



Home Office

# **Animals (Scientific Procedures) Act 1986**

Non-technical summaries for project  
licences granted 2016 that require a  
retrospective assessment



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# 1. Role of nitric oxide and reactive oxygen species in cardiac function

## Project duration

5 years 0 months

## Project purpose

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

## Key words

*No answer provided*

## 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
- Required at inspector's discretion

## Objectives and benefits

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

### What's the aim of this project?

The main objective of this project is to study how nitric oxide (NO) and reactive oxygen species (ROS) regulate cardiac function.

Our previous work and that of others have already identified changes in the production of these messenger molecules in situations where adverse left ventricular remodelling takes place leading to heart failure or heart rhythm disturbances.

Based on these results we will:

Test the beneficial effects of modifying NO and ROS signalling pathways on hypertrophy, inflammation, atrial fibrillation and diabetes.



Study other genes that will serve to identify novel molecular targets for future therapeutic interventions.

As these conditions are common (1 in 4 life-time risk of development) and often requiring long, and often acute hospital stays, the cost of caring for patients with heart failure (HF) and/or atrial fibrillation (AF- most common heart rhythm disorder) has been estimated to consume *ca.* 4% of the annual NHS budget in the UK. These data underscore the major public health burden posed by HF and AF in our society and the need for a better understanding of the mechanisms that control the evolution from myocardial remodelling to pump failure and rhythm disturbances.

The Framingham Heart Study was the first to demonstrate that diabetes (DM) and obesity are independent risk factors for developing AF. AF is associated with considerable morbidity, decreased quality of life, and increased mortality as a consequence of heart failure and thromboembolic events. AF accounts for 25-33% of all ischemic strokes. Diabetes is also a common condition and growing healthcare burden, particularly in developing countries. The total number of people with diabetes is estimated to rise from 366 million in 2011 to 552 million by 2030. A high proportion of patients (50-75%) will develop diabetic cardiomyopathy. An important need remains to further delineate the basic mechanisms of diabetic cardiomyopathy and to translate promising therapies in preclinical models to humans.

### **Retrospective assessment**

Published: 28 July 2021

#### **Is there a plan for this work to continue under another licence?**

Yes

#### **Did the project achieve its aims and if not, why not?**

Our programme of work was focused on studying the role of nitric oxide (NO) and reactive oxygen species (ROS) in the heart and disease. We invested most of our efforts experimenting with models of diabetes and atrial fibrillation. We have completed most of the work planned on these two areas and results have been published or submitted for publication.

The work planned to study the effects of inflammation and hypertrophy has not been completed and will be continued under the authority of a new project licence, together with new objectives.

Please relate this section to the stated objectives in the original application (including any amended or additional objectives as necessary)

Objective 1: Determine whether modulating myocardial or systemic inflammation, restoring myocardial NO-redox balance is sufficient to attenuate adverse myocardial remodelling and functional deterioration in response to stress or metabolic abnormalities.

We have shown the importance of the interaction of nitric oxide synthase with other counterparts to maintain atrial function and how this association can be impaired in disease. The loss of nitric oxide synthase in atrial fibrillation (AF) is responsible for the



maintenance of a pro-arrhythmic state in the heart. These experiments also demonstrated for the first time that the nitroso-redox balance is different between the right and left atria. Using mice carrying a mutated protein kinase A that is unresponsive to the redox environment we have shown that this protein is involved in the release of calcium from the lysosomes. This is a new role that allows PKA to regulate the cardiac response to ischaemia reperfusion.

Our data has also contributed to understand how nitric oxide synthase localisation regulates the downstream effects of NO, and therefore, cardiomyocyte function under physiological and pathological conditions.

Following our initial findings showing that the precursor of procalcitonin is increased in patients who develop atrial fibrillation after cardiac surgery, we have proven that paracrine signalling of cardiac calcitonin is a key regulator of atrial fibrogenesis and arrhythmia. Objective 2: Test new pharmacological approaches to treat diabetic cardiomyopathy and the increased susceptibility to atrial fibrillation and inflammation.

We have demonstrated that BH4 oral administration exerts cardioprotective effects in the presence of diabetes. We have been able to prove that it is possible to modify intracellular levels of nitric oxide in order to modify ventricular function and energy metabolism. We have also demonstrated that it is possible to increase NO availability in human cardiac tissue by oral BH4 supplementation opening the possibility of using this approach to treat cardiac disease. On the other hand, we proved that oral administration of BH4 does not protect mice from atrial arrhythmias.

In addition to the treatment with oral BH4, we demonstrated that high extracellular glucose promoted pro-inflammatory gene expression and pro-atherogenic functional characteristics, through glycolysis-dependent mechanisms.

Our experiments also suggest that BH4 acts as an anti-inflammatory molecule that can protect heart function in the presence of hypertrophy.

Objective 3: Characterise the interplay between known cardiovascular risk factors such as diabetes, inflammation and AF.

We have evaluated the effects of diabetes and inflammation on the development of AF.

Using optical mapping we have detected alterations in conduction velocity and action potential duration in specific areas of the diabetic atria suggesting that prolonged uncontrolled hyperglycaemia is sufficient to create a substrate favouring the induction of atrial arrhythmias. The causes driving these regional changes in the atria will evaluate in the new PPL.

**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.**

**What are the potential benefits that will derive from this project?**

By elucidating the mechanisms leading to an increased production/bioavailability of reactive oxygen species in the diseased myocardium, our research will increase our



understanding of myocardial NO-redox biology, identify new biomarkers of disease evolution and enable us to test whether specific interventions targeted to restore a normal myocardial NO-redox balance will prevent or retard the evolution towards HF and AF in chronically stressed hearts.

### **Species and numbers of animals expected to be used**

#### **What types and approximate numbers of animals will you use over the course of this project?**

We have estimated that a maximum of 10,900 mice will be used over a period of 5 years.

### **Predicted harms**

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

#### **In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?**

We expect that the level of severity will be moderate for the majority of mice. The experiments we are proposing include phenotyping new genetically modified mouse models as well as generating mouse models of human disease, notably diabetes and cardiac hypertrophy secondary to pressure overload (e.g., a situation that can be observed in patients with high arterial pressure or aortic valve stenosis) on which we could also test new therapeutic strategies.

Characterisation of these models would typically include non-invasive (e.g., echocardiography and/or magnetic resonance imaging) under anaesthesia and/or invasive (e.g. Left Ventricular haemodynamics or transoesophageal pacing) evaluation of cardiac function under terminal anaesthesia and ex-vivo investigations in myocardial tissue and cells.

Type 1 Diabetes will be induced by the administration of streptozotocin that is toxic to pancreatic beta cells, type 2 Diabetes by dietary modification or the use of transgenic models. These mice will experience high blood glucose, which will be monitored, and polyuria/polydipsia, therefore, they will have free access to water and absorbent bedding will be added to the cage whenever necessary. Cardiac hypertrophy will be induced by aortic banding or by administration of angiotensin II or isoproterenol via an osmotic minipump implanted subcutaneously. We routinely apply pre- and postoperative care to the mice that undergo procedures (anaesthesia/analgesia). After they recover from surgery most mice will be free from symptoms for the duration of the study. Occasionally animals will suddenly develop deep abdominal breathing, which is the first sign that they have developed heart failure. Suffering is minimised by using this as an immediate humane end-point. These animals are likely to experience a severity level that is severe. However, the majority of animals are killed humanely at the scientific endpoint without exhibiting any symptoms of heart failure.

At the end of these experiments the animals will be humanely killed and tissues collect for biochemical and histological analysis.



## Retrospective assessment

Published: 28 July 2021

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

Please describe the harms in terms of your reflective assessment of the pain, distress, suffering, or lasting harm you consider the animals have experienced. Do not simply list the procedures applied but describe the harms in terms of animal based outcomes e.g. animals experienced mild discomfort as subcutaneous tumours were allowed to grow to around 10mm in diameter before they were humanely killed.

Most of the mice used during this period were genetically altered animals maintained for breeding purposes and in protocols where we evaluated the function of the heart. These were not associated with lasting harm and were classified as sub-threshold or mild severity.

We used 1300 adult mice for the study of diabetes in a protocol with moderate severity. Half of these mice were injected with streptozotocin, an alkylating agent that targets insulin-producing beta cells reducing the production of insulin in the pancreas. These mice experienced mild discomfort due to the injections. Mice were normally kept between 6 weeks and 12 weeks on this protocol. During this time, some received additional general anaesthetics to allow for noninvasive imaging using ultrasound or testing if atria were prone to develop arrhythmia, which were not associated with additional adverse effects. Some received drugs in their food free from adverse-effects and blood samples were sometimes taken, which only caused transient discomfort. All animals were humanely killed at the end of the experiment under deep anaesthesia following removal of the heart for further biochemical analysis.

We induced changes in myocardial NO-redox state in 311 mice using gene transfer. Gene transfer was achieved using a sterile solution of a replication deficient adenovirus into the heart by thoracotomy and myocardial injection under direct visualisation. In this group, 6 mice died during surgery and therefore only experienced transient distress from induction of anaesthesia. After thoracotomy, animals were always given analgesia, fluid replacement as necessary and heat therapy to aid recovery. All animals that were allowed to recover from anaesthesia were given pain killers to minimise pain, nevertheless, it is likely they experienced some pain and discomfort as a result of the surgery, and therefore considered to have experienced moderate severity. Most of the mice recovered well with pain killers and body weight loss less than 10%. Only 6 mice experienced severe severity. One of them suffered wound breakdown, this was reported as an unexpected side effect to the HO and PPL was amended appropriately. The other 5 mice made slow recovery with weight loss greater than 15% and showing hunched body position or inactivity, symptoms that triggered humane endpoints.

## Replacement

### **State why you need to use animals and why you cannot use non-animal alternatives.**

Although we are actively engaged in the process of reduction, we recognise that at present, it would be impossible to model the behaviour of such a complex system in silico



or using cell lines. We are carrying out a number of complementary experiments in humans and surplus human tissue from patients undergoing cardiac surgery as well as developing a computational model of the healthy and diseased myocardium to guide our experiments and minimise the use of animals and *in vivo* experimentation.

In order to characterise the function of a specific gene on NO-redox balance we will initially make use of isolated cardiomyocytes and perfused heart preparations from mice, as these experiments are the least severe. To complete these first investigations and gain insights of the function of these genes in disease. We will need to use models of human disease (diabetes, hypertrophy).

## **Retrospective assessment**

Published: 28 July 2021

### **What, if any, non-animal alternatives were used or explored after the project started, how effective were they and are there any lessons worth sharing with others?**

We have developed computational models of the healthy and diseased myocardium to guide our animal experiments. This *in silico* work is focused on the modelling of the atrial action potential and ion currents.

We are using BioBank resources to evaluate the role of NOS1 adaptor protein in atrial fibrillation using Mendelian randomization.

In order to test the acute effects of tetrahydrobiopterin on cardiac function we are planning to recruit diabetic patients for a new clinical trial.

## **Reduction**

### **Explain how you will assure the use of minimum numbers of animals.**

The minimum numbers of animals required have been carefully reviewed by the funding agencies and have been based on power calculations.

Numbers have been reduced in single cell based experiments (cell shortening, calcium measurements and patch clamp, immunohistochemistry and molecular studies) due to the availability of three different cellular electrophysiology set ups as well as other equipment (e.g. confocal microscope/FRET system) on site, allowing us to have several scientists working on cardiomyocytes isolated from one heart.

To maximise the use of animals, we share our surplus tissue with other groups with non-cardiac research interests.

As part of the strategy to reduce the number of animals we are developing the optical mapping of the atria. This technique will allow us to assess cardiac electrical properties in perfused hearts instead of using live animals, thereby reducing the number of *in vivo* experiments.

## **Retrospective assessment**



Published: 28 July 2021

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

In addition to appropriate power calculations, numbers have been reduced in single cell based experiments (cell shortening, calcium measurements and patch clamp, immunohistochemistry and molecular studies) due to the availability of three different cellular electrophysiology set ups as well as other equipment (e.g. confocal microscope) on site, allowing us to have several scientists working on cardiomyocytes isolated from one heart.

Because of our interest in atrial fibrillation and heart failure we used both the atrial and the ventricular myocytes/tissue from the same heart and shared our models with groups investigating non-cardiac aspects of our murine models, thereby maximising the use of animals.

### **Refinement**

#### **Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.**

Surgical and imaging techniques are constantly refined and used by all the groups in our Department. Any step forward on the refinement of these procedures that leads to minimise welfare cost to the animals is actively sought and shared in our regular animal welfare meetings.

We have modified the protocol to induce type 1 diabetes in mice. This allows us to generate a less severe model where we can study early changes in the myocardium. The severity and variability of previous protocols have been significantly reduced. Diabetic mice are subject to regular screening (blood glucose measurements and body weight monitoring). Careful attention is given to excess consumption of water or frequent wetting of bedding material. Absorbent bedding is added whenever necessary.

Refinements have also reached other techniques such as the reduction in volume of blood required for glucose measurements or the delivery of drugs by using implants instead of multiple injections.

Strict humane end-points, evaluated by Vets, will be applied to minimise suffering of the animals.

We routinely apply pre- and post-operative care to the mice that undergo procedures, ie, analgesia, heat support, access to water-softened chow, subcutaneous fluids and oxygen.

Mice are allowed to recover in a heated chamber and checked after recovery. Recovery surgery is performed earlier in the day to allow sufficient monitoring within normal working hours.

### **Retrospective assessment**

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**With the knowledge you have now, could the choice of animals or models used have been improved at all? How did you minimise harm to animals during the project?**

We routinely apply pre and post-operative care to the mice that undergo procedures, ie, analgesia, heat support, access to water-softened chow, subcutaneous fluids and oxygen, application of liquid tears to the eyes and moistening the mouth after extubation, Isoflurane anaesthetic was used for induction and full anaesthesia during surgery, echocardiography and trans-oesophageal electrical stimulation (terminal procedure).

We have liaised with the technicians to perform tail vein injections as we did not have in house expertise for these experiments.

Diabetic mice were subjected to regular screening (blood glucose measurements and body weight monitoring). Careful attention was given to excess consumption of water or frequent wetting of bedding material. Absorbent bedding was added whenever necessary.

The number of diabetic mice within a holding cage was limited to 3-5, this to reduce any physical distress and guarantee free access to water without compromising social interactions between individuals.

Blood withdrawal has been reduced to a volume of approx. 1 to 2 microliters (min volume 0.6 microliters) by the use of strips and glucose meter.

We have established a method to recorded ventricular arrhythmias ex vivo.



## 2. Regulatory and Investigative Toxicology

### Project duration

5 years 0 months

### Project purpose

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

### Key words

Medicine, safety, toxicity, toxicology

### 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
- Required at inspector's discretion

### Objectives and benefits

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

#### What's the aim of this project?

Understanding how safe a potential new medicine is before it is given to humans is an essential part of medicine development. Although some information on safety can be obtained without using animals, some tests must be carried out using animals to better understand how these medicines might affect the human body. The objectives of this project are as follows:

- Identify the right potential medicines for development which are safe to give to people and are most likely to be able to treat the target illness.
- Identify any possible safety concerns and understand how these might arise and whether they could cause harm to patients or human volunteers in clinical trials.



Where possible, improve and refine our tests using animals to provide more relevant information to humans whilst minimising the use and impact on animals.

