration spreading beyond the toes, difficulty walking —will be monitored closely and supportive measures may be implemented in conjunction with the local veterinary surgeon, and if there is no improvement within 24 hours, then the animal will be humanely killed. All animals will be carefully and individually monitored after surgery.

With the ambition to further refine our models and methods, we are updating our knowledge via interactions with other scientists based in the UK and overseas. We also reviewing the literature andthe nc3rs.org.uk website.

The Matrigel plug angiogesis model allows us to study the in vivo angiogenic effects of substances in a mild severity context. It consists of implanting in the animal flank a plug containing proteins or non-cancerous cells modified before the implantation. The Matrigel, which will solidify as a small plug following injection into the animals. The plug is not expected to grow over time. The plug will be colonised by blood vessels, which grow more or less as responses to the tested substances and will be explanted after the death of the mice to quantify the blood vessels growth. The study typically last 2-4 weeks. The animals are not expected to experience major adverse events. We do not expect the mice will experience pain, discomfort or limited mobility.

Refinement: as a difference from some other groups, we do not use cancer cells to stimulate angiogenesis. Immunocompromised mice (used when human cells are implanted in the Matrigel) willbe housed in a barrier environment, thereby minimising the likelihood of infection.

High blood cholesterol and diabetes models are created by feeding animals (mice and rats) with high fat diets, and/or using a drug (streptozotocin, STZ), which damages the pancreas cells responsible for insulin production, and/or by using animals with genetic alterations that predisposes them to the development of these diseases. High blood cholesterol and/or diabetes will progressively cause small vessel disease (microangiopathy) in several organs (heart, limb, kidneys) and reduce thecardiac function (cardiomyopathy).

Refinement: The studies have been designed to end before animals develop severe diabetes and became symptomatic for cardiac complications. After diabetes onset, animals will be checked daily forsign of sufferance, dermatitis, or obesity-induced impaired mobility in animals with type-2 diabetes mellitus (T2DM) and regularly screened to assess urine and blood glucose levels and to monitor the evolution of cardiac changes (by echocardiography). To model type-1 diabetes mellitus (T1DM), othergroups have used STZ, compound which at high doses immediately kills the insulin producing β cells in the pancreas. We will use a refined protocol based on repeated injections of STZ at a low dose to induce T1DM in mice and rats. Used according to this refined protocol, STZ elicits an immune and inflammatory reaction in the pancreas, which more progressively damage the β cells. This is better tolerated by the animal and importantly mimics better the insurgence of human T1DM.

Acute Limb ischaemia (LI) model consists of the surgical occlusion of the femoral artery in one leg and it can be associated with the induction of a wound on the calf of the same leg (to mimic the humancondition of critical LI). The procedures are developed in under aseptic conditions, keeping the mouse under general anaesthesia. Our plan is to surgically induce limb ischaemia (LI) in one leg by ligating the femoral artery while the other leg, which remains well perfused, is used as an internal control. Afterthat we will inflict one circular skin laceration (4-5 mm diameter) in both the left and the right lower leg at the calf muscle level, to subsequently assess and compare wound healing capacity in hindlimbs withLI in comparison with “internal controls”. Blood flow reduction observed in the immediate post- operative hours/days in the ischaemic leg will naturally improve and normalise within 5 weeks after surgery. The calf skin laceration in both legs is expected to completely heal within the first 5 weeks following injury. The LI and wound healing models will be developed in healthy animals (both wild type and genetically altered, GA) and in animals with high cholesterol level and diabetes (both wild type andGA). Animals with diabetes and/or high cholesterol blood level are expected to require longer to reachblood flow recovery and closure of the skin wound. To accelerate the angiogenesis (new blood vessel formation) responses and improve tissue perfusion, test substances might be delivered locally (injections in the ischaemic leg muscles and topical application to the wounds), or systemically, either during the operation or at different times points before or after it.

Refinement: We induce limb ischaemia in one leg, only. The other leg is used as internal control whenmeasuring the post-surgery blood flow recovery to the animal foot and the local angiogenesis responses. The operations takes course under the most refined protocols for anaesthesia and analgesia. Animals are inspected daily and will be promptly humanely killed if they have foot necrosis.Animals are also killed if the wound is infected and does not respond to antibiotic treatment. When thelimb ischaemia model is developed on hypercholesterolemia and diabetic animals, the specific refinement strategies described above will be also apply.

The **Myocardial Ischaemia** with Coronary Occlusion model The ‘heart attack model’, also named myocardial infarction (MI) model is based on the ligation of one major coronary

artery, which delivers the blood to the heart. This intervention results in tissue death (infarction) due to inadequate blood supply to the affected area, after a couple of weeks the infarcted zone is progressively replaced by a fibrotic scar. The surviving myocardium is starved of blood flow and is therefore ischaemic. Substancesmight be delivered locally to the infarct border (typically by direct injection with a needle and syringe, orby application of medicated patches/scaffolds on the heart surface). New blood vessel formation can be triggered this way to improve tissue perfusion. Animals can die early (usually within 3 days) after theoperation (by cardiac rupture or fatal arrhythmia) and develop heart failure thereafter (typically, after 2 weeks post-MI). The MI model will be developed in healthy animals (both wild type and GA) and in animals with high cholesterol level and diabetes (both wild type and GA). Animals with diabetes areexpected to experience more severe adverse events.

Refinement: The MI model of coronary ligation is well validated, refined and already used by many experts in the field. During the course of this project, we aim to further refine the model by importing anew advanced technique recently published by another group. This new approach allows to occlude the coronary artery in mice without resorting to traumatic tissue incisions and open chest surgery, hence it proved to be less invasive and deadly.

Alternatively, we will induce MI by giving the animal a high dose of a drug (β-adrenoceptor agonist (such as isoproterenol – ISO or isoprenalin), which increases the heart rate. is also Upon ISO administration, ischaemia occurs due to the imbalance between cardiac stimulation and decreased coronary blood flow. This model, which is also called “type 2 MI” or “MINOCA” (Myocardial Ischaemiawith Nonobstructive Coronary Arteries).

Refinement: This model has been used for decades by many groups and is currently already used inour department. We are not planning to further refine it during the course of this project.

The animal models that will be used in studies carried over for over several weeks during which we willuse imaging methods at regular intervals to measure how the blood vessels in the heart and extremities function and grow, in response to the tested substances and the overall impact on the heartfunction and wound healing. Imaging will be done under anaesthesia from which the animal will recover.

The Applicant of this animal licence is already experienced with the animal models and methods included in this project, has used them in published scientific paper, and has been awarded research grants employing these models. The establishment staff are also experienced with the models (with exception of acute limb ischaemia). The models and methods proposed in this project have been usedfor many years by many groups, which have contributed to their progressive refinement. This has supported the capacity to prevent adverse effect and reduce the pain, suffering and distress caused byunavoidable adverse effects on the animals.

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

We propose to use mice since they are considered the least sentient mammalian species with genes that can be readily modified to alter expressions of key molecules, allowing for proof-of-principle studies of the heart and blood vessels. In addition, they represent the lowest group of mammals with acardiovascular system similar to man, and for which there are good models of heart attack and lower extremity arterial disease. In a few experiments, we will use rats because their larger size facilitates some surgical procedures and allows for the extraction or more biological material to be used for ex- vivo and in vitro studies.

For example, in the ‘living myocardial slice’ ex-vivo assay rats are humanely killed and their heart collected and placed in a cold solution and sliced using a vibratome, to producedmyocardial slices. These tissue preparations are cultured up to several days and used to investigate fundamental biology questions and to conduct pharmacological studies.

Similar to recuperating patients in hospitals, animals recovering from surgery will be closely monitoredusing individual daily health sheets for signs of distress and heat therapy. Animals receiving injections will be monitored daily by the operator (who holds a personal HO licence -PIL- to perform the work onanimal approved in this research programme). Where problems arise, we shall consult the named animal care and welfare officer (NACWO) and veterinary surgeon and offer pain relief, treatment, or cull animals as appropriate.

Most animals used in this project will die at the end of a terminal procedure without suffering since killing takes place whilst the animal is under full anaesthesia. A small proportion of animals that underwent surgery with recovery will be immediately killed if they show signs of uncontrolled pain, or signs of suffering that are greater than minor and transient or in any way compromises normal behaviour. As with man, deaths is expected in some of the animals subjected to experimental heart attack. All animals that have not undergone a terminal procedure will be humanely killed at the end ofthe protocol.

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

Surgical procedures on animals are performed on Mondays–Thursdays to allow for adequate monitoring in the subsequent 24-48 hr. We only perform surgery before 3pm to allow sufficient time foranimals to recover and be monitored and assessed before being returned to their holding room. Goodaseptic methods are in place to prevent bacterial infection of animals during and after surgery. Proper anaesthetic (Isoflurane) is used to avoid potential pain and distress during surgery. Mice are lying on aheated pad throughout surgery to prevent heat loss from being anaesthetised. Mice receive appropriate painkiller before surgery and for 24hr or longer if needed to reduce pain. Saline is given atthe end of the operation (through one injection under the skin) to counter risk of dehydration. Mice canrecover in a heated chamber following procedures under general anaesthesia (surgery), and their health/well-being is periodically checked after recovery. Mashed food is placed in the cage at floor level; food pellets and water are provided. Heat therapy is provided whenever necessary to improve recovery. Mice are checked daily during key periods, such as the first week post-operatively

Diabetic, hypercholesteraemic, obese mice used in this project are closely monitored through their lifetime. They are weighed once a week and require frequent monitoring for movements, behaviour, water and food consumption. At some point they will require more frequent cage change than normalmice and water addition, since moderate diabetes causes frequent urination and increases water consumption. Skin condition will be inspected regularly since obesity causes skin problems (rashes, infection, etc.).

Some animals may have an altered immune system making them more susceptible to infection. Animals with altered immune status will be housed in a barrier environment thereby minimising thelikelihood of compromising health.

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

The conduct of animal experiments is for the most part guided by the LASA, PREPARE, and 2019ARRIVE guidelines, and information found on the NC3Rs website.

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

We will constantly review our protocols and experimental design to reduce, refine and replace the use of animals in conjunction with the support of experienced NACWOs and the named veterinary surgeon (NVS). Additional support from the facility consists of dissemination/organisation of relevant event and conferences (e.g., 3R Advisory group, LASA or NC3Rs events, etc.). Training/assessments on the newest most refined techniques will be provided by the named training and competency officer (NTCO)and NVS. We will attend our quarterly animal facility's operation committee meeting which provides a forum for discussion on all matters related to animal welfare, care and use on the campus. We will keep informed about any advances by reading scientific publications and speaking with colleagues within the College and elsewhere.

A retrospective assessment of refinement will be due by 21 September 2028

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 forfuture work of this kind? During the project, how did you minimise harm to the animals?

16. Development of brain tumour models in mice for tumorigenicity screens and pre-clinical therapeutic trials.

Project duration

5 years 0 months

Project purpose

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

Key words

Medulloblastoma, Cancer, Therapy, Orthotopic

Animal types	Life stages
Mice	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 orscientific needs it's addressing.

What's the aim of this project?

Develop, investigate, and treat brain tumours (cancer) in mice.

A retrospective assessment of these aims will be due by 21 September 2028

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 advancedor 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?

Medulloblastoma is the most common malignant brain tumour of childhood with approximately 80 newcases diagnosed every year in the UK. Over the last 50 years, advances in standard treatments (i.e., surgical removal of tumour, radiotherapy, and chemotherapy) have led to long-term survival rates of approximately 70%. However, for survivors there is a burden of tumour and/or treatment related late- effects, with ~40% of long-term survivors suffering from ≥1 severe chronic medical problem.

Furthermore, we have identified biologically-defined groups of the disease which do not respond tocurrent therapies (e.g., MYC-driven medulloblastoma; < 10% survival). Moreover, medulloblastomacomes back (disease relapse) in 30% of patients and is usually fatal. Overall, medulloblastoma therefore accounts for a disproportionately high amount (~10%) of childhood cancer deaths.

This project will initially focus on developing new brain tumour mouse models of medulloblastoma. Wewill i) develop mouse models which mimic some of the known biological drivers of aggressive disease(e.g., MYC-driven medulloblastoma), ii) use a repertoire of brain tumour models to investigate additional candidate biological drivers of disease progression and relapse which we call tumorigenicity screens, and iii) use these findings to identify new treatments/combinations of treatments to be tested in our medulloblastoma mouse models. New treatment approaches will need to be tested in appropriate mouse models to understand whether they firstly cause harm (are toxic), and secondly arebeneficial i.e., are better at treating the tumour than our current therapies. These trials will deliver the essential evidence to progress these treatments into human clinical trials.

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

Development of a team at the establishment with a new suite of technical skills in animal work that willbe supportive of collaborations and can be shared globally with partners.

Development of the first medulloblastoma mouse models at the establishment, including potentiallynovel models and tumorigenicity screening systems.

Identification and/or evaluation of specific targeted treatments in these novel models.

Dissemination of pre-clinical information through national and international working groups, conferencepresentations and open publications.

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

In the short-medium term, the outputs will initially benefit the local scientific community at the establishment, followed by collaborators and the wider childhood cancer community.

Ultimately, the outputs will provide key information for the development of future early-phase clinical trials in children, which will be supported through the already established national and international networks of the applicant. Longer-term this project will provide the platform for routine development of brain tumour mouse models at the establishment including novel models from patient-derived tissue (human cancer cells which are taken directly from the patient tumour). This in turn will support the international effort todevelop suitable, workable mouse models for all childhood brain tumours.

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

I will continue to expand my collaborative networks in the field of both childhood brain tumour researchand mouse model development. Where appropriate early resources and findings will be made available to the wider research team and establishment through local group and institute research-in- progress meetings. Similarly, findings and resources will be shared with collaborators actively engaged with the project. This early sharing will maximise the impact of outputs, and aid in experimental refinement and validation.

Validated outputs (including negative findings) from this project will be disseminated to the scientific community through national and international working groups, conference presentations and publication in open access journals. Publication of novel discoveries will be announced, supported by the establishment's press office, to disseminate findings more widely and broadcast via social media. Activities to entice a varied audience will also be undertaken. This will include providing written piecesfor non-academic audiences, and I will also utilise additional outreach platforms to maximise the impact of this research.

Species and numbers of animals expected to be used

- Mice: 600

Predicted harms

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

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

Mice have been chosen for this project as the least sentient being that these models can be developedin. Mice importantly model the supporting tissue surrounding the tumour (tumour microenvironment), blood vessel development, and metastatic disease spread, all of which is observed and important in understanding the human disease. In treatment trials, mice will also display treatment side-effects which will provide vital information for selecting new treatments. Furthermore, mice can be genetically modified as appropriate and immunocompromised strains (mice without a working immune system) willbe used as they will be more likely to accept human cancer cells which are taken directly from the patient tumour (known as patient-derived samples). The age of the species has been selected to mostrepresent the age at which childhood tumours typically develop in humans and is also in line with collaborative experience and expertise within the field.

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

A mouse undergoing this study will be obtained from collaborative breeding programmes, breeding programmes at the establishment, or purchased commercially. Mice will be group

housed in individually ventilated cages, unless needing to be singly housed for welfare reasons, and maintainedin appropriate biosecurity conditions depending on the mouse strain. Their housing conditions will match those within the code of practice (e.g., enriched environment). The animals will be handled using a low-stress techniques (i.e., tunnel handling).

Subcutaneous model development

We will develop subcutaneous (tumour cells injected into the fat under the skin) mouse models as an adjunct to our orthotopic animal models (tumour cells injected into the relevant part of the body i.e., thebrain). Subcutaneous models will be particularly useful for testing new treatments that may not initiallyget into the brain and thus orthotopic models will not be helpful to understand whether these new treatments are beneficial and worth developing further so that they can ultimately be delivered to the brain.

Animals will be shaved and experience tumour cell injection into the flank into the fat under the skin (subcutaneously). Animals may require a general anaesthetic for this procedure. Animals will make afull recovery following this procedure and will be closely monitored using appropriate injection site inspection, tumour measurements with callipers, and where required non-invasive imaging under general anaesthetic. Animals will only experience tumour cell injection once. The animals will beterminally euthanised at an appropriate time point using humane methods.

Orthotopic model development

Animals will experience a surgical procedure under general anaesthetic, whereby they will have a small part of their head shaved, positioned in a surgical frame, a small hole made in their skull, andtumour cells injected through this hole into the relevant part of the body i.e., the brain (orthotopic). Animals will make a full recovery following this procedure and will be closely monitored using appropriate non-invasive imaging under general anaesthetic and scoring techniques for tumour development. Animals will only experience this surgical procedure once. The animals will be terminally euthanised at an appropriate time point using humane methods.

Treatment trials in tumour bearing mice (subcutaneous)

We will develop subcutaneous (tumour cells injected into the fat under the skin) mouse models as anadjunct to our orthotopic animal models (tumour cells injected into the relevant part of the brain).

Subcutaneous models will be particularly useful for testing new treatments that may not initially get intothe brain and thus orthotopic models will not be helpful to understand whether these new treatments are beneficial and worth developing further so that they can ultimately be delivered to the brain.

Animals will be shaved and experience tumour cell injection into the flank into the fat under the skin (subcutaneously). Animals may require a general anaesthetic for this procedure. Animals will make afull recovery following this procedure and will be closely monitored using appropriate injection site inspection, tumour measurements with callipers, and where required non-invasive imaging under general anaesthetic. Animals will only experience tumour cell injection once. Upon tumour development, animals will be entered into treatment trials. These will include, for example, the administration of a compound(s) or control by intravenous (into the vein) injection, intraperitoneal (thespace around the

abdominal organs) injection, subcutaneous (into the fat under the skin) injection, intramuscular (into the muscle and requiring a general anaesthetic) injection, gavage (direct administration into the gut using a tube) or via fluid/food alternation until an appropriate humane endpoint or a scientific purpose has been obtained. The administration method and frequency may vary depending on the compound. Animals will be terminally euthanised at an appropriate time pointusing humane methods.