## **Retrospective assessment**

Published: 9 August 2021

### **Is there a plan for this work to continue under another licence?**

Yes

### **Did the project achieve its aims and if not, why not?**

The programme of work conducted under the Project License met its aims to generate in vivo data relevant to human risk assessment (characterise and understand toxicity of potential new medicines). The decision-making outcomes included safety data which showed a favourable profile and provided reasons to believe in the potential new medicines' continued development, or conversely, adverse safety profiles which lead to terminations in development and the prevention of further in vivo studies. Studies conducted to support the company portfolio were as follows:

30 studies were conducted in rats (26), mice (3) and dogs (1) to understand toxicity (normally following 7 days dosing) of potential new medicines and enable the company to choose the most promising new medicine candidates for future development.

66 studies were conducted in rats (16), mice (7), dogs (41) and pigs (2) to understand toxicity (normally following 7 to 14 days dosing) to help choose doses of potential new medicines that may be used in subsequent studies.

52 studies were conducted in rats (24), dogs (23), mice (2) and rabbits (3), normally following single doses to understand the toxicity of potential new medicines and choose doses for subsequent studies primarily based on an assessment of the concentrations of the potential new medicine in the body over time (for example following changes in the test substances characteristics or dosing regimen to reduce toxicity or improve concentration profiles).

23 studies were conducted in rats (12), dogs (4), mice (5) and rabbits (2) to further investigate findings seen in humans or in other non-clinical studies, or to address specific project related questions related to the assessment of toxicity.

1 study was performed in the dog specifically to assess the possible irritancy of a potential new medicine when given by injection under the skin to help choose doses for subsequent studies.

5 studies aimed at developing or improving the methods used in our studies to understand toxicity of test substances were conducted in rats (4) and dogs (1) but did not lead to improved methods (for example studies to refine low volume blood sampling were associated with variability or sample wastage).

4 studies were conducted in rats (3) and mice (1) specifically to understand how potential new medicines affect the genetic material (chromosomes or DNA).

In some instances, the studies outlined above had more than one purpose (for example studies conducted to enable the company to choose the most promising new medicine



candidates for future development also assisted with selection of doses for subsequent studies, and some studies also included an assessment of the ability of potential new medicines to affect chromosomes or DNA using the same animals assessed for general toxicity effects, therefore, negating the need for separate studies). In some cases this maximised the information obtained from the animals without additional harms.

**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.**

**What are the potential benefits that will derive from this project?**

Achievement of the objectives will support development of safe, new medicines to improve health and quality of life of patients by generating high quality, regulatory acceptable data and will help to remove unsuitable candidates from the development pipeline at an early stage, thus minimising the use of animals and resources. The benefits gained by studies performed depends on the study purpose and type and include: Making decisions on whether potential new medicines are suitable for development as early as possible in the process to avoid wasting animals and money. We use the information generated during early studies to help to understand what we need to measure on future studies. To help us to decide the doses and endpoints to measure on early human studies to minimise the risk to human volunteers. To allow regulatory authorities to decide whether to allow the potential new medicine to be given to humans.

**Species and numbers of animals expected to be used**

**What types and approximate numbers of animals will you use over the course of this project?**

Over the 5 year period of the licence we expect to use approximately:

- 5500 rats
- 2000 mice
- 575 dogs
- 200 hamsters
- 200 rabbits
- 575 pigs

**Predicted harms**

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

**In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?**

Evaluation of safety to assess potential risk to humans requires the use high doses of a potential new medicine which can cause some adverse effects in animals. Adverse effects in animals are usually of mild or moderate severity. The most common effects will be loss of body weight or reduced weight gain, reduction in the amount of food the animals are



eating and clinical signs such as reduced activity, postural changes, changes in faeces and in some species, vomiting. No animals will intentionally experience severe adverse effects but because early studies may be the first time that a potential new medicine is given to animals, effects may occasionally be more severe than expected. Animals are monitored closely and animals which show signs toward the limit of moderate severity are humanely killed. Most safety studies require examination of blood and tissues from animals to see whether the potential new medicine has caused any damage to organs or tissues, so the majority of animals are humanely killed at the end of a study and subjected to post mortem examination. Samples of tissues are then examined microscopically. On some early studies animals are not required to be killed and provided they have not shown adverse effects they may be used again in a subsequent study.

## Retrospective assessment

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

Over the lifetime of the Project 181 studies were conducted in mice, rats, rabbits, pigs and dogs with actual severities shown in the following table:

#### Actual severities recorded over the 5 year review period

Species	Reuse	Number of mild procedures	Number of moderate procedures	Number of severe procedures	Total number of procedures
Mouse	No	746	87	5	838
Rat	No	1932	185	7	2124
Rabbit	No	43	1	1	45
Pig	No	4	0	0	4
-	Yes	7	0	0	7
Dog	No	313	56	1	370
-	Yes	102	20	0	122

#### Actual severities expressed as a percentage of the total number of procedures for each species

	Mild severity (% of the Species total)	Moderate severity (% of the total)	Severe severity (% of the total)
Mouse	89.0	10.4	0.6
Rat	91.0	8.7	0.3
Rabbit	95.6	2.2	2.2
Pig	100	0	0
Dog	84.3	15.4	0.2

Adverse effects related to procedures conducted on studies (for example administration of test substances, collection of blood samples) were assessed as mild severity with the animals experiencing no more than transient pain and no lasting harm.



The number of animals experiencing moderate adverse effects attributable to the test substances under investigation were relatively low (approximately 10% of the total number of procedures) over the 5-year review period which is considered to reflect the success of the proactive strategies used to manage study severities (e.g. selection of doses and close and frequent monitoring).

#### **Reasons for Moderate severity:**

The nature and severity of adverse effects in animals assessed as moderate severity were variable depending on the test substances under investigation. The more common adverse effects observed included, piloerection and partially closed eyes in rodents, vomiting, salivation and abnormal faeces in dogs and subdued behaviour/decreased activity/reluctance to move, hunched posture, body weight loss and reduction in food consumption in all species.

The number of animals experiencing severe adverse effects attributable to the test substances under investigation were very low (approximately 0.4% of the total number of procedures) over the 5-year review period which is also considered to reflect the success of the proactive strategies used to manage study severities.

#### **Reasons for Severe severity:**

##### Study 1 (mouse) : Single dose study to assess concentrations of the test substance in the body over time.

One mid dose animal died 30 minutes after dosing when returned to its cage following blood sample collection. Clinical signs comprised lying down (not responsive when approached), whole body shaking and post dose rapid breathing immediately prior to death. The cause of death was not established.

One high dose animal died 30 minutes after dosing when returned to its cage following blood sample collection. Clinical signs comprised of reluctant to move, collapsed, post dose rapid breathing, convulsing and loss of coordination immediately prior to death. The cause of death was not established.

##### Study 2 (mouse) : 15 day repeat dose tolerability screen

One high dose animal was found dead approximately 1.75 hours after its second dose. No abnormal clinical signs were noted prior to death. There were no noteworthy macroscopic or microscopic findings to confirm the cause of death.

##### Study 3 (mouse) : 7 day dose range finding study

One high dose animal died after accidentally becoming trapped in the cage lid prior to collection of a blood sample approximately 12 hours after its first dose.  
One intermediate dose animal was found dead in its cage approximately 2 hours after its fourth dose. No abnormal signs were noted prior to its death. At necropsy, minor inflammation on the outside of the oesophagus was noted but there was no perforation of the wall of the oesophagus in the tissue sections examined microscopically. Although inconclusive the death may have been related to an accidental misdose.

##### Study 4 (rat) : 14 day toxicity study



One high dose animal was found dead approximately 5 hours after its sixth dose. No abnormal signs were noted prior to its death. The cause of death was not established.

One high dose animal was euthanised approximately 5 hours after its sixth dose due to a deterioration in condition with clinical observations comprising subdued behaviour, reluctant to move, lying down, not responsive when approached and eyes half closed.

One high dose animal was found dead after its fifth dose. No significant clinical observations were noted prior to death (rubbing chin on cage floor was noted after its fourth dose and rubbing muzzle on cage floor was seen following its third dose). A microscopic examination of the animal did not establish the cause of death.

#### Study 5 (rat) : 7 day candidate selection toxicity study

One high dose animal died approximately 1 hour after its first dose when returned to its cage following blood sample collection. The animal showed moderate noisy breathing, gasping, collapsed in cage and convulsed prior to death. A microscopic examination of the animal did not establish the cause of death.

#### Study 6 (rat) : 7 day candidate selection toxicity study

One high dose animal was found dead prior to scheduled necropsy on the day after it was given its last of 7 doses. Rubbing chin on cage floor, signs of salivation around mouth and pawing, as well as irregular breathing, partially closed eyes, and fur staining around eyes and on the chin, were seen following its 7th dose. Rubbing chin on cage floor, pawing, signs of salivation around mouth and red staining around nose/snout were noted on days prior to its 7th dose. A microscopic examination of the animal did not establish the cause of death.

#### Study 7 (rat) : 28 day toxicity study

One control animal died whilst in the restraint tube used for blood collection 5 days prior to the study start. The animal showed no abnormal clinical observations prior to death. There were no noteworthy macroscopic or microscopic findings to confirm the cause of death.

#### Study 8 (rat) : 7 day candidate selection toxicity study

One high dose animal was found dead prior to its fourth dose. The only abnormal clinical observation was slightly decreased activity noted after its third dose. A microscopic examination of the animal was inconclusive although test substance related findings in the heart may have contributed to the death of the animal.

#### Study 9 (rabbit) : Investigative tolerability and ocular toxicity study

One control animal was found dead approximately two minutes after being given a second weekly dose. Prior to death no abnormal clinical observations were noted. The cause of death was not established.

#### Study 10 (dog) : Single or 14 day dose range finding study

One intermediate dose animal was euthanised approximately 3.5 hours after its third dose following clinical signs that rapidly progressed in severity including lying on its side, rapid



and irregular breathing, convulsion, retching, white foam around the mouth and blue skin discolouration. The animal showed no abnormal clinical signs following its second dose (1 pool of white/foamy vomit and 1 area of green faeces were noted in the pen following the first dose. Microscopic examination did not establish the cause for the rapid deterioration in condition of the animal.

## Replacement

**State why you need to use animals and why you cannot use non-animal alternatives.**

Whilst alternatives to in vivo animal models are being developed and are used where possible, there are currently no reliable models available for broad, primary toxicity screening and none that are acceptable to drug regulatory authorities, it is therefore necessary to screen for toxicity in animal models

### Retrospective assessment

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**What, if any, non-animal alternatives were used or explored after the project started, how effective were they and are there any lessons worth sharing with others?**

Regulatory Authorities require the safety of potential new medicines to be evaluated in mammalian species before they will give permission to conduct trials in humans. There are currently no reliable and robust animal alternatives acceptable to Regulatory Authorities for this purpose.

Whilst there has been much progress with the development of non-animal alternatives such as organoid cultures (three dimensional cellular structures held within a matrix gel) and experiments using many small pieces of isolated various tissues or cells, they are not yet fully characterised and/or validated to provide confidence to replace data from studies in animals because:

They are not yet at a stage where the relevance of the data generated from them for humans is fully understood.

They are not yet able to reproduce the level of complexity and integration of body systems within a living animal such as the ability of cells and organs to continuously communicate.

They are not yet adequately able to simulate absorption, distribution, metabolism and elimination of medicines throughout the body.

Consequently, non-animal alternatives were not used during the lifetime of this project to replace in vivo studies.

## Reduction

**Explain how you will assure the use of minimum numbers of animals.**



For safety 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. We have a track record of designing studies that provide us with the information we need to make decisions on the safety of our test substances (leading to continuing, or stopping, development).

For preliminary studies, small groups are acceptable because of the endpoints used give a clear answer. Where group sizes are sufficient data from definitive toxicity studies are analysed statistically. Statistical input is sought, where necessary, to strengthen the overall scientific quality and relevance of the studies to be performed, with sample size calculations performed for specific studies to determine the group size. Group sizes in dog and pig studies are usually insufficient for valid statistical analysis. However, because toxicity is the result of changes in multiple parameters, assessment is made by examination of data from each animal and by correlation of in-life and post mortem findings within an individual.

In order to minimise animal use, we will consider using animals on more than one study when this can be justified on welfare and scientific grounds.

### **Retrospective assessment**

Published: 9 August 2021

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

The studies conducted under this project were performed using animals to understand how effects relate to the dose of test substance administered. The number of groups of animals and number of animals in each group were based on core study designs that have been used extensively by the Company to characterise toxicity, successfully design follow on studies and enable the most promising new medicines to be developed further. The animal models are well established with large historical control databases and have a proven track record for provision of data aiding assessment of toxicity. The following steps were taken to minimise the number of animals used:

A large pool of scientists provided expertise to assist with the design of the studies and all studies underwent internal scientific and ethical review to ensure the best possible designs

Where possible (e.g. a suitable assay is available) blood samples were collected using microsampling technology which allows measurements to be made using reduced volumes of blood supporting a reduction in the number of animals required for some studies

Samples of tissues or body fluids were collected from some studies for use in further investigations potentially avoiding the need to use additional animals in investigative studies

When appropriate endpoints normally included in other study types were added to general toxicology studies to improve our understanding of the toxicity of a test substance and potentially avoid the need for additional studies (e.g. a detailed clinical observation assessment or an assessment of the potential to cause damage to DNA which are



normally conducted on studies aimed at evaluating safety pharmacology or genetic toxicology respectively)

The results from preliminary in vitro assays in mammalian cells to assess genetic toxicity were used to decide what measurements to include in subsequent studies evaluating genetic toxicity in animals (i.e. multiple measurements for assessing different types of genetic damage were combined into the same in vivo study in order to minimise the number of studies and reduce animal use)

Where appropriate, robust study design principles were used to maximise the likelihood of generating non-biased experimental results and limit the number of animals needed to generate good quality decision making data (e.g. randomisation of animals to treatment groups)

- Experimental work authorised by this project was performed to the general standards of Good Laboratory Practice guidelines in order to ensure data integrity and high quality standards

## Refinement

**Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.**

Regulatory guidelines state that toxicology studies in support of administration to man should be conducted in one rodent and one non-rodent species. Generally the rat is the rodent species of choice unless it is known to be an inappropriate model for man for the compound. The non-rodent species will be that likely to give the most satisfactory, reliable and regulatory acceptable results.

The pig will be used as the preferred non-rodent species in this licence, unless it is shown to be unsuitable based on scientific information available, when the dog will be used. Where evaluation of all information indicates that both the pig and dog are a suitable non rodent species, the pig will be chosen.

## Retrospective assessment

Published: 9 August 2021

**With the knowledge you have now, could the choice of animals or models used have been improved at all? How did you minimise harm to animals during the project?**

In order to generate relevant decision making information to support the development of new medicines it was necessary for studies conducted under this project to use the same experimental animal models to correlate with studies required by Regulatory Authorities.

The rodent and non rodent species used under this project are routinely used for toxicity assessment since they are known to be acceptable to Regulatory Authorities, there is a wide knowledge of their response to various test substances, and a wealth of background data exists within the pharmaceutical industry.



There are currently no non-animal models that are considered acceptable to drug regulatory authorities for this purpose. All studies conducted under this project were subject to scientific and ethical review to ensure the best possible designs.