Treatment trials in tumour bearing mice (orthotopic)

Animals will experience a surgical procedure under general anaesthetic, whereby they will have a small part of their head shaved, positioned in a surgical frame, a small hole made in their skull, and tumour cells injected through this hole into the relevant part of the body i.e., the brain (orthotopic). Animals will make a full recovery following this procedure and will be closely monitored using appropriate non-invasive imaging under general anaesthetic and scoring techniques for tumour development. Animals will only experience this surgical procedure once. Upon tumour development,animals will be entered into treatment trials. These will include, for example, the administration of a compound(s) or control by intravenous injection, intraperitoneal injection, subcutaneous injection, intramuscular injection (under general anaesthetic), gavage or via fluid/food alternation until an appropriate humane endpoint or a scientific purpose has been obtained. The administration method and frequency may vary depending on the compound. Animals will be terminally euthanised at anappropriate time point using humane methods.

Dose finding and toxicity studies in non-tumour bearing mice

Animals will be entered into dose finding and toxicity studies of novel compounds. Dose setting at a low dose in initially no more than three mice will be undertaken. If no toxicities are observed, a furtherthree mice may be tested at a higher dose and so on until an effective dose level is reached. If the initial dose produces evident toxicity, doses will be reduced. Compounds will be administered using intravenous injection, intraperitoneal injection, subcutaneous injection, intramuscular injection (under general anaesthetic), gavage or via fluid/food alternation. The administration method and frequency may vary depending on the compound. All animals will be monitored for weight, skin colouration, abdominal distention, hydration status and stool consistency a minimum of twice weekly. Animals willbe terminally euthanised at an appropriate time point using humane methods.

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

Subcutaneous model development and treatment trials

Animals will be assessed for pain both before and after tumour cell injection and where appropriate given pain relief. Where appropriate animals will be anaesthetised, shaved and tumour cells injected. Following tumour cell injection, animals will be closely monitored using appropriate injection site inspection, tumour measurements using callipers, and where required imaging (under general anaesthetic) for tumour development and tumour response to treatment. During this we anticipate animals may show signs of weight loss, poor grooming, poor intake of food and water. Animals will be scored against a local scoresheet which will define the humane endpoints and guide appropriate welfare interventions such as soaked diet or a heated environment. Veterinary care will be called uponwhere appropriate.

Orthotopic model development and treatment trials

Animals will be assessed for pain both before and after surgery, given pain relief during surgery beforetumour cell injection (perioperatively), and where appropriate given pain relief following surgery for as long as deemed necessary. Following recovery from surgery, animals will be monitored using imagingtechniques (under general anaesthetic) and scoring for tumour development and tumour response to treatment. During this we anticipate animals may show signs of weight loss, poor grooming, loss of balance, poor intake of food and water. Animals will be scored against a local scoresheet which will define the humane endpoints and guide appropriate welfare interventions such as soaked diet or a heated environment. Veterinary care will be called upon where appropriate.

Dose finding and toxicity studies in non-tumour bearing mice

All animals will be monitored for toxicities associated with novel compound(s). This will include monitoring for weight, skin colouration, abdominal distention, hydration status, and stool consistency aminimum of twice weekly. Animals will be scored against a local scoresheet which will define the humane endpoints and guide appropriate welfare interventions such as soaked diet or a heated environment. Veterinary care will be called upon where appropriate.

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 animaltype)?

Subcutaneous injections, tumorigenicity screens and treatment trials
100% of the animals are going to experience a moderate severity category.Orthotopic injections, tumorigenicity screens and treatment trials
100% of the animals are going to experience a moderate severity category. An unknown percentage ofanimals may experience a severe severity category.

Dose finding and toxicity studies in non-tumour bearing mice

Up to 30% of the animals may experience a moderate severity category.

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

- Killed

A retrospective assessment of these predicted harms will be due by 21 September 2028

The PPL holder will be required to disclose:

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

Animals (mice) support the development of these brain tumour models and subsequent treatment trialsbecause they have an appropriate tumour microenvironment (the tissue surrounding and supporting tumour development), model blood vessels development, metastatic disease spread, and exhibit similar treatment side-effects to what we would observe in humans.

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

2D cell lines (tumour cells that grow indefinitely as two-dimensional cells on a flat surface in a petri dishor flask under laboratory conditions).

3D cell lines (tumour cells that grow indefinitely as three-dimensional clusters of cells in a petri dish orflask under laboratory conditions).

Why were they not suitable?

2D cell lines have been and will continue to be utilised to shortlist appropriate compounds for testing inanimals. Animal tests are required to assess compound side-effects and tumour response. 2D cell lines do not grow in a representative tumour microenvironment, they do not model blood vessel development, metastatic disease spread, or exhibit treatment side-effects when compared to tumours in a mouse model. Therefore, cell line responses to treatments are not always reliable or reproducible in human clinical trials. 3D cell lines in medulloblastoma are yet to be developed in the establishment but are an aspiration of the applicant and wider research team. Internationally, efforts to develop 3D medulloblastoma cell cultures which more reliably represent the tumour microenvironment remain challenging. Furthermore,3D cultures still do not model blood vessel development, metastatic disease spread, or treatment side-effects in the way that mice models do.

A retrospective assessment of replacement will be due by 21 September 2028

The PPL holder will be required to disclose:

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

Reduction

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

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

For the establishment of new medulloblastoma brain tumour models (orthotopic or subcutaneous),three mice will be injected per medulloblastoma tumour type.

Upon establishment and characterisation of each model, appropriate models will be

selected for use, for example in our pre-clinical treatment trials. We have defined that for most treatment trial groups wewill require five animals per treatment arm with appropriate controls for both drug and animal. This is based on previously published data and our local experience and will provide results that are scientifically significant. Upon calculating the number of animals required a total number of animals to complete our pre-clinical treatment trials will be injected with tumour cells. For our dose findings and toxicity studies, three animals per dosing level will be required and we anticipate that no more than five dosing levels will be required per novel compound. This is again based on previously published data, our local experience, and will provide results that are scientificallyrelevant.

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

For model development, we will predominantly be using immunocompromised mice (animals without aworking immune system). These strains of mice have been selected as they are most likely to accept tumour tissue from another species, e.g., human, and therefore this will increase our chances of success and reduce the number of mice required for model development.

All treatment will first be tested in cell lines before being short-listed for our mouse treatment trials. Where possible treatment doses for mice will be obtained from previously published studies or workundertaken by our collaborators to reduce the number of dose finding studies required in mice.

Both sexes of mice will be used. Results for both sexes will be combined and analysed together, butsex will be assessed as a confounding factor. Only significant differences attributable to sex will be accounted for in future study designs.

Longer term I aspire to develop 3D cell cultures to better represent the tumour microenvironment, andthus reduce the number of animals required in future experiments.

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

We will obtain mice from either approved suppliers or internal/external collaborators. This will reduce the number of animals required as we will be using efficient breeding programmes and will only requestthe exact number of animals required. This will also support reproducibility and reduce any effects of genetic drift (small genetic changes that get passed on to subsequent generations of mice).

Our initial medulloblastoma model development will be undertaken in small numbers of mice to characterise each model and aid future selection of the most appropriate model(s) to take forward, for example, into treatment trials. Furthermore, our initial work with patient-derived tumours (i.e., cells taken directly from the human tumour and grown in mice) will be first undertaken in established patient-derived samples that already reliably grow in mice. This will reduce the initial number of mice required to develop these models in our hands.

Where possible treatment doses for mice will be obtained from previously published studies or work undertaken by our collaborators. Where this data is not available, we will use the minimal number ofmice possible (e.g., three mice per dosing level with an estimated maximum of five dosing levels) to establish the maximal tolerated dose of any novel compound.

Upon completion of our treatment trials, within the childhood brain tumour research community, it is now best practice when investigating new treatment approaches, to validate any findings in a secondappropriate mouse model which may be undertaken in a separate institution. We will follow this guidance where appropriate and work with our collaborators and international networks to prioritiseresults, and future translation into human clinical trials.

A retrospective assessment of reduction will be due by 21 September 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan 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 welfarecosts (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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Animals transported to the establishment will have a period of habituation (7-10 days). Animals will be handled using low-stress techniques (i.e., tunnel handling). We will have appropriate scoring and behavioural assessments in place to detect treatment toxicities, pain and response to pain relief whererequired. We will have tight humane endpoints in place to minimise suffering for the mice, for examplein those mice that develop orthotopic brain tumours, this includes regular imaging of mice to accuratelyassess both the size and growth rate of the tumours. Furthermore, those animals undergoing treatment are likely to experience a prolonged life and tumour reduction.

We will use regular inspection (subcutaneous tumours) and imaging techniques (subcutaneous and orthotopic tumours) to monitor for early tumour development alongside scoresheets to monitor animalwelfare. These scoresheets will be regularly reviewed and where appropriate refined to provide the most accurate assessment of animal welfare.

Previous in-house and collaborative experience has shown that for the injection of tumour cells subcutaneously, a 1:1 mixture of matrigel:tumour cell prevents tumour cells from dissipating in the flank, enabling more reliable tumour development and a reduction in engraftment failure. We will observe other refinements such as avoiding needle reuse, and those acquired from our collaborators. These will include the use of a surgical needle as opposed to a microdrill to create burr holes in the skull, this will minimise the damage to normal brain tissue. Tumour cell injection sites will be shaved which will improve asepsis and reduce the risk of infection. We will use dissolvable sutures for surgicalsites which will reduce the need for animal handling and further procedures to remove the sutures.

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

Less sentient beings such as zebra fish and nematodes (roundworm or threadworm), are not suitablealternatives to mice. They are not routinely established for use in the field of childhood brain tumour research. Furthermore, they do not mimic the tumour microenvironment, blood vessel development,metastatic disease spread, or treatment side-effects in the same way that mice models do.