Studies conducted under this project were managed to minimise harm to the animals as follows:

Prior to evaluation of a substance in studies under this licence considerable amounts of literature-based, in silico, in vitro and in some cases in vivo data will normally have been generated to assess the suitability of the substance for development

Substances used in this project were normally provided with analytical data confirming the structure and composition, stored under appropriate conditions and used within assigned expiry periods

Studies were conducted in facilities providing an optimal environment suitable for the species, with dedicated and competent staff performing the technical procedures, animal care and husbandry according to standard procedures. The purpose designed research and development facilities of the Company have independent accreditation for ensuring commitment to excellence in setting, achieving and maintaining high standards for animal care, welfare and animal use in science.

Formulations of test substances suitable for dosing were prepared by a dedicated team of dispensary scientists who optimised formulations based on the nature of the formulation and route of administration.

All animals were monitored closely after dosing with the frequency of observations related to the nature and intensity of any adverse effects observed. Where the condition of an animal gave cause for concern, observations were continued until clear signs of recovery were evident or further action was taken to ameliorate them (typically euthanasia) to ensure that effects remained within moderate severity classification.

Where previous information was limited prior to studies being conducted on this project they were performed with 'staggered starts' where the effects in animals following initial doses were assessed before commencement of dosing animals at higher, or lower, doses depending on the outcome of the initial dosed animals.

Additional refinements implemented during the course of the licence included:

Use of nesting material to provide environmental enrichment for rats without compromising regulatory requirements.

Use of additional nesting material as well as paper based nesting material to provide environmental enrichment to mice without compromising regulatory requirements.

Refining the way that mice are restrained for procedures by using a soft (e.g. sponge) rather than a rigid surface.

Handling of mice using tube/tunnel/cupping method.

Regular attendance at our scientific and ethical review forum by representatives from research statistics as part of the scientific and ethical review of studies.



Introduction of enhanced caging (large multi level cages with added enrichment including suspended perches) for a study with an extended duration. This will be considered for future studies where applicable.

Use of a flexible cannula instead of a needle and syringe to administer subcutaneous doses to dogs

Use of oral analgesia to reduce the effects of subcutaneous dosing of long acting formulations forming a subcutaneous depot

Discontinuation of 'oral sham dosing' in dogs (where animals are subject to the dosing procedure to get them acclimatised to it before being given a dose of test substance) as a result of effective handling procedures



### 3. Understanding mechanisms of fibrosis

#### Project duration

5 years 0 months

#### Project purpose

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

#### Key words

Fibrosis, scar, myofibroblasts, therapy, diagnosis

#### 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
- Required at inspector's discretion

#### Objectives and benefits

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

#### What's the aim of this project?

Fibrotic diseases are increasing and a major cause of morbidity and mortality worldwide. In some cases, end-stage diseases can be treated by transplantation; however, there is a huge shortage of donor organs; significant side-effects from immunosuppression; and focus on end-stage disease is too late. Urgent development of novel diagnostics to determine stage of disease and anti-fibrotic therapies are needed. This requires a better understanding of the underlying mechanisms of fibrosis to develop hypothesis based approaches to identify novel dynamic markers of disease and targeted strategies for therapeutic intervention. The aim of this project is to provide a greater understanding of the molecular mechanisms underlying chronic fibrotic diseases to instruct identification of novel diagnostic and therapeutic targets that can be used for patient benefit.

#### Retrospective assessment



Published: 13 April 2023

**Is there a plan for this work to continue under another licence? Yes**

**Did the project achieve its aims and if not, why not?**

The overall aim of this project licence was to provide a greater understanding of the molecular mechanisms underlying chronic fibrotic diseases to instruct identification of novel diagnostic and therapeutic targets that can be used for patient benefit. As inflammation and fibrosis predispose to cancer, factors with pro-inflammatory and pro-fibrogenic actions will also be therapeutic targets in cancer (e.g. HCC). In particular we believe the transcription factor, SOX9, or its up and down stream regulators, may be useful as diagnostic tools or targets for anti-fibrotic therapy.

Our programme of work was to build on the previous licence which was liver centric and explore the broad context of common pathways associated with organ fibrosis in this licence. In particular, to explore the role of the core factor SOX9.

We have achieved our aims and provide highlights below:

Critical and first discovery that the transcription factor, SOX9, is a major regulator of fibrosis through molecular investigation in vitro and in vivo studies through to human translation. This includes its use as a prognostic marker of human liver disease and downstream targets as markers of severity in patient serum.

Basic mechanistic discoveries on liver regeneration and progressive fibrosis/cirrhosis have translated cell biology and in vivo perturbation in mouse to innovation funding for population-level integrated clinical diagnostics.

Through transcriptomic approaches, identified novel pathways that can be targeted pharmacologically in vivo to improve fibrosis, demonstrating anti-fibrotic capability.

My lab considers organ fibrosis broadly as a unifying mechanism of disease and have established mechanisms related to heart, Lung and Kidney fibrosis.

Recognition and inclusion in studies of fibrosis due to COVID-19 infection in humans and Long-COVID19 study.

Developing a portfolio of complex in vivo models of fibrosis and regeneration across multiple organs, including technical demanding surgery models, cancer and aging (part of our new PPL).

- Broad work links to human development and includes stem cell platforms for regenerative medicine and large dataset integration to understand human development.

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

**What are the potential benefits that will derive from this project?**



Fibrosis is a common step in the progression of the majority of chronic diseases. However, there are no approved anti-fibrotic drugs and diagnosis remains poor. Our work in this area has already uncovered novel mechanisms implicated in broad organ fibrosis that are currently under discussion with pharmaceutical companies as novel diagnostic / therapeutic strategies in fibrosis. There are clear implications for patient benefit and this has only been achieved by proof of principle using both in vitro and in vivo models of disease.

### **Species and numbers of animals expected to be used**

#### **What types and approximate numbers of animals will you use over the course of this project?**

We will use rat but more often mouse, particularly because of the ability to use genetically modified strains. Over a period of 5 years, with funding and staff / students working on these projects, I would expect breeding numbers to reach approximately 10,000 mice using several different genetic strains and for experimental protocols ~1,500 rats and ~18,500 mice (a mix of wild type background and genetically modified animals drawn from those bred under this licence or other appropriate licences). Where possible we will try to use both sexes from our transgenic breeding, but this may not be appropriate as females can be resistant to developing liver fibrosis. However, we always aim to include any appropriate females in cell preparations for in vitro studies as support for the in vivo work.

### **Predicted harms**

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

**In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?**

In most instances, tissue from animals will be removed for studies in the laboratory. In some instances, animals will be treated with agents that cause an imbalance in regeneration and/or fibrosis. Although transient discomfort may occur at the time of administration the animals appear normal soon afterwards. Similar to humans, animals can sustain fibrotic injury for a long period of time with no apparent symptoms. In the rare scenario that an animal shows signs of organ failure the animal will be put down to ensure the animal does not exceed the severity limits set out in the project. Some animals will undergo surgery to induce fibrosis, but these are not life-threatening procedures. In general, animals will suffer moderate adverse effects from this, which are similar to and primarily associated with the surgical procedure, the effects of which will be alleviated with pain-killing drugs.

### **Retrospective assessment**

Published: 13 April 2023

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



For the majority of our procedures, animals experience mild to moderate discomfort overall. Our kidney fibrosis model (unilateral ureteral obstruction; UUO) is a surgical model however it is relatively straight forward to carry out and animals recover quickly following surgery. For all surgical models, pain relief is given post-surgery and recovery is facilitated by use of a warming cabinet. There is little systemic disease as one of the kidneys remains fully functional. Similarly, use of carbon tetrachloride to induce liver fibrosis remains a robust and well tolerated model. It is an extremely robust and reproducible procedure. Although transient discomfort may occur at the time of CCl<sub>4</sub> administration (intraperitoneal injection) the animals appear normal soon afterwards. Similar to humans, animals can sustain liver injury for a long period of time with no apparent symptoms. Immediate cessation of CCl<sub>4</sub> even in severe fibrosis leads to full recovery and repair with days due to the rapid regenerative response of the mouse liver. With bile duct ligation (BDL) surgery to induce liver fibrosis – the procedure would be associated with a moderate level of discomfort due to surgery. It is also our most technically challenging and requires a modest amount of time in surgery. However, following recovery in a warming cabinet mice tolerate the fibrotic process extremely well with moderate discomfort; even though the disease process is more rapid than CCL<sub>4</sub>, animals do not tend to show signs of liver failure and appear well throughout.

Partial hepatectomy is a technically demanding surgical model to investigate liver regeneration. Following surgery animals recover well and the liver rapidly regenerates and is completely normal within a week. As such this would constitute a moderate level of discomfort associated with the surgery but the physiological response to liver damage is minimal. We have recently started a model to induce hepatocellular carcinoma using a single injection of diethylnitrosamine (DEN) on a background of CCL<sub>4</sub> induced fibrosis. In this protocol animals experience mild discomfort associated with the IP injections but recover rapidly. Over 3-6 months animals develop liver tumours, however, in this context animals appear well with mild symptoms (i.e. the liver still functions well).

For bleomycin induced lung fibrosis, there is some discomfort associated with the intratracheal delivery of compound, however this is mild and short term. Although animals tend to experience significant weight loss due to the disease process, they generally appear well with mild-moderate levels of discomfort.

A breakdown of retrospective severity by procedure is as follows

Cell prep: 357 mice of which all were non-recovery.

Carbon tetrachloride: 308 mice of which 131 were mild and 177 were moderate

Bile Duct Ligation: 81 mice of which 30 were moderate, 47 were severe and 4 were nonrecovery

Unilateral Uteric Obstruction: 93 mice of which 91 were moderate and 2 were non-recovery

Partial hepatectomy: 98 mice of which 89 were moderate and 9 were non-recovery

Hepatocellular Carcinoma: 7 mice of which 3 were mild and 4 were moderate

Bleomycin : 317 mice of which 70 were mild, 174 were moderate and 73 were severe



## Replacement

### **State why you need to use animals and why you cannot use non-animal alternatives.**

Despite progress in understanding the biology of fibrotic diseases, these discoveries have been unsuccessfully translated into the clinic. Fibrotic diseases are complex which develop and resolve over many weeks; involving the organ, immune system, and cell-cell interactions. For this reason, it is not possible to study these events in isolation in an in vitro / ex vivo system.

### **Retrospective assessment**

Published: 13 April 2023

### **What, if any, non-animal alternatives were used or explored after the project started, how effective were they and are there any lessons worth sharing with others?**

As part of this licence we have used cell lines to help model molecular mechanisms discovered from our animal models – rather than always using primary cells taken from animals. We have also (in most cases) directly applied our knowledge to human sources of material.

There have been developments in the field of fibrosis research that involve the use of human organ slice culture models. However, at present these remain imperfect as they do not give a full physiological response that is achieved using animal models. For example, the positive and negative effects of inflammatory cell infiltrate, the fibrotic niche (which is a 3D environment) and cell based interactions is not reproduced. As a result, whilst these applications are a useful indicator that similar mechanisms exist in human models of the disease, animal models remain necessary to investigate mechanisms underlying fibrosis and response to potential therapeutics.

## Reduction

### **Explain how you will assure the use of minimum numbers of animals.**

Power calculations performed based on an important component of fibrosis (collagen deposition) indicate 6 animals per group are required to analyse the fibrotic processes. For example our experience of biological variability shows fibrotic livers of 6 weeks CCl<sub>4</sub> treated rats have a mean collagen (hydroxyproline) content of  $1.45 \pm 0.25$  (SD) mmol/g liver. Based on these data, accepting an 80% chance of detecting this difference at the level of  $p \geq 0.05$ , gives a sample size of  $16 / (1.74)^2 = 5.3$  animals per group.

Where possible we will make use of archived material and importantly make use of human cells and tissue to reduce animal use.

Through refining our technical skills, see below, we are also able to reduce animal numbers.

Animal breeding will take into account the power calculations required for the experimental protocols.



## Retrospective assessment

Published: 13 April 2023

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

Use of cell lines rather than primary cells extracted from animals is our first option where ever possible. Where in vitro work is required we try to maximise the use of a single animal by removing cells from several organs relevant to our studies (e.g. cell types from lung, liver and kidney).

Breeding was carefully monitored to ensure the minimum numbers of animals were used.

We have carried out statistical calculations for our models and have a great deal of experience having published in peer reviewed journals of the numbers required to ensure significant results are produced using both in vitro (cells in a dish) and in vivo (in the animal) experiments. For the majority of our work this typically requires 5 animals in 4 groups (to include control measure for both those undergoing fibrosis induction).

Any tissue and blood samples taken from rodents was carefully stored to limit the number of animals required for simple histological use or analysis of potential markers of disease identified over the duration of the project.

Overall, from our current (and previous) project licences we have gained a lot of experience and information to instruct our experiments. This includes detailed calculations and real experimental outcomes for number of animals required. In many cases we have archived control tissue from previous experiments so we can reduce animal numbers by only carrying out the treatment (for example only inducing fibrosis). For many of our experimental models we can only use male animals (many female animals are protected from fibrosis in several organs). However, in this broad project we can now use females for many of our experiments including all cell preparation and even some in vivo fibrosis experimental models. Where possible we make use of human cells and tissue to further reduce the need for animals. In addition, we are very well organised as a group and routinely make use of several tissues from one animal such as liver, lung, kidney and heart. These structures already in place in the group ensure we limit the number of animals used for this project.

## Refinement

### **Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.**

In the case of cellular studies, particularly for liver fibrosis, we will use rats as this allows a greater analysis of the mechanisms associated with the disease process compared to mouse. However, for in vivo studies, mice will be necessary based on the use of genetically modified strains.

To investigate the therapeutic potential of our findings in fibrotic disease in different organs from multiple etiologies, it is necessary to use more than one model of injury. We have



chosen established models of organ fibrosis that have good comparison with human disease and have been refined over many years in labs worldwide.

As evidence of limiting animal experimentation through refining our models, improved technical skills and post-operative care we have reduced the mortality of bile duct ligation from 30% to ~10% on our current liver fibrosis models. We will ensure similar refinement in all protocols (which are much less severe).

As further refinement, and in agreement with our resident statistician, we will seek additional statistical assistance as required to refine experiments.

### **Retrospective assessment**

Published: 13 April 2023

#### **With the knowledge you have now, could the choice of animals or models used have been improved at all? How did you minimise harm to animals during the project?**

To investigate human disease, it is important to use mammalian models to recapitulate as much as possible mechanisms that are likely to occur in human. For this reason, rodents were chosen as animals having the lowest degree of neurophysiological sensitivity to study these processes. In addition, in this license we were studying the role of different factors and proteins in the development and resolution of fibrosis. In order to achieve these aims we were using genetically altered mice which lacked the particular transcription factor or protein (e.g. SOX9). Hence these studies could only be performed in mice. We continue to maintain an excellent track record in ensuring high quality animal work, which includes several surgical models (bile duct ligation, partial hepatectomy and unilateral ureteral obstruction) and severe protocols (bile duct ligation and bleomycin lung injury). This is through technical excellence and high level understanding of the procedures and pathophysiology underlying the disease. In most cases, any transgenic models used in procedures only occurs following a rigorous hypothesis based approach based on significant in vitro (often including human) data.

In my group we have a significant number of clinicians (including surgeons) who are aligned to the various specific organ fibrosis projects. This continues to grow and is a major advantage to high quality practice and care. As an example, for our lung fibrosis work, the clinician developed the model ensuring equivalent bleomycin delivery into both lungs. Prior to this several groups in Manchester were experiencing poor fibrotic induction (patchy fibrosis) requiring additional animals for this protocol (10 plus). He also very quickly understood the process of chronic disease in the lung that led to our amendment for a severe model based on weight loss. This ensured all animals were fully used in this protocol where, for the most part, the animals overall experienced moderate severity. We have previously provided details on our bile duct ligation model (also a severe protocol). In our hands this remains one of our most robust models with excellent dynamics of fibrosis. We feel this is down to surgical excellence and use of a warming cabinet post surgery. The standard mortality reported for this model is 30-40% but remains around 10-15% in our lab. There are few labs in the world are able to carry out this high degree of surgery with consistent results.

More generally, we follow the best practice guidance to ensure our experiments are carried out in the most refined way. This includes: prior to any animal experiment, details



are described in detail and discussed with our unit to ensure best practice is taking place within the licence. We have standard operating procedures in place for complex experiments such as those requiring anaesthesia and surgery. We follow LASA guidelines (<https://www.lasa.co.uk/>) including animal welfare, administration of substances and aseptic technique. We also ensure our personal research areas are up-to-date through monthly literature searches to refine any experimental models we use. We are also part of an extensive national fibrosis network on broad organ fibrosis where all involved are open to discussion over how to improve methodologies in practice. This has often resulted in shortened experiments in recognition that the same outcome can be achieved in a much reduce time span. This will continue and is an excellent platform.



## 4. Regulatory Ecotoxicology

### 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 aims mentioned in purpose (b)
- Protection of the natural environment in the interests of the health or welfare of man or animals.

### Key words

Ecotoxicology, Regulatory, Safety, Chemical, Pharmaceutical

## 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
- Required at inspector's discretion

## Objectives and benefits

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

### What's the aim of this project?

To allow the identification of hazards associated with the manufacture, transport and use of industrial chemicals, agrochemicals, pharmaceuticals, microbial pesticides and biocides such that their possible adverse effects on humans, wildlife and the natural environment can be determined. This will allow regulatory authorities to classify and label these substances, recommend safe handling procedures, and impose risk reduction measures if required such that the benefits provided by the substances can be safely achieved. Specifically this project will assess the ecotoxicological effects of these substances to fish following a single (acute) or series of exposures (chronic).

### Retrospective assessment

Published: 15 September 2022



**Is there a plan for this work to continue under another licence? Yes**

**Did the project achieve its aims and if not, why not?**

Studies that were conducted under the authority of this project licence were completed satisfactorily such that they were suitable for submission in order to satisfy a regulatory requirement and for suitable risk assessments to be made by the Sponsoring companies.

**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.**

**What are the potential benefits that will derive from this project?**

The main benefit of this project is the development of data to support the risk assessment of chemicals such that any detrimental effects on the environment can be minimised.

**Species and numbers of animals expected to be used**

**What types and approximate numbers of animals will you use over the course of this project?**

A variety of fish species including rainbow trout, fathead minnow, common carp, bluegill sunfish, sheepshead minnow, medaka and turbot are expected to be used. The total number of fish used over the 5-year licence period is expected to be approximately 125,000.

## **Predicted harms**

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

**In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?**

The individual studies undertaken involve exposure of groups of fish to varying concentrations of the chemical to assess the effect that the chemical may have on survival and/or growth of the fish and any interaction the chemical may have with the endocrine system. Adverse effects ranging from mild discomfort through to death are expected during the course of this project. However in the majority of exposed fish adverse effects will only be mild. The programme of work will be designed in accordance with the principles of the 3Rs in order to minimise animal use and severity of procedures. Tiered testing strategies will be implemented, so that the results of one study can be used to refine the remaining studies in the programme thus minimising the severity of any adverse effects. All fish that are exhibiting significant toxic effects, and those surviving to the end of each test, will be humanely killed as soon as possible to avoid unnecessary suffering.

**Retrospective assessment**

Published: 15 September 2022



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

Due to the nature of acute toxicity testing (in relation to determining LC<sub>50</sub> values) deaths are inevitable and to be expected due to the nature of the testing being undertaken. However, if the animals were considered unlikely to survive then steps were taken to alleviate suffering at the earliest opportunity. For acute studies with a severe severity category a total of 1569 fish were used with 437 of these being classed as severe, the majority of these 437 fish would have been found dead or in a moribund state.

A total of 167 Common Carp were used during the project duration.  
A total of 1338 Rainbow Trout were used during the project duration.  
A total of 64 Fathead Minnow were used during the project duration.

For acute toxicity limit tests a moderate severity was given in the Project Licence as deaths were not to be expected on this study type. 753 fish were used in total with 6 of these being classed as severe, these 6 fish would have been found dead or in a moribund state. 33 fish were classed as moderate (showing observations such as lethargy and loss of equilibrium) with 714 being mild (as they were not fed throughout the 96-hour test). <1% were expected to be in the severe category for these protocols with an overall of 0.8% being noted as severe throughout the project duration.

A total of 14 Common Carp were used during the project duration.  
A total of 697 Rainbow Trout were used during the project duration.  
A total of 42 Fathead Minnow were used during the project duration.

For fish juvenile growth tests, a moderate severity was given in the Project Licence as deaths were not to be expected on this study type. 94 fish were used in total with 1 of these being classed as severe, this fish was found dead. 93 fish were classed as sub-threshold (as no effects were apparent). <10% were expected to be in the severe category for these protocols with an overall of ~1% being noted as severe throughout the project duration.

A total of 94 Rainbow Trout were used during the project duration.

For fish, early life stage toxicity tests a moderate severity was given in the Project Licence due to the low neurological sensitivity at the life stage being assessed, meaning that deaths noted were classed as a moderate effect. 19043 fish were used in total with 1663 of these being classed as moderate, these fish were found dead. 69 fish were classed as mild with observations such as lethargy being present, the remaining fish were all classed as subthreshold. 5% to 10% (definitive test to range-finding test respectively) were expected to be in the moderate category for these protocols with an overall of 6% to 10% being noted as moderate (range-finding test to definitive test respectively) throughout the project duration.

A total of 19043 Fathead Minnow were used during the project duration.

For Microbial Pesticide Toxicity Testing to Fish a severe severity was given in the Project Licence. 270 fish were used in total with 25 of these being classed as severe, these fish were found dead. 29 fish were classed as moderate with observations such as lethargy and loss of equilibrium being present. Adverse effects due to exposure to the test item



were expected in approximately 10% of exposed fish with an overall of 20% being noted throughout the project duration.

A total of 270 Rainbow Trout were used during the project duration.

The Range-Finding Test for Dose Level Selection in Fish, Short Term Reproduction Assay was given a severe severity rating in the Project Licence as deaths were to be expected. 312 fish were used in total with none of these being classed as severe. 6 fish were classed as moderate with observations such as lethargy and loss of equilibrium being present. 32 fish were classed as mild with observations such as increased pigmentation being present.

The remaining 274 fish were classed as sub-threshold as no effects were apparent.

A total of 312 fathead Minnow were used during the project duration.

The definitive Fish, Short Term Reproduction Assay was given a moderate severity rating in the Project Licence as deaths were not to be expected. 30 fish were used in total with 2 of these being classed as severe with these being found dead, the remaining 28 fish were classed as sub-threshold as no effects were apparent.

A total of 30 fathead Minnow were used during the project duration.

Fish numbers used throughout the project were in line with the Protocols within the project licence.

## Replacement

### **State why you need to use animals and why you cannot use non-animal alternatives.**

Current regulations e.g. REACH, require the use of fish to assess potential environmental effects. Non-animal alternatives have not yet been sufficiently validated for acceptance by various regulatory authorities and hence cannot be used to replace animal testing in this context.

### **Retrospective assessment**

Published: 15 September 2022

### **What, if any, non-animal alternatives were used or explored after the project started, how effective were they and are there any lessons worth sharing with others?**

Due to the nature of the testing requirements and results that are required there are currently no alternative methods to the use of in vivo studies for these study types. The complex interactions involved in evaluating toxicity in the species on this licence are such that these effects can, currently, only be effectively studied in live animals.

The use of fish cell lines has been investigated as a replacement for acute fish testing (CEFIC-LRI; ECO8-CELLSens project), however ECVAM have recommended that further evaluation (ongoing) of reproducibility, predictive capacity and applicability domain is required before its use as a replacement can be confirmed.



The zebrafish embryo toxicity test (ZFET) which has been published as OECD TG 236 “Fish Embryo acute toxicity test” also has the potential to be used as a replacement for acute toxicity testing, however, a recent report commissioned by the European Chemicals Agency (ECHA) again recommends that additional scientific investigations into its applicability domain are undertaken before its use to replace short-term fish toxicity studies.

Therefore, it was not possible to replace the use of animals in any protocol within this project licence.

## Reduction

### **Explain how you will assure the use of minimum numbers of animals.**

The number of animals used in regulatory toxicology studies is specified in the relevant Test Guidelines and is the minimum that is sufficient to allow meaningful interpretation and submission to a range of regulatory authorities. The use of the specified numbers of animals ensures that the data generated will be acceptable to regulatory authorities and hence will minimise the need for subsequent duplication or supplementary testing. Where possible the results of QSAR predictions, physico-chemical testing and non-animal tests will be used to aid in the prediction of toxicity hence reducing the number of animals required to satisfy the regulatory requirement, e.g. by performing Threshold tests.

### **Retrospective assessment**

Published: 15 September 2022

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

The number of animals used throughout these projects are specified in the relevant Test Guidelines and are the minimum that is sufficient to allow meaningful interpretation and submission to a range of regulatory authorities. The use numbers used ensured that the data generated was acceptable to regulatory authorities and hence minimised the need for subsequent duplication or supplementary testing.

For acute fish studies, the use of “limit tests” at the highest attainable test concentration or the maximum test concentration given in the test guidelines where appropriate were used to demonstrate the absence of lethality (these used one test group plus control(s)). Wherever possible acute fish studies were conducted according to the threshold approach as recommended by REACH and OECD Guidance Document 126 thus negating the requirement for conducting a full LC50 determination using up to 5 test groups plus control(s).

The use of control and solvent controls (where necessary) is pre-disposed by the test guidelines, however the use of common control groups for concurrent studies is employed whenever possible as a means of minimizing the overall numbers of animals used. The company's recommended approach to evaluating existing information on toxicology or for the determination of the toxic potential of test materials with little or no testing history, is to use a sequential testing strategy where possible, starting with non-vertebrate studies (algae, plants and invertebrates). Therefore, the results of each study, as it becomes



available, was considered in terms of the other proposed studies. The testing programme could then be altered or studies omitted based on the previous results generated.

Before any programme of work was undertaken, the available information on the chemical or chemical preparation was gathered and reviewed. Sources of information included the Sponsor of the work, scientific literature, toxicological databases, expert knowledge-based systems, and data from other studies. If the specific objective could be achieved on the basis of this information, then testing in animals was not undertaken for that specific endpoint.

Experience accumulated in the ecotoxicological assessment of specific classes of chemical or types of chemical preparation, were used in refinement of the work programme thus reducing the numbers of animals required to satisfy the protocol requirements.

In order to minimize the occurrence of acute toxicity and/or sub-lethal effects, wherever possible computer generated structure activity relationships were used to identify possible toxicity and studies with invertebrates (for example *Daphnia magna*) were conducted first.

The results of these preliminary investigations allowed the use of the minimum numbers of fish that was consistent with the aims of the protocol and prevented exposure of fish to excessively high test item concentrations that may cause adverse effects.

## Refinement

**Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.**

The fish species used have been selected in accordance with the relevant Test Guidelines and the age ranges of the fish are such that they are of the lowest neurophysiological sensitivity that will allow evaluation of the specific endpoints.

The species selected are representative of wild species. The data generated is therefore designed to protect these representative species in the environment thereby minimising larger scale environmental effects of tested chemicals.

Any fish that are showing a significant departure from the animal's normal state of health or well-being will be identified and humanely killed.

## Retrospective assessment

Published: 15 September 2022

**With the knowledge you have now, could the choice of animals or models used have been improved at all? How did you minimise harm to animals during the project?**

As the tests conducted are required to fulfil a regulatory requirement the test species to be used are clearly defined. However, the most appropriate species, particularly in terms of species sensitivity and availability of background data, as well as the species having the lowest neurophysiological sensitivity, were chosen for each testing strategy.



All scientific procedures using animals were performed in accordance with UK Good Laboratory Practice regulations. Standard Operating Procedures define animal welfare practices and experimental procedures. Technicians who undertook the work were fully trained and deemed competent to conduct the procedures required throughout the duration of each test. Technicians are familiar with the signs of pain, discomfort or distress in the species with which they are working and training records are maintained to document training levels, retraining and competence.

Systems of care and accommodation were provided for animals on test that enhanced their welfare. Environmental enrichment was provided where possible, unless precluded on scientific grounds.

Each test was designed and conducted in every case such that any actual or potential pain, discomfort, or distress to the animals was minimised or alleviated by choosing the earliest endpoint that was consistent with the scientific objectives of each study. Before starting each study, consideration was given to relevant background data or information supplied with the test material, together with databases for similar chemicals or substances or previous experience with other sponsor products. Thus, the type and severity of the abnormal signs of toxicity, particularly in terms of time of occurrence in relation to time of dosing could be anticipated.

Studies that were considered likely to cause significant acute adverse effects were started as early in the working day as possible. This ensured that the critical period for the animals occurred during normal working hours, this allowed the frequency of observations to be increased, allowing for intervention by technicians to alleviate any pain or distress.



## 5. Understanding and Treating Cardiovascular Disease

### Project duration

5 years 0 months

### Project purpose

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

### Key words

cardiovascular development, heart repair

### 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
- Required at inspector's discretion

### Objectives and benefits

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

#### What's the aim of this project?

This project will investigate how the heart and blood vessels function in health and disease, and how we can use this knowledge to improve outcomes for patients. These include patients who have an inherited condition that results in blood vessel problems that can also affect heart function. In addition, we aim to develop treatments for patients with a heart attack. Patients now have an increased chance of surviving a heart attack, providing they reach a suitable clinic in a rapid time frame due to improved intervention at the acute stage. However, heart attack patients who are discharged from hospital have an increased risk of developing heart failure over subsequent months and years. This (together with our ageing population) means there is a growing number of patients in the western world who develop heart failure. There are now more than 0.5 million patients living with heart failure in the UK. There is no effective treatment (other than heart transplant) and the disease will get progressively worse. Better treatments for patients at the acute stage of a heart attack



will reduce damage to the heart and thereby reduce the risk of later progression to heart failure. The work on this licence aims to address this issue using mice to model myocardial infarction.

### **Retrospective assessment**

Published: 15 September 2022

#### **Is there a plan for this work to continue under another licence?**

No

#### **Did the project achieve its aims and if not, why not?**

This project completed its aims. We gained an in depth understanding of the cellular and molecular pathologies that develop in a mouse model of an inherited vascular disorder affecting 1 in 5000 people. We showed rescue of this disorder with an antibody therapy. Studies of genes required for cardiovascular protection after a heart attack and medicines for improvement of outcomes have also been completed in this study, using mouse models. We showed that stem cells derived from the heart and delivered during a heart attack promotes some beneficial outcomes. We next showed that a natural protein and its molecular mimic delivered immediately after a heart attack effectively reduces the amount of heart injury and leads to a 50% reduction in scar size. A benefit of this magnitude reduces the risk of progression to heart failure, the major risk to patients following a heart attack. A genetic change to the receptor of this beneficial protein was used to prove the molecular specificity of the therapy. In parallel studies, we showed that loss of lymphocyte activity leads to a 60% reduction in scar size following a heart attack. We also used our genetic mouse models to investigate the role of a related gene associated with activated leukocytes, which is important in determining outcomes in heart attack patients.