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

All animals will receive appropriate peri-operative pain management. Animals will be housed in an enriched environment and handled using low-stress techniques (i.e., tunnel handling). Any animal thatdemonstrates vocalisation or sensitivity during treatment will be assessed by a NACWO/vet before continuing treatment. Where appropriate we will use the most refined/least invasive method of compound administration while gaining scientific results. We will refine our surgical techniques and models by working with the named veterinary surgeon (NVS) to improve asepsis and use the most up-to-date anaesthesia and pain management protocols.

During the development of our medulloblastoma brain tumour models all new PILs to the technique willundergo a period of cadaver training supervised by local and/or external experts. We have already refined our surgical technique through the use of a surgical needle, as opposed to a microdrill to make our burr holes. Use of imaging is also a refinement for better monitoring of tumour establishment and growth rate. Use of scoresheets has further refined our techniques for the monitoring of mice.

Where required we will include pilot studies to refine, establish and select appropriate tumour models to take forward into tumorigenicity screens and treatment trials. Longer term when we are looking to develop new in-house patient-derived models, where appropriate, we will undertake pilot studies to determine the minimal number tumour cells required to develop a brain tumour, thus reducing the post-surgical pain and/or inflammation experienced by the mice. During the length of this project, we will continue to work closely with our established collaborators to introduce new refinements where appropriate.

What published best practice guidance will you follow to ensure experiments are conducted inthe 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 standards for refinements discoveredduring the project licence.

Code of practice for housing and care of animals bred, supplied or used for scientific purposesLASA guidelines
RSPCA Animals in science guidelinesUFAW publications
NC3Rs and procedures with care website

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

The local Animal Welfare Ethical Review Body (AWERB) named information officer, named animal careand welfare officer (NACWO), named training and competency officer (NTCO) and veterinary team regularly inform and disseminate improvements and recent studies

involving replacement, reduction, and refinement (3Rs). During the 1-, 3- and 5-year review of the project licence, I will update on implementation and/or the consideration of the 3Rs that has occurred during the previous period, alongside a review of the linked training plan, scoresheets etc. with a particular focus on refinement.

Alongside this review process I will also encourage my team to explore new ways to observe the 3Rs,supporting small grant applications for example to advance this work.

A retrospective assessment of refinement will be due by 21 September 2028

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 forfuture work of this kind? During the project, how did you minimise harm to the animals?

17. Safety Testing of Medicinal Products Using Non-Human Primates

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 aimsmentioned in paragraph (b)

Key words

Non Human Primate, Regulatory, Safety Assessment

Animal types	Life stages
Cynomolgus macaques	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 non-human primates

Objectives and benefits

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

What's the aim of this project?

This project licence authorises the conduct of studies in laboratory non-human primates (NHP) toevaluate the safety, quality and effectiveness of medicinal products for the avoidance, prevention,diagnosis or treatment of debilitating or potentially life-threatening conditions in man, in terms of general toxicity and whole body system exposure.

A retrospective assessment of these aims will be due by 22 September 2028

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 advancedor 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?

New medicines have the potential to benefit in new or improved disease treatments. Before potential new medicines are administered to humans their safety must be evaluated. This testing is a mandatory legal requirement and provides information on risks to people taking new medicines. Often,the new medicines we test on this programme will be highly specific for a molecular target or receptor, which often make them less likely to have side effects than traditional medicines.

The test substances examined under this licence may include: chemical pharmaceuticals, biological pharmaceutical products derived from biotechnology (such as; gene therapies and monoclonal antibodies, stem cells and other therapeutic cell lines, cellular fractions, vaccines, serums and allergens). Substances with potential medical utility other than therapy (e.g. diagnostic imaging agents,liposomal encapsulation, nanoparticle carriers) and substances associated with drug candidates (e.g. metabolites, impurities, excipients, degradation products, placebos and/or novel vehicles for clinical trials) may also be investigated under this project licence.

The primary aims of this project are to support the development of these medicinal products through acquisition of data to 1) Support selection of new candidate molecules for further evaluation and development. 2) Demonstrate the safety-hazard profile of a new medicinal product prior to the initiationof clinical trials involving humans 3) Demonstrate the hazard profile of a medicinal product, in order to meet the regulatory requirements for marketing authorisation. Further aims include validation of new experimental conditions including the collection of blood/tissues to support drug development and thevalidation of non-animal alternative methodology.

These studies are run to satisfy the requirements of UK/EU (and sometimes international regulatory authorities) who are independent of governments) which require the testing of pharmaceuticals in a non-rodent species. At present there are no alternatives that don’t use animals that are scientifically, ethically or legally acceptable as replacements for systemic toxicity assessment. In addition, we onlyuse NHPs when no other species is suitable based on the nature of the drug (so if a drug can be tested in say a rodent, dog or a pig, and we would expect an equal outcome to the study then we would use them instead of a primate). These studies are performed prior to potential new drugs advancing into human clinical trials.

Primates will only be used where scientific justification shows that the purpose of the programme ofwork cannot be achieved by the use of animals that are not primates.

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

The overall benefit of this project is that it supports the development of safe, new medicines to improvethe health and quality of life of human patients by generating high quality data that is acceptable to regulatory authorities and enables internal decision making within our client's organisations.
Achievement of the objectives of this licence will enable safe development candidates to progress andwill also help to remove unsuitable candidates from the development pipeline

at an early stage, thus saving animals and resources.

Study reports will be included in regulatory submissions to allow regulatory authorities to make judgements on whether to permit clinical studies or to licence a drug. Global guidelines recognise thatthe justification for animal-based regulatory toxicology and safety testing is the need for regulatory authorities to have sufficient information to assess the risks to which humans are exposed to by new drugs.

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

Patients will benefit from these studies as this work will contribute to the development of new drugsthat help alleviate human conditions. These new drugs may work better in the clinic, relieve or curediseases and have better side effect profiles. We may, by our work, also contribute to better knowledge and understanding of these types of drugs, and that knowledge may be used to developfurther new drugs.

One of the key benefits is the production of data that is required by regulatory authorities, to ensuremedicines can be dosed safely to humans. These drugs that will be tested are for debilitating or lifethreatening human conditions, in some cases where there is an unmet clinical need to treat such conditions.

In addition, the models on this project may be used to assess the safety or other in life properties of anew drug, and find a dose that causes no effect. This is important when planning future trials in humans, to make sure any starting dose in a clinical trial is safe for the patients taking it.

Our customers will also benefit, as the data we generate will allow them to progress their new drugsinto clinical trial, or otherwise if they are found to have adverse side effects.

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

The work will be shared with customers who will use it to determine their future strategy, or for submission in documents required by regulatory authorities. Whilst we have no direct control over what happens to the data after we have shared it, we trust from information given to us that it is usedfor, or to support, regulatory purposes (e.g. to show that a certain chemical is safe for human exposure).

Where appropriate, we collaborate with our customers to share data we have produced in the form ofScientific publications that are in the public domain.

We are able to advise our customers on which studies are required in their development programme and on suitable study designs, based on our experience and on knowledge gained from previous post-registration feedback from customers and/or regulators, leading to focussed and effective studies.

It is difficult to predict how the benefits of any work done on this project will be seen in the future due toconfidentiality issues. However, this work will contribute to the safety of chemicals that the public and animals are exposed to.

Species and numbers of animals expected to be used

- Cynomolgus macaques: 5300

Predicted harms

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

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

We are using adult non-human primates on these studies. We only use non-human primates as a non-rodent species, when other non-rodent species (like dogs and/or pigs) are unsuitable to get the answers we need from the studies.

This is often because for the type of pharmaceuticals we are testing (for example 'biologics', peptidesor antibodies) we can only see any toxic effects if we use primates, maybe because the biological target of the drug is only present in a primate, or maybe because the requirements of the study meanwe can only use primates to get the results we need that will satisfy global regulatory bodies.

We are not allowed to use primates by law unless there is no other animal we can use that will give usthe results we need to satisfy the regulatory authorities.

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

Animals are dosed by the intended/likely route of human or animal exposure (for example oral administration, injection, infusion or inhalation), and observed regularly to monitor appearance,behaviour and clinical health.

Some animals may undergo a surgical procedure under general anaesthesia, eg placement of a deep vein catheter for intravenous infusion, or implantation of a monitoring device or minipump. Investigativeprocedures carried out in these studies are similar to diagnostic procedures that might be used medically to monitor progress of a human patient and include, for example, collection of blood and urine samples for laboratory investigations, or ECG monitoring to assess heart rate/function, or examination of the eyes using an instrument similar to those used by opticians. Animals undergoing surgery receive the same sort of care as a patient would in hospital. We discuss their pain relief and use of antibiotics with a veterinary surgeon before we start. We administer drugs as necessary and give them plenty of time to recover from surgery before we use them in experiments. These surgical procedures are carried out only for essential purposes.

Typically, on this project, animals are dosed over a period of time with test substances, and usually sampled (e.g. blood or urine) before having tissues taken after they have been humanely killed for extensive toxicology analysis. Studies would range from a single dose, to repeat-dose studies whichcan last up to 1, 3 or rarely 12 months. Study durations are dependent on the specific regulatory testbeing performed. Some animals are left dose free for a few weeks after dosing is complete to see if any effects of the test substances can be reversed.