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

#### **What are the potential benefits that will derive from this project?**

Studies to further increase our understanding of the disease mechanisms will underpin improved treatments for two groups of patients: (i) those with an inherited vascular disorder that affects approximately 1/5000 people; and (ii) those who survive a heart attack that subsequently progresses to heart failure.

Based on our advances in understanding we will use drug treatments that can completely or partially rescue the inherited vascular disorder. We will also use small molecule inhibitors for delivery at an early stage following a heart attack to reduce heart injury. Our goal is to improve long term outcomes for both these patient groups.

#### **Species and numbers of animals expected to be used**

**What types and approximate numbers of animals will you use over the course of this project?**



Mice : approximately 3,000 adults and 300 neonates per year.

The majority of the adult mice are used in breeding programmes, and because of the silent nature of the gene alterations, they are indistinguishable from wild type mice

## **Predicted harms**

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

**In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?**

Inactivation of target genes may lead to appearance of clinical symptoms due to the development of abnormal blood vessels. Delivery of substances to rescue the clinical problems will be given via the least stressful method possible eg via the food or drinking water. Alternatively, where injection is required, multiple doses will be given using a surgically implanted minipump. On rare occasions the drug may be delivered locally in the eye of young mice, and implants may be placed beneath the skin to monitor blood vessel development. Ligating a coronary artery will be used to model a heart attack. The surgery is complex and on some occasions this can lead to respiratory distress or intra-operative bleeding. If this occurs the animals are humanely killed without recovery from anaesthetic. A small proportion of animals may later develop fatal disturbance to the heart rhythm or rupture of the heart which leads to sudden death. Some animals will be imaged using MRI or fluorescent methods, and these imaging methods are not normally associated with adverse effects. We keep within moderate severity limits and all animals are humanely killed at the end of the work.

### **Retrospective assessment**

Published: 15 September 2022

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

Inactivation of selected target genes led to the development of abnormal blood vessels, but mice appeared well and were humanely killed prior to developing adverse affects. The principal harms arose in work using mouse surgery to induce a heart attack, a model that primarily used wild type mice. A total of 837 mice were subject to a heart attack during over the course of this licence. This surgical procedure was performed under general anaesthesia with post-operative analgesia to minimise the pain. However, animals will have suffered some distress despite pain killers being given (immediately after surgery, plus in an edible jelly form overnight, and again on the day following surgery). In a few cases, mild and transient pain was also caused by taking blood samples. All animals were humanely killed at the end of the experiment and the heart tissue was taken for further examination in the laboratory. Every animal was carefully monitored for 2 hours after surgery to ensure a good recovery from the operation. Animals that did not show a good recovery were humanely killed but still provided useful scientific information as the 2 hour time point corresponds to when the white blood cells are responding to the heart attack. Approximately 10% animals died during surgery when they were fully anaesthetised, whilst 74% animals successfully reached the scientific endpoint. A minority of animals (<4%) died



in the hours after surgery during the immediate overnight period with no cause identified at post-mortem. A likely cause is sudden death due to cardiac arrhythmia which is associated with a heart attack and is harm of short duration.

## Replacement

### **State why you need to use animals and why you cannot use non-animal alternatives.**

Unfortunately there are no suitable cell culture systems that can be used to replace in vivo models of cardiovascular development and disease, due to the complexity of the processes involved. Some organs on a chip are in development, but are not sufficiently advanced to model a beating heart, mature blood vessels, inflammation, blood flow and tissue repair. Mice are being used in this project because this is the simplest organism that has a similar heart and blood vessels to human, and that can be used to investigate the roles of different genes in cardiovascular development and disease. Alternative less sentient animals such as Zebrafish are not suitable for this work because they are so evolutionarily distant from human that it would be difficult to translate any of our findings. For example they are cold blooded, they have only two heart chambers instead of four; they have no lungs; and they show endogenous regeneration of the heart following injury, a property that adult mammals do not possess. In some cases we use mouse embryos for analysis. All the work is complemented by cell culture work, for example when investigating processes that occur within individual cells, the effect of bioactive substances will be tested in culture prior to in vivo work.

### **Retrospective assessment**

Published: 15 September 2022

### **What, if any, non-animal alternatives were used or explored after the project started, how effective were they and are there any lessons worth sharing with others?**

Tissues/organs were collected from our mouse lines. Samples of whole heart, lung, and blood were used to maximise the information we can obtain from each mouse, and does not involve an additional welfare cost to the animal. The tissues obtained were used for protein expression analysis by immunofluorescent staining, cardiac infarct staining and histology, and gene expression analysis. This work informed our understanding of disease mechanisms taking place in our mice models of cardiovascular disease. For some experiments cell lines were generated to understand the molecular roles of selected genes of interest.

## Reduction

### **Explain how you will assure the use of minimum numbers of animals.**

We use the minimum number of animals required for our experiments and regularly consult a statistician for advice. We do pilot work so that power calculations can be done in advance of full experiments. We use online tools for power analyses (eg <https://eda.nc3rs.org.uk/> as discussed recently in Nature. 2016; 531(7592):128) to predict group sizes needed to detect differences with statistical significance based on pilot data. This key feature of good experimental design makes analyses more efficient and minimises the risks of overpowered or underpowered (and therefore wasteful)



experiments. Group sizes, gender, strain and age are matched for control and experimental groups. Sources of variability will be identified and minimised wherever possible. With this aim in mind, we have made particular effort to establish a priori exclusion criteria for mice undergoing a surgical myocardial infarction by measuring infarct size by blanching area of the myocardium. This allows us to exclude animals with small infarcts, reducing variability and allowing us to use smaller group sizes for studies. In another example, variability in the matrigel plug experiments is minimised by using small syringes to generate equal plug sizes. In addition, variability in vascular phenotypes following gene activation with tamoxifen is minimised by ensuring the optimised tamoxifen dose is used. Data is collected by researchers blinded to treatment wherever possible and with experimental details recorded following the ARRIVE guidelines.

## **Retrospective assessment**

Published: 15 September 2022

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

Variability in vascular phenotypes following gene activation was minimised by ensuring the same optimised tamoxifen dose was used. To investigate the outcomes after a heart attack, the number of animals required per group was minimised because an experienced surgeon performed all the work and performs ligation at exactly the same site on the artery. In addition mice were gender, strain and age matched. This all reduces intergroup variability enabling smaller group sizes to be used. Pilot studies were performed in order to do power analyses to establish the correct group sizes and avoid wasteful experiments.

## **Refinement**

### **Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.**

In terms of general mouse handling we have moved to the tunnel method to reduce stress when removing a (post weaning) mouse from its cage. When pups are removed (eg for injection), they are removed within their nest and returned the same way, ensuring mother returns to feed them. The genetically modified mice that we use generally carry 'hidden' mutations, such that almost all of animals in protocol 1 are completely healthy until they are given the inducer (eg tamoxifen) to activate the mutation, reducing any clinical effects to the absolute minimum necessary for the project.

As this is a continuation of a project licence that has already been running for over 9 years, protocols are already established for the majority of the work described in this application, and numerous refinements that we have introduced are summarised in the sections at the end of each protocol. For example 17 refinements have been added to the work in protocol 8. LASA surgical guidelines are carefully followed in all surgical procedures, and LASA guidelines are followed for blood sampling and injection volumes. Delivery of bioactive substances is now a major focus to rescue disease symptoms and to this end osmotic pumps are used instead of multiple IP injections. By using appropriate anaesthesia and analgesics in the procedures to alleviate pain and discomfort, the protocols cause the minimum possible discomfort to the animals. All PIL holders working under this PPL will only work independently for any single procedure after their competency has been



confirmed by the named competency officer or deputy (see supporting document describing training and competencies). A body condition scoring system (Hickman and Swan JAALAS 2010) will be used to check animals following procedures with recovery and is accompanied by twice daily checks. A more detailed follow up of twice daily checks and daily weighing is used where a short term loss of weight is anticipated following the procedure and therefore it is important to establish that normal weight is regained. Where animals are to be killed for reasons unrelated to the scientific endpoints of the work described here, the NACWO will be consulted to establish whether the animal tissues would be of value in other studies.

## **Retrospective assessment**

Published: 15 September 2022

### **With the knowledge you have now, could the choice of animals or models used have been improved at all? How did you minimise harm to animals during the project?**

The genetically modified mice that we use generally carry 'hidden' mutations, such that they are completely healthy until they are given tamoxifen to activate the mutation, reducing any clinical effects to the absolute minimum necessary for the project. Myocardial infarction surgery was conducted using aseptic technique and physiological support such as supplementary heat and fluids. Analgesia was provided routinely before and after surgery. We reached out to surgeons performing similar procedures to share our experience and discuss refinements. We also performed an extensive literature search to ensure we were using the most optimal methods. As a result over 31 refinements were introduced during this work. Importantly, we use a transient ligation model which is a far better representation of the clinical situation of heart attack patients compared with a permanent ligation model. The majority of deaths associated with the surgical induction of myocardial infarction occur in the intraoperative period whilst the animal is still under general anaesthesia. Careful monitoring is applied post-operatively using a score sheet to identify key clinical signs and minimise suffering associated with adverse effects. Any animal failing to show good mobility and responsiveness by 2 hours after surgery is humanely killed to reduce the risk of animal harms. We have not experienced any problems with wound healing or infection over the course of this project, thanks to the expert skill and care of our experienced surgeon.



## 6. Small animal models of cardiovascular disease

### Project duration

5 years 0 months

### Project purpose

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

### Key words

Ischaemia, Therapy, Cardiovascular Disease

### 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
- Required at inspector's discretion

### Objectives and benefits

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

### What's the aim of this project?

This programme of work has 2 main objectives:  
to determine whether a novel compound which blocks the function of a specific heart cell membrane protein (ion channel) has a beneficial effect during a heart attack and  
to determine whether a non-invasive protocol of temporarily disrupting blood flow to the limbs can modify re-modelling of the heart caused by high blood pressure

### Retrospective assessment

Published: 12 January 2022

### Is there a plan for this work to continue under another licence?

No



### **Did the project achieve its aims and if not, why not?**

There was no work carried out on this Project Licence. The project licence was originally taken over by the current licence holder from a previous licensee. At the time of taking over the licence, the funding to carry out the experiments was not in place. Funding was attained in 2019, however this grant funding was transferred to another institution along with the move of the licence holder. The licence was to be transferred, however the covid situation meant that this did not happen. The licence expired before it was possible for us to do the experiments.

**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.**

### **What are the potential benefits that will derive from this project?**

This project aims to improve our understanding of how the heart is damaged during a heart attack, and also to assess the efficacy of a novel group of compounds to treat the immediate muscle damage and reduce the chronic adverse effects of a heart attack on the structure and function of the heart, which leads to heart failure. The project will also investigate the links between a newly identified protein on the cell surface in the heart (Kir6.1) and the hearts own in-built protection mechanisms. One way that this is thought to be activated is by bursts of exercise. This project will investigate how short periods of exercise can protect the heart from damage. Finally, it will also assess the potential for a non-invasive procedure (remote ischaemic conditioning – achieved by repeated temporary interruption of blood flow to a large muscle bed, e.g. a limb, using a tourniquet) to modify the enlargement of the heart due to high blood pressure or diabetes, which are among the most significant risks for heart disease.

### **Species and numbers of animals expected to be used**

**What types and approximate numbers of animals will you use over the course of this project?**

1,100 rats and 1,250 mice over 5 years

### **Predicted harms**

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

**In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?**

Models of myocardial infarction (heart attack) and persistent hypertension (high blood pressure) are prepared surgically with full recovery of consciousness; therefore there are both anaesthetic and postop pain risks to the animals. Anaesthesia will be carefully monitored until full recovery occurs, and postop pain will be treated appropriately for as long as necessary. There is a small risk of intraoperative or immediate post-operative death in the animals undergoing the heart attack procedure, however these animals will



likely die under full general anaesthesia therefore no suffering is expected to occur. Severity is graded in the 'severe' range for the heart attack model and 'moderate' for the high blood pressure models. Longer-term consequences, including the development of heart failure, will be routinely assessed by clinical signs and imaging where appropriate. Remote ischaemic conditioning (temporary interruption of blood flow to a muscle bed, e.g. a hind limb) should have no risk other than repeated sedation, and would normally be considered to be of mild/moderate severity. However animals transferred from the surgical protocols may be at increased risk of death during this procedure, therefore under these circumstances, this protocol has been classified as 'severe'. Induction of diabetes is expected to be of 'moderate' severity and will be used for additional studies. It may be combined with the models already described (heart attack, high blood pressure, remote ischaemic conditioning) conferring some additional risk, but this will be mitigated by increased monitoring whenever necessary. Exercise conditioning (protocol 5) is considered to be of moderate severity. All animals in this protocol will be purchased specifically for this protocol and will not be from other protocols in this licence. Animals from this protocol will be used in protocol 1 or for tissue harvesting following a schedule 1 procedure. No knockout animals will be used in this protocol. The severity for protocol 6, GA breeding, will be mild, however the severity for the second breeding protocol (7) is graded as 'severe' due to the fact that Kir6.1 knockout animals are prone to sudden cardiac death. As such, knockout animals that are bred in protocol 7, and used in protocol 1, will be maintained under terminal anaesthesia during the reperfusion phase and killed at the end of the procedure. All animals will be humanely euthanised at the end of the experiments by trained staff.

### **Retrospective assessment**

Published: 12 January 2022

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

NIL Return - no work has been completed under this PPL

### **Replacement**

**State why you need to use animals and why you cannot use non-animal alternatives.**

Diseases of the heart and circulatory system are multi-faceted and rely on interaction between several body systems. These include complex biochemistry, hormone changes and blood pressure effects and cannot be replicated in vitro.

### **Retrospective assessment**

Published: 12 January 2022

**What, if any, non-animal alternatives were used or explored after the project started, how effective were they and are there any lessons worth sharing with others? NIL**

Return - no work has been completed under this PPL

### **Reduction**



### **Explain how you will assure the use of minimum numbers of animals.**

Appropriate statistical tests will be applied to all experimental procedures. Once the proposed models are validated (pilot data) power calculations will be used to determine group sizes.

### **Retrospective assessment**

Published: 12 January 2022

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

NIL Return - no work has been completed under this PPL

### **Refinement**

### **Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.**

The techniques described are widely used in rats and I have substantial experience in this species. They are established in the scientific community as excellent clinical models and data generated are considered applicable to human disease. Welfare costs to the animals are significant but will be mitigated by extensive use of pain relief and by the use of sedation/anaesthesia whenever it is required. To investigate the effects of elevated glucose on cardiovascular function, we have refined our surgical protocol to look at acute hyperglycaemia, rather than streptozotocin-induced diabetes. This will have significant benefits to the animal by reducing the need for the induction of diabetes to achieve the experimental aims

### **Retrospective assessment**

Published: 12 January 2022

### **With the knowledge you have now, could the choice of animals or models used have been improved at all? How did you minimise harm to animals during the project?**

NIL Return - no work has been completed under this PPL



## 7. Calcium-permeable channels and their associated mechanisms and therapeutic potential

### Project duration

5 years 0 months

### Project purpose

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

### Key words

Blood vessel, Cardiovascular disease, Cancer, Diabetes, Calcium channels

## 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
- Required at inspector's discretion

## Objectives and benefits

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

### What's the aim of this project?

The overall aim of this project licence is to identify molecular mechanisms that could be the basis for new therapies aimed at addressing cardiovascular disease and cancer in the absence or presence of the metabolic syndrome.