Dosing of animals is commonly done orally using a flexible tube or by capsule. Other common routesinclude by injection using a syringe and needle, maybe directly into a vein or under the skin.

Blood samples are usually taken from easily accessible veins. We are limited to how much blood wecan take at once or, cumulatively, over a month. If we need a large blood sample,

we would do this when the animal is anaesthetised and we would not let them recover consciousness.

Where possible, we try to take as many of the tissues and samples we need after the animals havebeen humanely killed after all dosing had been completed.

If we need to take a urine sample for analysis, we would put an animal into a special collection cagewhich is smaller than their normal cage. The animal can still move around.

Other more unusual tests might include assessment of retinal function, assessment of neural function, taking small samples of tissue under general anaesthesia, collection/examination of body fluids such as tear fluid or semen, collection under general anaesthesia and examination of lung washings or spinal fluid, body temperature by rectal thermometer. A minimal degree of restraint or confinement maybe required for some procedures. Where appropriate, positive reinforcement training (using treat rewards) is used to encourage co-operation in (and minimise any stress of) handling/procedures.

Some animals may be used on procedure on more than one occasion (re-use); such re-use is limitedand strict criteria are applied, eg veterinary examination indicates that it is appropriate to do so. Mostanimals are humanely killed at the end of the study to allow detailed examination of the organs, something required by the regulatory authorities to evaluate if there have been any toxic effects on organs and tissues.

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

When dosing an animal by injection or taking blood, the amount of pain an animal feels is similar to what a patient would feel having an injection done by a doctor. If we have to repeatedly inject animalsusing a needle and syringe, we would choose different sites to do this where possible. If we can take blood samples when an animal is deeply unconscious, then we do. If we need to take repeated bloodsamples or need to dose repeatedly then we try and use different sites. Of course, everyone who performs these procedures are trained to a high standard and hold a UK personal licence outlining their competency in the procedure.

Typically we can do this in the animal’s home cage though . Occasionally we may need to take a urinesample for analysis, so we would then put an animal into a special cage which is smaller than their normal cage. The animal can still move around however, and we'd normally introduce an animal to thiscage to acclimatise them to it. . Virtually every animal will get used to their new cage within about 15 minutes and are fine.

Generally, if we have to use any equipment to help us get the results we need, we acclimatise our animals to it so they get used to it and tolerate the procedure when we start dosing them. So, we carefully introduce them to things like restraint gradually, for short periods at first, and usually they accept it after a while. And if they don't acclimatise, we take them off the studies, to stop causing anyharm.

Dosing with pharmaceuticals may cause adverse effects in some studies. A percentage (~46% based on last project) may show transient subtle to mild clinical signs. Moderate signs of adverse effects maybe seen in some animals (~53% based on last project), usually in the higher dose groups. Lethality and/or severe effects are not study objectives in any of the protocols within this licence, and are not expected.

We observe our animals at least twice a day, and the people who do this know the signs when an animal is ill. If an animal is ill, we would check it more frequently, and consult vets and other senioranimal care staff for advice and guidance in its care.

Most animals are expected to experience no, or only mild, adverse effects during the course of the study such as slight weight loss. A small percentage of animals may show more significant adverse effects, such as more marked weight loss, reduced activity, vomiting or tremors. No animals would beexpected to die or to suffer prolonged adverse effects as a result of the procedures, and where necessary early humane end-points are applied, under veterinary guidance as necessary, to prevent this; such end-points might include interventions to discontinue dosing, or to provide supportive treatments, or if necessary to humanely kill the animal.

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

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

On the last project, about 46% of animals experienced mild severity, and around 53% of animals wereclassified as having experienced moderate severity. The moderate severities in the last project were either due to treatment-related signs of moderate severity (mostly in preliminary studies) or because asurgical procedure, e.g. cannulation, was involved.

It's impossible to predict the proportion of severities expected on a service licence like this, as this will be dependent on what study types we are asked to perform, however, a distribution between ‘mild’ and‘moderate’ severities similar to those in the last project are anticipated.

All protocols on this licence are classified Mild or Moderate only, there is no intention to perform anyprocedures that are Severe in nature.

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 22 September 2028

The PPL holder will be required to disclose:

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

Replacement

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

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

Pharmaceutical testing is a mandatory regulatory and legal requirement and provides information on risks to people taking new medicines. At present there are no alternatives that don't use animals that are scientifically, ethically or legally acceptable as replacements for systemic toxicity assessment. TheNHP (e.g. macaques) is a recognised non-rodent species suitable for toxicity studies, and is only usedwhen the dog or pig is shown to be unsuitable.

In vitro and in silico methods (test tube work not using animals and computational methods) are usedin combination with animal studies to inform study designs and assist in understanding of potential toxicity but cannot yet replace in vivo (animal) studies (except on some rare occasions) and only withapproval from global regulatory agencies.

We maintain a constant awareness of regulatory guidance and ensure that where non-invasive methods exist which fulfil the regulatory requirement, they are used in preference to animal studies.

The regulatory requirements for testing in the NHP are mainly for UK/EU regulators, but occasionallyother regulators in other countries like the US for example. If the requirements for these non-UK/EU tests are over and above the requirements for a UK/EU regulators, or the test required is more severe,then we consult the Home Office to ask for prospective authority to run such tests.

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

There are currently no scientific and legally acceptable evaluations of systemic toxicity which will satisfy regulatory requirements and provide sufficient safety data other than use of animals, though validated in vitro tests for specific organs are used wherever possible. As new in vitro methods becomeavailable and achieve regulatory acceptance during the course of this project they will be validated andused to replace in vivo procedures. Where available, review of scientific articles, non-animal methods and other animal data such as metabolism and pharmacology information will be utilised to reduce animal use.

As a specially protected species, the non-human primate is selected for safety assessment studiesonly after careful determination that it is the most biologically appropriate species with relevance toman, and that there is no other acceptable candidate species that is not a primate.

Why were they not suitable?

Although there are test tube tests that can model some parts of how drugs get into our bodies, and how our body deals with them, and can identify undesirable effects, for example, there is no series of test tube tests that brings all these complex happenings together, like we see in animals and humans.

That's why we need to test the new drugs in animals, as they have similar physiology and processesas humans, and that testing gives us a good idea what may happen if they were ever tested in, or exposed to humans.

However, in all cases we will assess whether data already exists or can be generated in other ways other than the use of animals, and that we will ensure that animal reduction, replacement or refinementstrategies and alternatives provided in the regulatory guidance will be considered, and animal use avoided where possible.

A retrospective assessment of replacement will be due by 22 September 2028

The PPL holder will be required to disclose:

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

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen 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 we have used are based on figures of previous usage from previous projects, or a projection thereof (based on estimated incidence) based on requests received from customers in the past. It is, however, impossible to accurately predict the number of studies that may be performed, inthe circumstances.

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

Studies are designed to provide maximal data and statistical power (where appropriate) from the minimum number of animals considering that it is better to increase the number of animals used toachieve the objective than to use too few animals and risk having to repeat the study.

For regulatory studies, guidelines require the number of groups and animals per group to be adequateto clearly demonstrate the presence or absence of an effect of the test substance; core study designs are based on international guidelines where these exist. Otherwise reference is made to standard study designs with input from the Department of Statistics, where appropriate, to identify the optimum number balancing the need to achieve study objectives while avoiding excessive animal use. These internal designs are reviewed and updated in line with changing external guidelines and internal refinements that either minimise numbers or reduce severity.

Whenever possible, common species of animals are used such that a large amount of controlbackground data is available. This reduces the need for large control groups.

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

We will try to get as many outputs as we can from a single animal where possible, without adversely affecting its welfare. So if we need to take several different samples, for example, we will often do thatin the same animal, rather than using separate ones, when possible.

Before our main studies, we use smaller groups of animals in exploratory studies to get an idea of the doses we need to use for the main studies. These preliminary studies are

important as they give us confidence that the doses we are using are correct prior to testing them in bigger groups of animals asrequired by global regulators.

A retrospective assessment of reduction will be due by 22 September 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan 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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use adult non-human primates (macaques). We only use non-human primates whenother species (rats, mice and other large animals like dogs and/or pigs) are unsuitable for scientific reasons.

The models we use are the least invasive procedures, for the least amount of time necessary to get the information we need. They are carried out using standard and recognised techniques by fully trained staff. We also have veterinary clinicians on hand for advice and on the occasions we have toanaesthetise the animals and for general advice on animal welfare.

If we have to repeatedly inject animals or withdraw blood using a needle and syringe, we would choosedifferent sites to do this where possible to minimise local adverse effects. Where appropriate we placetemporary cannulas in blood vessels to reduce the number of needle punctures necessary. If we can take blood samples when an animal is deeply unconscious, then we do so.

For all surgical procedures pain relief will always be provided. Surgical procedures will be carried outaseptically and to at least the Home Office minimum standards for aseptic surgery, and in accordancewith the principles set out in the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017) (LASA is the Laboratory Animal Science Association). Basically any animals who undego surgery will get the same standard of care as a patient who needed surgery in hospital.

For situations involving restraint procedures (e.g. in a restraint device or in a metabolism cage) the animals are habituated to this equipment starting with short periods, then building up. Most animalshabituate fine to this equipment, but if they don't (rare) we remove them from the study.