### Retrospective assessment

Published: 10 May 2022

### Is there a plan for this work to continue under another licence?

Yes

### Did the project achieve its aims and if not, why not?



Yes.

We determined the:

types of calcium-permeable channel that are important in various physiological and pathophysiological processes, especially in the cardiovascular and metabolic systems and cancer; associated mechanisms of these channels, such as downstream pathways that lead to biological effects; the therapeutic potential of the identified new mechanisms, including formation of a spinout company aimed at delivering a new therapeutic drug.

**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.**

**What are the potential benefits that will derive from this project?**

One potential benefit of the work is to provide fundamental biological understanding about calcium ion signalling mechanisms in mammalian biology. A second potential benefit is the foundation for new treatments for cardiovascular diseases and cancers which are common causes of premature death and disability, the most common causes of death world-wide, and the causes of over half of deaths in the western world, despite current-day treatments.

A particular concern is the confounding factor of the metabolic syndrome which is characterised by obesity, diabetes, greater cardiovascular disease and cancer risk, and resistance to current therapeutic agents.

**Species and numbers of animals expected to be used**

**What types and approximate numbers of animals will you use over the course of this project?**

Over the 5 years duration of the licence it is estimated that 28,000 mice will be used.

**Predicted harms**

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

**In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?**

The experiments will involve making modifications to calcium channel mechanisms using genetics or drug-like substances. It is our hypothesis that modification of the mechanisms could be a basis for potential novel therapeutic agents, so we expect that the modifications will have beneficial rather than adverse effects.

In some cases we may unexpectedly observe adverse effects of modification leading to mild or moderate, or perhaps in some cases severe, severity levels. Animals will be promptly killed using a Schedule 1 procedure if there is severe adverse effect. In some cases moderate severity might also be considered to be unacceptable and Schedule 1 will



therefore also be applied. We have, for example, found that genetic disruption of one of the target mechanisms is lethal in mice at around embryonic day 10. For studies of this mechanism we will use embryos or conditionally induce the modification only in a specific cell type in the adult animal. In some cases we expect to be able to achieve a subtle modification of Piezo1 which is not lethal and does not lead to a severe phenotype; in these cases we will study adult mice if the severity level is mild or moderate. At the end or if there is unexpected adverse severity level, mice will be killed by Schedule 1 procedure.

In some experiments we will mimic human disease in mice using genetic or dietary approaches or substance administration or through a surgical procedure or by injecting cells under the skin or systemically. It is expected that the severity level will be mild or moderate. There is the possibility to reach severe adverse effects with procedures of this type, such as with the induction of aneurysm which can be lethal as it is in humans, but we will minimise the risk of lethality by optimising the dose of the inducing agent. Lethality will not be a deliberate end-point of the studies.

As part of our aim to develop novel therapeutic agents we will test novel chemicals to determine the maximum tolerated dose. At the end of the experiments or if there is toxicity, animals will be killed by Schedule 1 procedure

## **Retrospective assessment**

Published: 10 May 2022

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

We bred genetically-modified mice and then made measurements from experimental mice from these colonies (e.g., by collecting blood, insulin tolerance testing, non-invasively imaging or determining metabolic status by CLAMS) or measurements after Schedule 1 killing (e.g., for myography or histology). The genetic modifications we used had relatively subtle effects and the animals did not appear to suffer. We induced metabolic syndrome by high fat diet and so animals became obese, but they appeared to suffer no more than humans in this condition.

For aneurysm studies we focussed on the elastase model to minimize severity. Using Protocol 10 (MTD) we found a toxic effect of one substance and so these studies were carefully managed with detailed oversight from the Named Veterinary Surgeon, who co-authored the publication. For the surgical procedures including the Severe AngII-induced aneurysm (AAA) of Protocol 9 we undertook aseptic surgery, administered appropriate analgesia and regularly inspected the animals post-operatively and intervened to reduce suffering. We undertook *in vivo* imaging to reduce the number of animals required in a particular experiment (i.e. no need for serial sacrifice where possible). For the higher risk aneurysm (AAA) experiments (Protocols 8 and 9, 253 animals in total), 10 animals experienced severe suffering; all were on Protocol 9, which has Severe severity limit. 72 animals underwent AAA formation under Protocol 9 with 62 experiencing moderate severity only.

## **Replacement**

**State why you need to use animals and why you cannot use non-animal alternatives.**



Cardiovascular disease and cancer are complex disorders. Whilst we can conduct much of our work in cells in the laboratory, we cannot fully recreate all the processes which occur in the body to cause diseases like heart attacks or aneurysms. In order to identify new ways of preventing and treating these diseases in humans, it is necessary to carry out some aspects of our research in animals.

## **Retrospective assessment**

Published: 10 May 2022

### **What, if any, non-animal alternatives were used or explored after the project started, how effective were they and are there any lessons worth sharing with others?**

We incorporated computer-based molecular modelling and computer-based analysis of patient data sets to help maximize the value and quality of our decision-making for animal studies and potentially avoid the need for animal studies in the future.

We incorporated human placenta tissue (vascular) studies as a means of avoiding animal studies in this aspect of our research and improving the relevance to human disease. We achieved this through use of human tissue samples obtained from the local hospital; not only placenta but also liver, adipose tissue and skeletal muscle. We introduced new approaches based on computer-based studies of proteins and human data sets (i.e., analysis of UK Biobank data).

We assembled a team of about 15 laboratory researchers who work together to optimize animal usage and experimental design and to maximize the volume and quality of scientific data obtained from each animal.

The team performs parallel in vitro lab studies to avoid animal studies where possible.

We restructured the group so that it comprises mostly only experienced postdoctoral researchers, in order to maximize the quality of the animal studies and the data obtained.

By working with a professional biostatistician, we optimize the design of all our animal studies and maximize the quality of data interpretation.

Where possible we incorporated osmotic mini-pump protocols to minimize injection and gavage protocols, and new imaging methods to enable non-invasive data collection

Where possible we used the latest genetic modification methods that allowed conditional disruption only at adult stage or a subtle modification that we had determined through extensive preparatory studies to be likely to have only mild or moderate effects (which turned out to be the case).

## **Reduction**

### **Explain how you will assure the use of minimum numbers of animals.**

We are able to minimise the number of animals used by careful design of our studies, combining scans, blood tests and detailed examination of tissues collected after the animals are humanely killed.



## **Retrospective assessment**

Published: 10 May 2022

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

We avoided the need for mouse placental vascular studies by obtaining human placental tissue and studying the blood vessels from these placentas instead. In this way we have reduced the number of animals used. We maximized the quality of our statistical approach to minimize the number of animals required. We organized animal studies collectively in our group to maximize data collection from individual mice and therefore reduce the number of animals used. We designed experiments based on previous knowledge of variance in data from experiments of this type or estimates of variance based on published data. Using these variance values and by specifying the acceptable size of effect we calculated the number of animals required. We use conventional statistical methodologies for much of our data analysis (e.g. t-tests) but also more sophisticated statistical modelling as guided by our professional statistician in the team.

## **Refinement**

### **Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.**

We have chosen the mouse as it is relatively straightforward to alter the genes of mice in order to study the effects of specific proteins in normal function and disease. The mouse is one of the lowest order mammals in which it is appropriate to study human disease.

## **Retrospective assessment**

Published: 10 May 2022

### **With the knowledge you have now, could the choice of animals or models used have been improved at all? How did you minimise harm to animals during the project?**

We introduced new imaging and metabolic measurement methods for non-invasive characterization of mice to improve the quality of the results and minimize suffering. We focused where possible on exercise studies that involved inclusion of a running wheel in the cage, thus improving animal welfare.



## 8. Regulation of glucose homeostasis in vivo

### Project duration

5 years 0 months

### Project purpose

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

### Key words

Diabetes, islets of Langerhans, insulin secretion, insulin action

### 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
- Required at inspector's discretion

### Objectives and benefits

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

#### What's the aim of this project?

Diabetes is a multisystem disease, involving insulin secretion deficit in response to elevated blood glucose, and insulin resistance in multiple tissues, including the liver, the adipose tissue, the brain, the placenta (for gestational diabetes), and the enteroendocrine cells of the intestines. Also diabetes complications cause stroke and heart attacks, kidney failure, neuropathy and limb amputation and blindness due to retinopathy. More specifically this project aims to understand the molecular mechanisms behind pancreatic beta cell failure during the progression of obesity and insulin resistance in order to provide invaluable data to healthcare professionals and pharmaceutical companies to develop new tools and drugs to limit the devastating effects of this disease. Another objective of this project is to implement, develop and validate the technique of islets transplantation in the



anterior chamber of the eye for serial imaging which will provide an instrumental tool for following the damages caused to the islets by diabetogenic insults and the potential recovery induced by new drugs and treatments.

Although originally developed as a weight loss intervention, bariatric surgery can improve various metabolic co-morbidities, particularly type 2 diabetes. Mechanistic research has demonstrated that changes in gastrointestinal physiology can play a role in the effects of surgery on diabetes. The exact mechanism of action, however, remains unclear. This project aims to develop the three main bariatric surgery models in rodents and explore the mechanisms of diabetes remission facilitating the group's expertise, including the islets transplantation in the anterior chamber of the eye.

### **Retrospective assessment**

Published: 23 May 2022

#### **Is there a plan for this work to continue under another licence?**

Yes

#### **Did the project achieve its aims and if not, why not?**

*No answer provided.*

**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.**

#### **What are the potential benefits that will derive from this project?**

Diabetes is one of the most challenging socio-health emergencies of the third millennium. About 350 million people worldwide are estimated to be diabetic (50% of whom are undiagnosed), but this number is rapidly increasing due to aging populations and sedentary lifestyles, with the prospective of exceeding 500 million cases in 2030. Every year, 1.5 million deaths can be directly attributed to diabetes. In Western countries, the economic cost of diabetes can exceed 15% of the budget of national health systems. Therefore, impact of innovative methodologies and technologies for diabetes management can be extremely high.

This basic research is trying to identify molecular signalling pathways involved in protecting the human body against the devastating effects of over-nutrition, physical inactivity and obesity, contributing to the development of type 2 diabetes and its crippling complications. Moreover, the most recent guidelines accept that bariatric surgery, and more specifically gastric bypass, causes diabetes remission in 80% of the cases, making the operation the closest we have been to a long-term treatment. It is therefore essential to decipher this observation to find the missing link between pancreas, liver and the gut so as to be able to create more specific drug targets that could allow us to replicate the effect of bariatric surgery.

Overall, the immediate beneficiaries will be academics working in the field of cell biology, but this research has the potential to interest pharmacological drug companies and to



translate into clinical research by identifying potential drug targets to protect the pancreatic beta cell and the liver against glucolipototoxicity. The patients suffering from diabetes are ultimately going to benefit from this research.

### **Species and numbers of animals expected to be used**

#### **What types and approximate numbers of animals will you use over the course of this project?**

The licence allows for 11700 mice and 6700 rats over a period of 5 years, spread over 6 research groups. The numbers have been adjusted according to the newly calculated numbers for Protocol 8.

### **Predicted harms**

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

#### **In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?**

Animals will be rendered diabetic either by genetic modification, administration of drug or a high fat Westernised diet. The severity of diabetes will be investigated by serial blood tests and glucose (sugar) administration either orally or by injection. It can also be necessary to perform some surgery on selected animals. All the procedures in this licence except 8 are classified as either mild or moderate and are done under local, general or terminal anaesthesia where appropriate to minimise stress and suffering of the animals. Procedure 8 is classified as severe and the number of animals that will undergo bariatric surgery will be closely monitored and humanely killed if suffering or distress is observed (assessed by lack of grooming, eye/ nose dark discharge and pigment, feeding behaviour, decreased bowel movements, urination, blood in faeces in the first 3 days) All the animals will be humanely killed at the end of the procedures. We will always consult with the vet and animal care staff to make sure we are up to date in applying the most refined methods.

### **Retrospective assessment**

Published: 23 May 2022

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

All the procedures in this licence were classified as mild or moderate with exception to protocol 8, which is classified as severe.

No unexpected harm to animals has occurred while carrying out protocols 1-6 apart from the mild discomfort during the immobilisation and injections that were required to assess glucose tolerance (Protocol 6).

No unexpected harm to animals has occurred while carrying out protocol 7, apart from initial eye rubbing/ discomfort in animals on the first day. Imaging did not cause any further issues to the animals.



Although protocol 6 is classified as moderate, most animals didn't undergo most of the steps described in the protocol. Accordingly, only ~5% of the animals underwent actual moderate severity. Typically this involved mice fed a high-fat diet and submitted to multiple experiments that individually caused only mild discomfort, such as injections and blood sampling. Others underwent protocol 7, including ~4 sessions of imaging under anaesthesia and blood sampling.

In protocol 8, 30% of operated mice were in initial distress due to the severe gastrointestinal surgery. In some cases this included weight loss, diarrhoea, poor wound healing and lack of appetite. This was resolved in most animals within the first week, as the stomach healed. In early experiments, surgery wounds did not heal well in obese animals and this was further refined by using antibiotics for longer, in addition to intrasite gel (see details under "refinement"). Following this period, animals healed well and regained their body weight. Only 79 mice (~0.6%) underwent this severe protocol. Most of the animals used in this project underwent sub-threshold severity (~7425 mice, 58%) which consisted in breeding and ear sampling for identification. This was required to generate transgenic mice for experiments or they were used for tissue sampling following termination by schedule 1. A high percentage of the animals (~4546mice, 35%) only experienced mild severity, often by being submitted to only one or two procedures involving mild discomfort such as blood sampling.

## Replacement

### **State why you need to use animals and why you cannot use non-animal alternatives.**

The maintenance of normal blood glucose requires the interplay between hormonal secretion from the islets of Langerhans and hormonal action on peripheral tissues such as the liver, the skeletal muscle and adipose tissues. Neuronal and endocrine outputs from the gut in response to changes in hormonal signalling and nutrient availability also modify the net effect on blood glucose. Such complex interrelations cannot be reproduced *in vitro* and require a whole living organism.

For bariatric surgery, this project will also require the use of a number of normal and diabetic animals to form surgical and control cohorts. This is essential as it is currently the only way we can replicate different types of gastric bypass and assess the progression of diabetes re emission day by day. Moreover, it has been demonstrated in the past that hormonal mechanisms associated with glucoregulatory gut function are not always direct eg. Gut- liver- pancreas axis for glucose homeostasis. It is therefore impossible to fully facilitate cell lines and explants to replicate the effect of bariatric surgery in vitro without an initial in vivo assessment. This type of research will require extensive access to post-operational intestinal tissue in specific days during the study, which is not feasible with human subjects who undergo bariatric surgery. Cell lines will be used in potential insulin sensitivity and receptor activation studies, to ensure a significant reduction of animals where necessary.

### **Retrospective assessment**

Published: 23 May 2022

**What, if any, non-animal alternatives were used or explored after the project started, how effective were they and are there any lessons worth sharing with others?**



They were not new non-animal alternatives that became available after this project licence was granted that could substitute the suggested experiments with animals to achieve the objectives proposed here

## Reduction

### **Explain how you will assure the use of minimum numbers of animals.**

Animal usage is based on careful power calculations, performed with G\*Power. For example, for glucose tolerance tests, we may need a total requirement of 22 animals (11 per genotype) per experiment. This requirement is based on a typical standard deviation in the measurement of blood glucose of ~4 mmol/L. So to detect abnormal glucose tolerance (20% difference compared to a normal 30 min peak of ~ 26 mmol/L during glucose tolerance tests in 12-week old WT C57BL/6 mice on high fat diet in our hands), the effect size  $d = 1.3$ , requiring a group of 11 mice per group to detect a change at a significance level ( $\alpha$ ) of 0.05 with 80 % power. [Campbell, M.J., Julious, S.A., & Altman, D.G. Estimating sample sizes for binary, ordered categorical, and continuous outcomes in two group comparisons. *BMJ* **311**, 1145-1148 (1995)]

### **Retrospective assessment**

Published: 23 May 2022

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

The planned measures for reduction took place:

Power calculations were performed throughout the project whenever possible to assure we were using enough animals to achieve adequate statistical power (which is the ability to find a significant effect in a sample given that there is an effect in the population from which the sample was drawn) and that no more animals than those strictly necessary were used in the experiments.

We didn't observe any rates of rederivation success below the expected normal values.

Rederivation is the transfer of embryos to female mice that are free of pathogens.

For protocol 7 (Islet Transplantation in the anterior chamber of the eye and imaging of the transplanted islets), this technique allowed us to obtain experimental information in a noninvasive manner. For some experiments, imaging endpoints allowed the same animal to be used as its own control or to be investigated over time, instead of requiring a group of animals per time point studied, thus minimising the number of animals used.

The number of animals used in protocol 8 was lower than anticipated since the refinement of the technique (see below) translated in lower mortality (~20%).

## Refinement

**Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.**



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

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

All the procedures in this application are done under local, general or terminal anaesthesia where appropriate to minimise stress and suffering of the animals.

A researcher with over 6 years of extensive microsurgery training and 2 years of experience in intestinal microsurgery will be performing the bariatric procedures. The researcher has already optimised the procedures in previous work appointments, in terms of pre, intra and postoperative care and will be consulting with the NVS on optimal animal care.

### **Retrospective assessment**

Published: 23 May 2022

#### **With the knowledge you have now, could the choice of animals or models used have been improved at all? How did you minimise harm to animals during the project?**

This licence was planned to study all aspects of diabetes in vivo using mouse and rat models. However, our team's expertise is greatly focussed on the study of the islets of Langerhans in genetically modified mice, and no experiments were performed in rats. Though rats of the relevant strain might need to be used in the future to achieve similar objectives due to the fact that they give a better yield of blood and tissues per animal than mice, the number of animals requested in future licences will be (/has been) lower. The experimental models used for protocols 1-6 had already been extensively refined:

We used several genetically modified models in which the genetic alteration was restricted to adults following treatment with specific drugs (such as tamoxifen or doxycycline), thus bypassing any potential detrimental effects of genetic alteration during development.

The use of diets to induce type 2 diabetes (T2D) allowed us to apply the least severe model of diabetes to meet our scientific objectives thanks to a continuous monitoring of the development of diabetes in the animals. We used a "westernised" diet, consisting of high sugar and/or high fat content, which is well tolerated but leads to obesity and type 2 diabetes over time.

For protocol 7, islet transplantation in the cornea (eye) and imaging are the less invasive methods that have allowed us to study islet function in vivo. Only mild discomfort has been associated with this model.

Protocol 8 is classified as severe but it has undergone important refinement since the first cohort was operated in July 2018, where peritonitis (infection of the inner layer of the abdomen) led to low survival of the animals (~30%). We have since refined the procedure using lean mice, giving them liquid diet for 3 days prior and 5 days post surgery, which



allowed better emptying prior to partial resection of the stomach, giving them antibiotics for 5 days post operatively and changing the suturing style. No animals were found dead as they were closely monitored. Two small cohorts of lean mice reached 70% survival and the latest cohort of high fat diet-fed mice (~50 animals) was at 85% survival. More specifically this is what has been discussed/agreed with the vets since the initiation of this project:

Mice are no longer fasted pre-operatively

Three days of liquid diet pre-operatively (sugar water) – to allow for faster and easier gastric emptying intra-operatively, and ensuring no food is caught between the suture lines.

Swabs: Pack the stomach off with moist swabs prior to gastric incision. Ensure that all swab pieces are removed at the end of the surgery and if gross contamination occurs remove swab and replace with a clean one.

Suture pattern: We are using a specific pattern of suture known as ("inverting pattern, Cushing's pattern or Lembert). This encloses the edges of the stomach reducing the chances of leakage and infection. 2-3 layers of suture should be placed to create a sufficient seal. Care should be taken to avoid penetrating the suture material into the stomach cavity although this will be very difficult in a mouse.

Mice are on liquid diet 10 days post- operatively, solid diet re introduced on day 7. This is still sugar water, due to ensure milk based diet solidifying in the stomach immediately after surgery, as no gut mobility takes place for the first 48 hrs. As a result, this increased tension on the gastric sutures when normal feeding behaviour resumed, causing ruptures.

Bedding is changed to softer and more absorbent material that is kinder to the abdominal wound.

A course of 5 days injection antibiotic (ciprofloxacin 0.1mg/kg or Baytril) is given to avoid peritonitis.

Skin sutures: during the last HFD cohort, we observed poor wound healing (expected from animals with high sugar levels in blood) which resulted in culling 3 animals as we are not allowed to close a wound more than once. This was not observed in lean animals, and pathology examination showed bacteria characteristic of faeces contamination of the wound, potentially because the increased body weight forces the large abdomen to rub on the cage floor. We have since discussed with the NVS and agreed that specific sutures ("interrupted intradermal") will be used. Moreover, a layer of glue will be applied over the wound to ensure sealing. As a precaution, antibiotics will be given in water for 5 days following the end of their 5 days injection course at a 20mg/kg dose.

Bariatric procedures are widely published and refined and following a learning curve can have a survival rate of 80%. Multiple learning curve papers and video protocols for all three procedures (RYGB, VSG, DJB) have been published, providing vital protocol information to improve animal welfare, increase survival rate and minimize stress that could affect research objectives. This makes these procedures the more refined of the group of gastrointestinal surgeries, which allows us to confirm the success of the model by comparing the phenotype presented in our cohorts to published data.



## 9. Analysis of skin carcinogenesis in transgenic mice

### Project duration

5 years 0 months

### Project purpose

- Basic research

### Key words

Carcinogenesis, Skin, Transgenic, Mechanism

### 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
- Required at inspector's discretion

### Objectives and benefits

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

### What's the aim of this project?

The incidence of non-melanoma skin cancer [squamous cell carcinoma SCC] is rising steadily. As for all cancers, skin tumour development is a multi-step process and for patients, the most significant stage in clinical terms is the conversion of benign tumours to malignancy and their subsequent increased risk of becoming metastatic. Whether a specific benign tumour possess the potential for progression is unclear, as this depends upon a complex combination of the types of genes mutated and precisely when they are acquired; as each mutation creates a unique tumour context with differing malignant potential. This cancer causing mechanism is also pitted against efforts of protective systems that have evolved to resist the cancer causing process [carcinogenesis] at each stage. To study this problem, genetic engineering technology has been coupled to the classic mouse skin model of multistage carcinogenesis, a mainstay of cancer research for more than 100 years, to create transgenic mice that develop cancer but only after treatment with topical steroids. The model is designed to assess the effects of multiple, genetic insults in well-defined, precancerous stages through to malignant tumours. The approach exploits genetic engineering techniques so that activation of cancer causing genes or inactivation of cancer preventing [tumour suppressor genes (TSGs)] occurs exclusively in the epidermis of skin. Further, an on/off gene switch system is incorporated



into the transgenic mouse design, to elicit highly localised disease but only following topical treatment with a steroid. By testing key genes thought to be responsible for causing tumours [the “driver” mutations] and studying their cooperation, the stage at which mutations become active can be identified.

The genes chosen for analysis represent commonly mutated tumour suppressor genes that help regulate skin cell growth and correct differentiation; and provide the protective systems that normally cope with mutations occurring naturally e.g. from daily sun exposure.

On going analysis of these multi-gene transgenic mice, will aid in identifying the relevant molecules involved in tumour progression or its inhibition. This approach may highlight a potential Achilles heel appropriate for targeted therapy, equally applicable to other cancer types.

### **Retrospective assessment**

Published: 15 September 2022

#### **Is there a plan for this work to continue under another licence?**

Yes

#### **Did the project achieve its aims and if not, why not?**

The incidence of skin cancer is rising steadily, and the most significant stage in clinical terms for any cancer patient is the conversion of non-malignant, benign tumours [in skin called papillomas] to malignant ones called carcinomas; and their risk of spreading called metastasis. Our research involved studying the activation of cancer-causing genes [called oncogenes] or inactivation of cancer preventing genes [called tumour suppressor genes (TSGs)] in the skin of genetically modified [transgenic] mice. Using this real life transgenic mouse approach, we tested which are the key genes thought to be responsible for causing tumours [the “driver” mutations] and by studying their co-operation, we assessed the stage at which these mutations become active to start cancer and focused on which mutations caused benign tumours to become malignant carcinomas or expressed genes that inhibited this process and limited tumour progression.

This approach required topical treatment of mouse skin with a steroid which then activated the specific genetic mutation[s] and was designed to ensure tumours appeared only in the skin and prevent disease in the internal organs. Thus any tumours produced were localised to areas of treated skin; e.g. the ear tip, and this approach assessed effects of several mutations in pre-cancerous stages that lead to benign and later malignant tumours.

Three main themes were investigated in this research:

The first theme investigated the effect of mutations in genes that were involved in driving cell division [the proto-oncogenes that mutate to become oncogenes] and loss of genes that normally cause cells to stop dividing i.e. the tumour suppressor genes [TSG]. Both sets of genes are prime targets for mutations that lead to cancer formation and we chose to assess two common oncogenes and three TSGs whose functions are often lost in human cancers.



In summary, we found that typically combinations of two mutant genes induced a benign, wartlike papilloma that either regressed or persisted but did not become malignant. This result therefore gave the platform to study which mutations make this tumour persist and not regress, and we successfully identified which extra mutations caused a benign tumour to become malignant and which combination caused an early malignant tumour to progress and become an aggressive carcinoma. We also found that one specific combination formed an odd benign skin tumour called a keratoacanthoma, which is benign but can be confused with carcinoma.

Collectively, our results showed that skin was quite resistant to cancer formation requiring four possibly five potent genetic mutations to achieve malignancy and thus a platform to try to target these genes in new therapies. Employing these benign tumours therefore, a second theme investigated whether protection of an important TSG called p53 could inhibit cancer formation in our mice, with the idea that identification of these molecules may become a good therapeutic target. The p53 TSG has roles in stopping cell division, to give time to make sure no mutations occurred e.g. those arising from exposure to UV in sunlight; and was one of the key TSGs studied above, were we found that its loss caused malignancy. However, in normal skin, p53 has to be carefully regulated to ensure there is sufficient cell growth to continually replace the skin cells that differentiate to form the outer skin layers and maintain the barrier functions. Again this was a prime target for failure, as observed in our earlier experiments, but had the potential to inhibit the cancer process if p53 levels could be protected.

The first aspect to inhibit tumour formation explored the effect of increased expression of a gene which acts to increase expression of p53; whilst the second approach explored effects of preventing removal of p53. However, when we created transgenic mice that increased expression of the gene that regulated p53 levels, it gave unexpected results and instead of inhibiting tumour formation it co-operated with our oncogenes to drive benign tumour formation and their conversion to malignancy. Moreover, one combination resulted in the formation of a novel aggressive hair follicle cancer whether p53 was present or not; suggesting that we had discovered an important pathway involved in the development of this rare skin cancer type in humans. This result indicated that this hair follicle tumour type was unusual in that it was independent of p53 status- hence p53 loss or engineered increased expression had little effect. These results highlighted the complexity of cancer formation and showed that any given mutation may have different roles in the formation of different tumours; with significant consequences for design of appropriate targeted therapies.

The second aspect explored experimental therapies geared to restoration of p53 function, which is lost in many human cancers, and gave a more logical outcome. These experiments are still underway, but early results showed that initially, p53 protection does indeed stop the formation of benign tumours in the majority of mice-but for a period. As in some older mice the increased protection from p53 became circumvented: either by mutations in p53 itself that cause loss of the TSG functions or that the transformed cells take alternate cancer causing pathways that avoid a role for p53; as seen with the hair follicle tumours. Also these extra events needed to bypass this initial protection resulted in a more aggressive cancer.

A third final theme investigated the failure of cancer cells to remain attached to their neighbour, which is a key feature that allows invasion of the surrounding tissue in malignant tumours. One approach investigated mutation in one of the proteins that



regulate the process of normal cell movement e.g. as the skin grows and replenishes itself every month, cells shuffle into their different layers [called differentiation]; and a second approach investigated direct effects of disrupting the molecules that form part of the zip which helps hold cells together. Thus, these normal functions which interact with the molecules that hold cells together are prime candidates for failure in the cancer causing process.

The first experiments investigated mutations in a gene that regulated this cell movement, changing the rigidity and stiffness to aid migration e.g. to allow cells to move and close the gap following a wound to complete the healing process. We found that this mutation induced benign tumour conversion to malignancy and now gave an enhanced ability to migrate and wiggle away from the benign tissues. This increased cell motility helped the tumour cells invade and induced a rapid progression to aggressive carcinoma. We also found that this event needed the benign tumour stage; as earlier, the skin was able to respond to this altered tension and stiffness in these corrupted cells and prevent their migration by inducing p53. Thus, this migration ability was a late stage event and is now considered a prime target for therapeutic intervention to combat tumour invasion.

In order to wiggle and detach from a benign tumour tissue, there needed to be changes in the molecules that originally glued the cells in place. Therefore, a final set of experiments investigated this failed cell-cell adhesion by deregulating a molecule that helps anchor cell membrane proteins that zip cells together. When cells normally need to divide, these molecules unzip and the anchoring protein then takes this information to the nucleus. Thus, this message informs the nucleus that division needs to take place and helps coordinate the expression of the correct genes involved in cell division. Again these are prime candidates for failure leading to the formation of cancer.

We found that when we mutated the anchoring protein, this induced a hair follicle tumour [trichilemmomas] similar to hair follicle tumours produced in Cowden's Disease; a familial cancer syndrome in humans. However, overall we observed that mouse skin was very sensitive to this mutation that caused excess cell division and the skin responded by inducing their differentiation to become the keratinised, dead outer layer of skin. This compensatory response spread into untreated areas and resulted in a very keratotic skin.

Whilst this inhibited benign tumour formation in certain combinations, the degree of keratosis produced was deemed to approach the limits of a moderate severity and these experiments ceased. Instead, in on-going experiments we have mutated the protein that forms the teeth of the zip and this has not induced this harmful keratotic response, allowing us to continue our experiments to investigate the failure in cell adhesion and tumour invasion.

**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.**

**What are the potential benefits that will derive from this project?**

It is envisaged that analysis of how the progression mechanism unfolds in a real life situation will identify changes to additional key genes to aid in the design of novel therapeutics; whilst the animals themselves may provide a future technology platform able to stringently test novel generic therapeutics or those directly tailored to the actual causal



mutations underlying cancer formation in both skin and be relevant to other tissues such as breast and colon carcinogenesis.

### **Species and numbers of animals expected to be used**

#### **What types and approximate numbers of animals will you use over the course of this project?**

GA mice; up to 7000 over 5 years.