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

Non-human primates are only used when no other species is suitable to get the information we need.In fact, we have to prove that the primate is the only species that will give us the answer we need (instead of rodents or dogs or pigs) that will translate to the effect we would see in man.

Most of these studies require repeat dosing for days, weeks or months, to assess potential adverseeffects in man, so it is not practical to perform them under terminal anaesthesia.

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

Animal welfare is of utmost importance and Good Surgical Practice will be observed for any animal undergoing surgical procedures. Surgery will be conducted using aseptic techniques (to prevent infection) which meet at least the standards set out in the Home Office Minimum Standards for Aseptic Surgery. Before we start surgery, we agree with a veterinary surgeon what pain killers or antibiotics the animals need both before and after the surgery. When recovering from surgery, we give the animals extra heat and monitor them closely until they start behaving normally again. We then checkthem at least twice daily before they go on study.

During dosing and restraint, animals are constantly and closely watched for signs of distress.

All procedures are subject to ongoing assessment and technique improvement and we participate incross-company working parties on best practice. Animals are regularly reviewed for general health and a veterinary surgeon are on call at all times to assess and relieve any adverse events.

Refinements to improve the animals experience include but are not limited to group housing, environmental enrichment, including novel toys and foods, human interaction, acclimatisation and training to procedures, to move around the cage and to leave the cage voluntarily as required, forageopportunity and calming measures such as stroking/gentle talking are used to help animals have a better experience of restraint.

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

Regulatory guidelines (ICH)

Note for Guidance on Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies S3A(1994)

Pharmacokinetics: Guidance for Repeated Dose Tissue Distribution Studies (S3B) (1994)

Duration of Chronic Toxicity Testing in Animals (Rodent and Non Rodent Toxicity Testing) S4 (1998)Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals S6(R1) (2011)
Safety Pharmacology Studies For Human Pharmaceuticals S7A (2000)Immunotoxicity Studies for Human Pharmaceuticals S8 (2005) Nonclinical Evaluation for Anticancer Pharmaceuticals S9 (2009)
Guidance on Non-clinical Safety Studies for the Conduct of Human Clinical Trials and MarketingAuthorisation for Pharmaceuticals M3(R2) (2009)

OECD Guidelines

OECD 409 – Repeated Dose 90-Day Oral Toxicity in Non-Rodents (1998)OECD 417 – Toxicokinetics (2010)
D 452 – Chronic Toxicity Studies (2009)

Summary of Considerations in the Report from the OECD Expert Groups on Short Term and LongTerm Toxicology (2006)

Other guidelines

Notes for guidance on repeated dose toxicity. Committee for Proprietary Medicinal Products (CPMP),2010.

Guideline on the evaluation of control samples in non-clinical safety studies: checking for contamination with a test substance. Committee for Medicinal Products for Human Use (CHMP), 2005.

LASA/NC3Rs: Guidance on dose level selection for regulatory general toxicology studies forpharmaceuticals.

Notes for guidance on non-clinical local tolerance testing of medicinal products. CPMP, 2001.LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017) Diehl et al. A good practice guide to the administration of substances and removal of blood, includingroutes and volumes. Journal of Applied Toxicology: 21, 15-23 (2001)

Non-Rodent Selections in Pharmaceutical Toxicology (Smith & Trennery, ABPI ‘Points to consider’,2002)

Gad et al. Tolerable levels of nonclinical vehicles and formulations used in studies by multiple routes inmultiple species with notes on methods to improve utility. International Journal of Toxicology: 1-84 (2016)

NC3Rs: Overview of 3Rs opportunities in drug discovery and development using non-human primates(Drug Discovery Today, 2017)

NC3Rs: Recommendations from a global cross-company data sharing initiative on the incorporation ofrecovery phase animals in safety assessment studies to support first-in-human clinical trials (Regulatory Toxicology & Pharmacology, 2014)

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 Named Information Officer, colleagues in AnimalTechnology, and by attending appropriate training courses and conferences, or getting feedback fromsuch events.

A retrospective assessment of refinement will be due by 22 September 2028

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 forfuture work of this kind? During the project, how did you minimise harm to

the animals?

18. Development and validation of in vivo preclinical models to substantially refine and reduce the number of animals subjected to severe procedures in snakebite envenoming research

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, ortheir effects, in man, animals or plants
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

Antivenom, Venom, snakebite envenoming, neglected tropical disease, refinement

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 orscientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop a refined mouse model of envenoming which accurately reflectsreal-world scenarios and will allow:

- flexibility in testing different therapeutic formats (antivenoms and other drugs) at differenttimepoints after envenoming,
- substantial reduction of the total number of animals required for robust efficacy testing,

- a maximum ‘moderate’ severity rating to be applied, once established, and validated.

A retrospective assessment of these aims will be due by 29 September 2028

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 advancedor 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?

Snakebite is estimated to kill approximately 140,000 and maim a further 400,000 people every year.Globally, all snakebite envenoming therapies, both existing and in development, are assessed for efficacy using a 40-year-old, World Health Organization (WHO)-endorsed model of “neutralisation ofvenom lethality”. Whilst simple, this assay is not reflective of human envenoming and requires large numbers of mice (n=25/experiment/venom) to be subjected to highly distressing severe procedures. The objective of this proposal is to develop and validate a new *in vivo* model of envenoming which more accurately reflects a human snakebite scenario, which will have a maximum severity limit of ‘moderate’, and will require considerably fewer mice/experiment. Ultimately the new assay will providemore pathologically relevant data on the efficacy of current and future envenoming therapies.

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

The development of a suitable, fully validated and established model of envenoming has been identified as an urgent need in the development and assessment of treatments for envenoming (Knudsen, et al,*Toxins* 2020, 12(9), 528). The development of envenoming models has been progressing ad-hoc in many recent publications, but differences in experimental methods and lack ofpublished data on the development of these models hampers confidence and their wide-spread adoption, especially by that of regulatory authorities. Furthermore, many of the newer models in use still rely on the requirement for establishing median lethal doses and nearly all rely on the use of lethality as an endpoint. These assays are becoming challenging to justify to authorities regulating animal experimentation in increasingly more countries.

Here, by focusing on optimising and fully validating a new model of envenoming, which is more reflective of real-world envenoming, and by providing full transparency in its development and demonstration of its reproducibility, we hope to develop an assay that can be used by academics, industry, and key stakeholders globally. Importantly, it will aid in the progression of the next generationof envenoming therapies, which will be required to progress through more rigorous pre-clinical testing,as compared to existing envenoming therapies, before progressing to human clinical trials.

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

Potential 3Rs impact: Our laboratory routinely uses 300-800 mice/year in severe rated conventionalassays, which equates to approximately 3-5% of the UK total of mice used in

severe experiments.

Establishment of a new, refined assay would reduce severe mouse use in our laboratory to 0, substantially reducing total UK academic severe mouse use values, and result in using an estimated40% fewer mice/experiment at a lower maximum severity limit.

Academically, the conventional assays and their modifications are used routinely. To provide context, arecent review of pre-clinical testing of antivenoms for just sub-Saharan Africa, examining only the neutralisation of lethality (thus excluding the mouse determination of lethal doses required for the assay) identified 18 papers in which 3,930 mice were used (Ainsworth, et al 2020, *PLOS Neglected Tropical Diseases* 14(8): e0008579). If these experiments were to be repeated using the proposed model, we estimate this would require 15 mice/experiment maximum, 40% fewer mice than used previously (a saving of 1572 mice).

Industrially, the WHO recommends full *in vivo* testing of each batch of new or existing antivenoms. There are approximately 50 antivenom manufacturers worldwide, producing in the region of 120 antivenoms. It is difficult to accurately assess the global numbers of mice used in the current conventional assay, as it will vary substantially on the basis of how many venoms the antivenom is indicated for. However, a personal communication with a single antivenom manufacturer detailed theuse of 2,000 mice per month to fulfill batch release criteria. Thus, numbers of mice globally subjected to this severe assay annually for fulfillment of antivenom batch release regulatory requirements by antivenom manufacturers is likely to number into several hundreds of thousands, annually. If all industrial manufacturing were to adopt the proposed model, after establishment, and if performed in the same manner as proposed, we would expect an estimated 40% reduction in mouse numbers usedworldwide.

Potential snakebite victim impact: As results from murine pre -clinical efficacy assays remain the primary means to assess/predict antivenom performance prior to their licensed use in humans, despitesuch animal assays not accurately reflecting a snakebite or snakebite pathology, products lacking efficacy still find their way to market. Improved, more robust models of assessing antivenom efficacy will have the potential to reduce the number of poorly efficacious antivenom available, leading to greater confidence in therapeutic efficacy and will aid in reduction of death and morbidity due to snakebite.

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

In addition to publication of protocols, we will perform three key activities to encourage wider uptake ofthe improved model.

Presentation at key international conferences. At least one of three key international conferences onvenom biology will be targeted; The bi-annual Gordon Research Conference on Venom Evolution, Function and Biomedical Application, The bi-annual International Society on Toxinology conference, orthe Annual Oxford Venoms and Toxins conference. These three conferences are well attended by key stakeholders, including academia, industry, governments and the WHO. In addition to orally presentingthe model, we will approach organisers to hold breakout/target sessions on venom *in vivo* experiments.

We will commission a professional scientific communicator to generate simple, clear trainingmaterials for application of the new technique in other laboratories, in addition to encouraging adequate reporting and improved consideration of the 3Rs.

We will organise a virtual workshop to communicate a) the new model, b) to discuss the wider issues with *in vivo* venom pre-clinical testing and c) to discuss the need for more systemic adoption of3Rs principles in animal envenoming research. We will invite potential end users, antivenom manufacturers, clinical specialists, regional/global patient stakeholders (e.g. WHO/MSF/Ministries of Health) and key regulatory bodies (e.g. WHO, UK Medicines and Healthcare products Regulatory Agency, etc) to actively contribute to the discussion.

Species and numbers of animals expected to be used:

- Mice: 2400

Predicted harms

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

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

Adult (18-22g) male CD1 mice have been the model of choice for envenoming studies globally. To reduce variables and to build on extensive prior literature and expertise gained using these models, wewill develop our new model of envenoming using CD1 mice. The main alteration from the existing normwill be to use animals from both sexes in these experiments. By using a strain that is commonly used globally in such experiments, it will be much easier to encourage uptake and implement the refined protocol in these laboratories once established.

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

Mice will receive an injection of venom, which if left untreated, would lead to death within a specified time frame. Mimicking real-life envenoming scenarios, mice will be treated with a therapy (antivenomor other) after a specified delay, in order to reflect the time it takes for snakebite patients to reach hospital.

The experiments will last no longer than 1 day, and will terminate early if animals progress to humaneendpoints to ensure no animal succumbs to the effect of envenoming.

With the exception of a small number of mice at the outset of the project or working with a previously untested venom, all other mice will be given long-lasting pain relief prior to venom injections. These mice are to assess the effect of pain relief on envenoming progression, as previous experiments havedemonstrated some pain relievening drugs can worsen or accelerate envenoming (ibuprofen, asprin, buprenorphine).

We also plan to explore a further refined assay, performing similar experiments with mice under terminal anaesthesia, to allow detailed monitoring of vital signs during envenoming and treatment, withthe animal being unaware of any venom effects. It is currently not possible to complete the majority of this work in terminally anaesthtised mice as our ability to assess response to treatment currently relieson the behavouir of conscious animals. It is hoped the pilot work performed in this project using terminally anaesthetised mice will enable progression to a greater proportion of future assessment of envenoming therapies being performed in this manner.

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

Despite the use of long-lasting analgesia, we expect the mice to feel some pain and discomfort duringthe procedure. During the study, it is likely that some envenomed mice may experience the effects of the specific venom they have received. This can include difficulty with breathing or mobility, and reluctance to display normal behavioral traits, such as eating and nesting. We expect the effects of enevenoming to either i) subside due to successful intervention, ii) persist at a moderate severity untilthe end of the short experiment, or iii) progress in severity to a humane endpoint, where the mice will be humanely euthanized.

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 animaltype)?

Initally, during pilot study experiments, where we attempt to optimise envenoming route, dose and perform detailed observation on progression of envenoming (allowing for identification and validation ofearly humane endpoints), we expect the severity limit to be classed as severe. We expect about 20% of the mice to be returned under a severe category. However, once the optimal route, venom dose andearly humane end points have been established for a particular venom, enabling the experiment category using that venom to be rated as moderate, we will seek to amend the severity category of future experimentation with that venom to moderate.

For mice under general anaesthesia, the severity limit will be non-recovery, i.e. the animals willenvenomed and treated and subsequently put to sleep whilst under anaesthesia.

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

- Killed

A retrospective assessment of these predicted harms will be due by 29 September 2028

The PPL holder will be required to disclose:

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

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

Snake venoms contain a multitude of toxins that work simultaneously on many physiological systems to synergistically cause pathology/death. Consequently, it has proven impossible (despite extensive efforts by our laboratory – see the section

"Replacement") to devise i*n vitro* tests that accurately reflectvenom lethality or neutralising agent efficacy.

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

We and others have considered many other models for pre-clinical testing of envenoming, and continue to do so. These include - 'antivenomic' methods, chick embryo models, wax-moth larvaemodels, *ex vivo* human and mouse tissues and chick-bi venter preparations.

Why were they not suitable?

Despite extensive attempts, the vast majority of *in vitro* approaches developed have not been able to provide a satisfactory correlation to *in vivo* efficacy models. This is likely due to the way snake venomsimpart their effect, with different toxins found in the same venom acting on different targets in different organ systems simultaneously. There is therefore the requirement for any therapy to be able to neutralise pathologies in multiple compartments simultaneously, and thus animal models, despite their drawbacks, remain essential for efficacy outputs, as they are considerably more informative than singlereadout *in vitro* or limited *ex vivo* models.

It is clear that murine models of envenoming will remain essential for regulation, translation and clinicaldevelopment of snakebite therapeutics into the future. Due to the inevitability that mice will continue to be used in these experiments, it is ethically justified that attempts to refine and reduce their use are robustly explored.

A retrospective assessment of replacement will be due by 29 September 2028

The PPL holder will be required to disclose:

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

Reduction

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

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

All the numbers below are estimates based on assumptions and will be refined as pilot and variabilitydata becomes available. Fot the total project, we estimate we will use 2100 mice.

<u>Protocol 1</u>
We have estimated approximately 1480 mice for the development and validation of the envenomingmodel proposed in this project (protocol 1).

100 mice for the establishment and validation of reference physiological ranges in CD1 mice,including validation of analgesia on reference ranges and validation of point of care tests.

300 mice for Development of a novel *in vivo* model of murine envenoming. We estimate to investigate five injection routes - assuming 15 mice/route = 75 mice x 4 venoms = 300 mice

1080 mice for examining therapeutic efficacy in the model. We estimate 15 mice/therapy/venom.We wish to establish efficacy of 6 antivenom and 3 small molecule inhibitors (9 therapy total) vs.6 venoms = 15 x 9 x 6 = 810.

Protocol 2

We have estimated approximately 620 mice for the development and validation of the non-recoveryenvenoming model proposed in this project (protocol 2)

100 mice for the establishment and validation of references physiological ranges in CD1 micewhilst under anaethesia

300 mice for development of a novel *in vivo* non recovery model of murine envenoming. We estimate to investigate five injection routes - assuming 15 mice/route = 75 mice x 4 venoms =300 mice

270 mice for establishing the efficacy of therapeutics in the non recovery model of envenoming with 2 venoms and 9 thearpeutics (as above). Therefore 15 mice x 9 therapeutics x 2 venoms =270 mice.

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

The main step we have taken is to reduce the number of mice required for assessment of a therapy. The current median effective dose assay requires 25 mice per venom per antivenom. By taking an alternative minimum anticipated biological effect level (MABEL) and gold standard approach, we are confident we can reduce the number of animals required to demonstrate therapeutic efficacy overall by40%

Experimental numbers will be refined as pilot and variability data becomes available, in consultationwith specialist statisticians and through use of tools, such as the NC3Rs Experimental Design Assistant.

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

We are taking the following measures to optimse the number of animals used in our project.

Pilot studies to validate reference values, dose routes and variation in the new model will beperformed. Results from these pilot experiments will be used to inform improved statistical analysis in order to optimize the number of animals used.

A mouse envenoming tissue bank will be established and made available for academics to use, with the objective of reducing unnecessary mouse experimentation in the future. This will includeplasma for coagulation and immunoprofiling in response to different venoms, tissues for RNA profiling and spleens for naive B-cell mouse library controls.

Finally, fresh mouse skin will be made available to colleagues to aid development of *ex vivo*
mouse models of local envenoming.

A retrospective assessment of reduction will be due by 29 September 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan 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 welfarecosts (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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be developing a new animal model of envenoming during this project. The current widely usedmodel is rated as 'severe', does not use analgesia and requires large numbers of mice. The model weintend to develop will use analgesia by default, and be based around the discovery and validation of early humane endpoints. We hope this will enable a study whereby the highest severity level experienced by mice will be 'moderate', thus ameliorating the substantial pain, suffering and distress experienced by many animals in the current and globally used assay.

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

Models of envenoming in non-sentient or less-sentinet animals have been developed (such as *ex ovo*,*in ovo*, wax moth larvae or zebrafish larvae). However, they frequently lack one or more requirements for demonstrating therapeutic efficacy against venoms which simultaneously targets multiple systems.

For example, *in ovo* models are unable to demonstrate the neutralisation of one of the most medically relevant venom components (three finger toxins), while many insect models lack the vertebrate- specific targets of many venoms and typically require substantially more venom to induce insect death.

The reality is that despite extensive efforts over the past two decades, no murine alternative model ofenvenoming has been developed that satisfies the requirements of regulators, and is unlikely to change in the near future. As these murine models remain the primary mode of assessment of envenoming therapies prior to their use in humans, it is essential that models used can provide an accurate representation of envenoming, which is currently not-possible with less-sentient or more immature life stages. However, in this project, we are exploring the development of an assay using terminally anesthetised mice, with a view to this refinement being implemented more widely in future.

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

This project is centered on refining the currently globally used standard envenoming protocols. The major refinement will be for improved and validated monitoring which will enable the implementation ofendpoints at a lower severity level than is widely practiced. Other refinements will be for the absolute requirement for analgesia use in any developed protocol. Experiments using terminal anethesia have not been performed previously, and will provide valuable information about potential further refinementusing this "less sentient" model.

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

We will follow current LASA publications on best practice and NC3R's continuously updated guidancedocuments which can be accessed via their respective web pages.

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

This work is funded by the NC3Rs, and as such we are obliged to not only remained informed aboutadvances in the 3Rs and implement them, but to champion them to wider audiences as well.

We will stay informed through subscribing to both the NC3Rs and LASA websites, routinely attendingNC3Rs seminars, and attending (and presenting) at suitable conferences (e.g. International Conference on Laboratory Animal Science and Welfare).

A retrospective assessment of refinement will be due by 29 September 2028

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 forfuture work of this kind? During the project, how did you minimise harm to the animals?

19. Developing minimally invasive interventional cardiology techniques for studying and treating atrial fibrillation and other arrhythmias in the horse

Project duration

5 years 0 months

Project purpose

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

Key words

Horse, Heart, Electrophysiology, Therapy

Animal types	Life stages
Horses	adult, aged
Ponies	adult, 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.

- Uses Cats, Dogs or Equidae

Objectives and benefits

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

What's the aim of this project?

Heart rhythm abnormalities in the horse have a rare but very important impact on horse welfare andhuman safety.

The aim of this project is to develop safe and minimally invasive imaging techniques to investigate theelectrical and contractile activity of the heart, so allowing better evaluation and treatments for heart rhythm abnormalities in horses. Such techniques are routine in

humans, and sometimes used in dogs,but the technology is only recently sufficiently advanced to allow their use in horses.

A retrospective assessment of these aims will be due by 30 September 2028

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 advancedor 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?

Treatments for rhythm abnormalities in horses are very basic and lag well behind the options for humans or even dogs. For example, while many horses with persistent atrial fibrillation can be treatedand successfully return safely to their previous use, recurrence is a problem in a large proportion, withfigures of up to 64% reported. Intermittent rhythm abnormalities can be particularly frustrating to evaluate and treat. Treatments to prevent recurrence of persistent AF and better evaluate and treat these intermittent problems are therefore required so that horses can be restored to normal heart function. While some horses can be left in atrial fibrillation or tolerate abnormal rhythms, restoringnormal heart function is optimal to improve welfare and is likely to reduce the likelihood of sudden cardiac collapse and/or death.

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

By the end of this project we should have:

- developed minimally invasive techniques that allow us to safely investigate and better treatabnormal rhythms (including atrial fibrillation) in live horses
- developed a better understanding of the physiology of the normal horse heart
- developed laboratory based techniques that allow us to investigate heart rhythm abnormalities inthe horse

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

The primary benefits will be horse welfare and human (owner) safety. Atrial fibrillation (AF) is the mostcommon heart rhythm abnormality in the horse (prevalence up to 5%) and usually causes dullness, unexpected poor performance or unwillingness to exercise. It can also predispose to exercise-inducedbleeding from the nose and it may be one of the causes of sudden death in horses. While collapse and sudden death is rare it has serious impact for owners and rider safety

A better understanding of diseases like AF in the horse will also benefit our understanding of AF in thehuman and dog. The horse is unusual in that it has a high prevalence of this condition, without significant evidence of underlying heart disease. A better knowledge of the physiology of the equine heart and the development of pathologies such as atrial fibrillation will bring tangible benefits to our general understanding of AF in particular.

Once the techniques are developed and proven to be safe and effective, they will be offered as a cinical service to horse owning clients. This substantially increases the options for treatment of arrhythmias in the horse, providing as yet unavailable treatment for intermittent rhythm abnormalitiesand perhaps preventing the recurrence of problems like atrial fibrillation (up to 64% recurrence following treatment according to some studies).

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

We will collaborate with others in the UK and the world doing similar work to ensure that the development of these techniques is optimal. Experiences will be published, whether successful orunsuccessful.

Species and numbers of animals expected to be used:

- Horses: 8-12
- Ponies: 8-12

Predicted harms

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

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

Only horses are suitable as subjects for this study due to the need to develop this as a clinical technique for horses. The physical size and species-specific differences in heart anatomy and physiology from other large animals (e.g. bovine) make this necessary. Lone (also known as idiopathic) atrial fibrillation appears to be unique to horses and some human athletes. As a consequence, the information gained during development of these techniques, from horses without AF, will be crucial to our understanding of this important disorder. Bovine species for example do notdevelop AF without significant underlying disease.

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

This study will use normal horses that are destined for euthanasia due to chronic intractable problems that are affecting their health and/or welfare. Veterinary clinicians with no interest in this study, throughdiscussion with the owners, will make the decision for euthanasia. Any horses with acute clinical conditions where immediate euthanasia is required, or those horses with conditons that preclude travelfor euthanasia, will be excluded from the study. There will be informed client consent. There will be noeconomic incentive to the owners, other than the fact that the costs of euthanasia will be borne by the project. Horses will have intravenous catheters placed as per normal for euthanasia. Instead of euthanasia, a general anaesthetic will be given to render the horses unconscious, without stopping theheart. Up to six further intravenous or intra-arterial catheters will then be placed while the horse is anaesthetised to allow internal access to the heart. Electrodes will be used to detect electrical activity and contraction patterns within the right heart. The electrodes will then be placed into the left heart, again to detect electrical activity and contraction. Finally special electrodes will be used to cauterise areas in the heart that are known to cause arrhythmias in humans. The total time of the procedure willbe maximum 6 hours, after which the horse will be euthanised.

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

Adverse effects will be minimal. A single intravenous catheter in the left jugular vein will be placed byan experienced veterinary clinician and personal licence holder. All subsequent vascular access will be under general anaesthesia, so the horse will be unaware of the procedures. If there are any adverse effects from the procedure, e.g. it induces dangerous heart rhythm problems or serious bleeding, the horse will immediately be euthanised as planned.

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 animaltype)?

All horses will be non-recovery.

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

- Killed

A retrospective assessment of these predicted harms will be due by 30 September 2028

The PPL holder will be required to disclose:

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

Replacement

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

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

The proposed techniques must be developed in live horses to enable translation into a safe andeffective technique for use in clinical practice.

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

Post mortem specimens

Why were they not suitable?

No realistic and complex enough models exist for the horse heart; and post mortem specimens would not accurately represent the live technique proposed for treatment. We aim to also develop a laboratory model that uses hearts dissected after death. This should provide us with a convenient toolfor investigation the basic electrophysiology of the equine heart, minimising use of future live horses. Comparison of this laboratory technique/tissue with the live, but anesthetised, animal work is thereforecrucial.

A retrospective assessment of replacement will be due by 30 September 2028

The PPL holder will be required to disclose:

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

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen 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?

Because these techniques are new and the purpose of this project is development, it is very difficult toestimate the number of animals required. Experience from developing other clinical techniques wouldsuggest that 8-12 animals should allow acquisition of the necessary skills and data. After this numberwe will also have knowledge of the inherent variation in horse heart tissue and effects of the procedure.

Should the necessary expertise and knowledge be acquired before this upper number is reached, oralternatively in the unlikely event that the technique be deemed impossible, the project work will be stopped.

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

A minimal number of experimental animals will be used, allowing development of the technique while also generating scientifically useful data. One of the team is an experienced human electrophysiologist who has already translated skills to experimental large animals (pigs). The applicantis an experienced equine cardiologist. Sequential experiments will be performed to a set degree of confidence in the technique, while also gaining knowledge of inherent tissue electrophysiological variation and procedure effects, estimated to be maximum 12 animals.

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

Some aspects of the techniques will be optimised prior to experimental work in either clinical cases (non-invasively) or in post mortem specimens. For example optimising cardiac imaging to enhance placement of necessary electrodes, practising the transfer of electrodes from the right side of the heartthrough the septum to the left. Although not equal to the live situation, this preparatory work will maximise the chances of effective development in the live horses.

A retrospective assessment of reduction will be due by 30 September 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything otherscan 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 welfarecosts (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 modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Only horses are suitable as subjects for this study due to the need to develop this as a clinical tool for horses. The physical size and species-specific differences in cardiac anatomy and physiology from other large animals (e.g. bovine) make this necessary. The choice of horses already destined for euthanasia and the non-recovery protocol will minimise suffering while also allowing us to measure theeffects and the outcomes of our techniques. From the horse perspective, this is no different from the intended euthanasia, with one intravenous catheter inserted before the horse is rendered unconsciousfor the procedure.

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

Non-recovery anaesthesia protocol opted for in this study to minimise harm. Immature animals wouldnot be appropriate, nor would other species for reasons already covered.

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

As per our usual protocol for anaesthesia and/or euthanasia of clinical cases, a single intravenous catheter in the left jugular vein will be placed by an experienced veterinary clinician. For horses that are averse to intravenous injections, we have in-house behaviour expertise to help minimise stress andtrain horses, e.g using overshadowing techniques. All subsequent vascular access will be under deep general anaesthesia. The horses will be anaesthetised by highly trained anaesthetists, with significant experience in equine anaesthesia, ensuring they maintain unconsciousness until euthanasia.

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

The following sites provide excellent resources from which we will seek guidance for best practice inrefining the experimental protocol: https://nc3rs.org.uk/ ; and https://norecopa.no/prepare

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

Maintaining awareness of these concepts and advances by using sites such as:

https://nc3rs.org.uk/

A retrospective assessment of refinement will be due by 30 September 2028

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 forfuture work of this kind?
- During the project, how did you minimise harm to the animals?