### **Predicted harms**

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

#### **In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?**

Certain transgene(s) expression elicits a persistent thickened dry, scaly skin in adult mice. The hair is also soft, sparse and shortened. At localised sites [e.g. ear tip] treated with the inducing hormone; wart-like benign tumours appear. Changes in benign tumour size may lead to malignant conversion: typified by a change from rounded, cauliflower-like appearance of papilloma clusters, to a solid, uniform tumour with a typical concave indentation. This is the end point; unless contra-indications apply e.g. benign tumour size, skin location which impedes natural functions. The majority of mice [>90%] are biopsied following humane killing for genetic analysis.

Such adverse effects would be closely monitored to adhere to the current recommended guidelines (Workman et al 2010. BJC; [www.NC3Rs.org.uk](http://www.NC3Rs.org.uk)).

On occasion certain genes can elicit a greater degree of hyperplasia and hyperkeratosis resulting in a more thickened, scaly skin in juveniles and give adults with a hunched appearance. This phenotype can result in mice that are smaller than normal, mainly due to competition from littermates. Thus, a scoring system is applied to help monitor the developing phenotypes exhibited and on rare occasions, such hyperkeratotic mice that are deemed scientifically essential may be maintained for a short period to observed e.g if this increased differentiation blocks tumour formation.

### **Retrospective assessment**

Published: 15 September 2022

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

The mouse skin model of multistage cancer formation is considered to be a very useful approach to study the formation of cancer. An advantage is that these tumours are easily observed, and any changes are easily followed over time; from early pre-cancer stages that manifest as a scaly rash, to wart-like benign tumours that may regress or progress into fully malignant tumours (termed carcinomas). Our ability to target expression of mutant genes specifically to the skin avoids disease in the internal organs, and the use of



a gene switch means that the mutations remain dormant until mouse skin is treated with a steroid.

For the past 30 years individual transgenic mouse genotypes have been established on previous project licences and routinely bred to create new genotype combinations. In these mice, one of the genes is the gene switch regulator which is turned on when adult mouse skin was painted with a steroid and expression of the target gene occurs in these areas of treated skin. Thus, any potential tumour formation was highly localised by painting an ear tip or lower back and these areas either developed tumours or tumour growth was inhibited depending upon which genes were activated. Thus in the breeding protocol the requirement for this topical treatment meant that the majority of breeders did not exhibit a phenotype and harm was restricted to analysis of mouse genotype.

Here juvenile mice [~ 24 days old] were ID tagged on the ear and a tiny piece of tail tissue was removed for DNA analysis to identify which mice contain the correct genes. In the majority of mice used in breeding [pa: 913 + 937+ 1225 + 531 + 595 = 4201] 80% remained disease free [termed sub-threshold; approx. 3360 mice] with the remaining 20% [ approx. 820 mice ] exhibiting a mild, ear keratosis or a benign ear tumour by 6 months of age.

For protocol 2, the induction of tumours, tagged mice [2814 mice] were anaesthetised and treated topically with a steroid at the ear tip and lower back and maintained for up to 6 months to assess tumour inhibition or tumour progression. Here a scoring system was adopted to prevent tumours exceeding moderate severity limits. Any animals that exceed the clinical signs associated with the moderate were promptly and humanely killed. In this protocol effects remained sub-threshold [540 mice] whilst other individuals exhibited a scaly/keratotic skin and the hair was sometimes sparse but no tumours appeared and remained mild [653 mice]. These mice were designated score 0 and maintained for up to 6 months and humanely killed by a schedule 1 method.

In the moderate category [1621 mice], approx. 90% of mice [1460 individuals] exhibited a score 1: where treated mice produced a scaly skin and by 8-10 weeks produced a benign tumour. Depending on the genetic mutations, this tumour could become malignant over the subsequent weeks or regress. Malignancy was typified by a change from the cauliflower-like appearance of a benign tumour, to a tumour with a typical concave indentation [i.e. volcanolike] indicating carcinoma. These mice were monitored thrice weekly for any adverse effects and adhered to the recommended guidelines for mouse cancer models (Workman et al 2010. BJC; [www.NC3Rs.org.uk](http://www.NC3Rs.org.uk)). Benign tumour conversion was considered the end point unless other issues appeared e.g., tumour size/location, and these mice were maintained for up to 6 months to give the time to assess any progression/regression, and killed by schedule 1 methods prior to skin/tumour biopsy.

Approximately 10% of the moderate category in protocol 2 mice [162 individuals] exhibited a score 2, where treated mice produced a more significant level of thickened, scaly skin with some hair loss. Such individuals were quite obvious by 2-3 weeks post steroid treatment, and typically exhibited a more rapid appearance of benign tumours [6-8 weeks], which could become malignant over the subsequent two to four weeks when animals were promptly and humanely killed. Hence, these mice were monitored daily to assess their overall condition and ensure that the moderate severity level was not exceeded. Again, conversion to malignancy was the end point unless tumour size or severity of the skin phenotype approached the limits of a moderate protocol. The latter was assessed on a



case by case basis and relied on the PI experience of the previous 30 years. Mice were killed by schedule 1 and tumours biopsied. Adverse effects adhered to the recommended guidelines for cancer models (Workman et al 2010. BJC; www.NC3Rs.org.uk).

NB : During the course of the cell adhesion experiments, it became clear that mice were highly sensitive to mutation in the anchoring protein and gave immediate score 2 individuals. This unexpected increased skin phenotype rapidly spread into untreated areas such that two mice were deemed to exceed the moderate category and were immediately euthanized. Initially, this PPL was altered to include a severe category in a new protocol as this differentiation response to excess cell division also inhibited tumour formation. However, it became clear that this approach would lead to an unavoidable level of harm and these experiments were discontinued. No animals were transferred to this new protocol.

## Replacement

### **State why you need to use animals and why you cannot use non-animal alternatives.**

The objectives of this project cannot be achieved without the use of live, genetically altered [GA] animals.

It is the goal of the project to establish inducible transgenic mouse skin models to verify causal roles for multiple, relevant mutations that drive stage-specific tumour progression, explore the subsequent mechanism and identify putative compensatory sentinel systems that have evolved to inhibit carcinogenesis. Unlike cell culture systems, transgenic mice offer the possibility to determine the influence of factors critical to malignant progression such as blood supply, an intact immune system, hormonal and cell-mediated growth controls, together with the physical barriers that inhibit tumour cell growth and invasion. However, experiments will utilise skin cells from transgenic mice for tissue culture experiments and the results will be compared to the mouse data. This will assess whether this approach accurately mimics tumour progression sufficiently well to replace the need for monitoring malignant progression in adults.

### **Retrospective assessment**

Published: 15 September 2022

### **What, if any, non-animal alternatives were used or explored after the project started, how effective were they and are there any lessons worth sharing with others?**

The objectives of this project could not be achieved without the use of genetically modified [GM] animals. The investigation identified roles for mutated genes that drive or inhibited tumour formation and focussed on the conversion of benign tumours to malignancy. We were able to identify when the mutations exerted their effects and identify sentinel systems inhibited this process and may become valuable targets for new therapeutic approaches. Unlike cells cultured in dishes, mice offer the possibility to determine the influence of factors critical to cancer formation, such as an increased blood supply or an intact immune system, together with the physical barriers that are present and inhibit tumour cell invasion. However, complimentary experiments were performed in tissue culture that utilised skin keratinocytes [the cells that become carcinomas] and assessed their benign or malignant potential e.g. we investigated the ability of cultured tumour cells to invade a gel-like matrix in the development of a 3-D "living skin". These tissue culture experiments often



gave contrasting results compared to the GM animals; possibly due to the sentinel anticancer responses deployed by a complete tissue.

## Reduction

### **Explain how you will assure the use of minimum numbers of animals.**

Considerable experience has been gained in previous experiments. This monitoring permit experiments to be terminated as soon as significant data has been obtained, thus minimising suffering whilst obtaining meaningful data. Whilst generating these cohorts requires breeding of multiple GM lines, breeding strategies are carefully structured and rigorously monitored to ensure that only the minimum numbers are generated. In addition, given the requirement for topical steroid treatments, animals carrying multiple target transgenes can be routinely maintained; again reducing overall numbers. Untreated breeders act as additional controls and since several genotypes have already been characterised repeating these comparison controls is unnecessary; thus further reducing numbers required.

### **Retrospective assessment**

Published: 15 September 2022

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

Considerable experience has been gained over the past 30 years and has distilled the numbers required to obtain meaningful data to [up to] 15 animals per genotype in repeat experiments. Given the requirement for topical treatment to activate the mutations, e.g. treating each ear tip and a small dorsal area, this has the capacity to produce up to 90 biopsies. This requirement for topical treatment to induce the activation of the mutations meant an individual breeder could carry several latent mutations without developing any tumours, thus reducing overall numbers required. Breeding strategies were carefully structured and rigorously monitored to ensure that only the minimum numbers were generated and treated cohorts were carefully monitored so that experiments were terminated as soon as significant data was obtained. In addition, normal breeders act as untreated comparison controls that detect the appearance of any non-specific, unexpected events.

Further, several genotypes have been characterised in previous PPLs, thus repeating these as comparison controls was unnecessary, further reducing numbers. The total number of GM animals utilised was also determined by each outcome; i.e. in certain instances malignant conversion occurred with loss of one TSG and subsequent cohorts were not required; and contra-indications also dictated cessation of certain experiments.

## Refinement

### **Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.**

Ideally a transgenic mouse model system should be able to assess multiple, stage-specific genetic insults and accurately mimic the discrete tumour pathologies observed in human



carcinogenesis; yet be designed to minimise disease wherever possible. Mouse skin is an ideal target tissue being the classic model for multi-stage carcinogenesis. Here a major advantage of skin models is their accessibility, which not only facilitates induction of localised disease but also allows macroscopic observation of cancer causing events without invasive procedures. A major strength employs a skin-specific inducible gene switch system that prevents disease during animal development or unnecessary disease in internal organs. Thus, during breeding and juvenile development, tumours do not appear; and moreover, as disease can be highly localised [e.g. treatment of ear tip] suffering is minimised.

## **Retrospective assessment**

Published: 15 September 2022

### **With the knowledge you have now, could the choice of animals or models used have been improved at all? How did you minimise harm to animals during the project?**

Ideally any system designed to accurately mimic cancer formation in humans should be able to assess multiple, stage-specific mutations. Mouse skin considered one of the most useful systems to study tumour progression as it accurately mimicked the tumour pathologies observed in humans skin cancers; yet was designed to minimise disease and harm where possible. Here, the use of a skin specific, inducible gene switch system that required steroid treatment to activate the latent mutations meant that animals were mainly disease free e.g. during breeding. Topical treatment prevented disease in the internal organs and during animal development, juveniles were tumour free until skin was treated and tumours were localised [e.g. treatment of ear tip]. Another major advantage of skin was its accessibility which allowed macroscopic observation of cancer progression without invasive procedures. In being one of the classic models employed to study carcinogenesis, many relevant papers have been published and the results produced in this PPL cycle compared these data and lead to new experiments able to acquire/share appropriate mice/reagents with colleagues.



This non-technical summary is currently not accessible due to a technical issue. We are working to resolve this, and it will be added as soon as possible. We appreciate your patience and understanding as we address this matter.

## 10. Metabolism and Toxicity in 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 aims mentioned in purpose (b)

### Key words

Pharmaceutical, Regulatory, Primate

### 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.

- Required at inspector's discretion

### Objectives and benefits

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

#### What's the aim of this project?

This project enables the programme of regulatory metabolism, immunology and toxicology studies in the non-human primate (macaques and marmosets), and the validation or investigative studies which enable the regulatory programme.

#### **A retrospective assessment of these aims will be due by 30 May 2022**

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.**

### **What are the potential benefits that will derive from this project?**

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. At present there are no alternatives that don't use animals that are scientifically, ethically or legally acceptable as replacements for systemic toxicity assessment.

### **Species and numbers of animals expected to be used**

#### **What types and approximate numbers of animals will you use over the course of this project?**

Macaques (cynomolgus and rhesus) 7000 Marmosets 500 Over 5 years.

### **Predicted harms**

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

#### **In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?**

Procedures carried out during these studies include:

- : Dosing (eg by oral administration, injection, infusion, insufflation),
- : Blood sampling or collection of urine for measurement of different components as changes in these may serve as early indicators of toxicity. Doctors for similar reasons often take blood and urine samples from humans.
- : ECG monitoring to assess changes in heart function (e.g. number of heart beats per minute). This technique is also used by doctors to assess heart function in humans.
- : Examination of the eyes using a similar device to that used by opticians
- : Examination of more unusual parameters, eg retinography, placement of subcutaneous vascular access port or tissue biopsy under general anaesthesia, seminology (sampling by direct stimulation), body temperature by rectal thermometer (such as a doctor might use for a small child).

A degree of restraint or confinement may be required for some procedures. The animals are trained using positive reinforcement (treat rewards) to move about the cages for handling/procedures, and to sit in restraint chairs.

Some animals are re-used, but most animals are humanely killed at the end of the study by an overdose of anaesthetic to allow detailed examination of the organs.

The majority of animals are expected to have mild adverse effects such as slight weight loss. A small percentage of animals may show more significant adverse effects e.g. more marked weight loss, or changes in appearance or behaviour (e.g. reduced activity)



indicative of moderate severity. Humane end-points are applied, under veterinary guidance as necessary.

### **A retrospective assessment of these predicted harms will be due by 30 May 2022**

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 why you need to use animals and why you cannot use non-animal alternatives.**

Pharmaceutical testing is a mandatory 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.

In vitro and in silico methods are used in combination with animal studies to inform study designs and assist in understanding of potential toxicity but cannot yet replace in vivo studies.

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.

### **A retrospective assessment of replacement will be due by 30 May 2022**

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 you will assure the use of minimum numbers of animals.**

The numbers of animals used in any particular study are generally linked directly to those indicated in the published regulatory guidelines. Animal numbers are kept to the minimum commensurate with meeting the objective of each study.

### **A retrospective assessment of reduction will be due by 30 May 2022**

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**



**Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.**

We use non-human primates when other species (dogs and/or pigs) are unsuitable by one or more of the following criteria:

: kinetic or metabolic differences from man;

: species specific pharmacological or toxicological response,

Or when only primates are suitable by the following criteria:

: relevant toxicity or pharmacology which is only shown in a primate

: study design requires assessment of effects on organ systems or receptors for which primates are the only relevant model.

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 and ameliorate adverse events.

**A retrospective assessment of refinement will be due by 30 May 2022**

The PPL holder will be required to disclose:

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



## 11. Antibody Production for Research, Diagnosis and Therapy

### 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 aims mentioned in purpose (b)

### Key words

Antibodies, sheep, goats, hens, donkeys

## 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.

- Required at inspector's discretion

## Objectives and benefits

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

### What's the aim of this project?

Farm animals (sheep, goats and hens) and donkeys will be injected with minute quantities of proteins, peptides or haptens conjugated to a carrier protein to stimulate the production of antibodies. Blood donations will be taken and the antibodies from the blood serum will be used to produce antivenoms to snake bites, or treatments for other life-threatening conditions such as serious infections or drug overdose. Antibodies are also required for use in a vast range of laboratory tests for the diagnosis and monitoring of disease.

### Retrospective assessment

Published: 15 September 2022

### Is there a plan for this work to continue under another licence?

Yes



### **Did the project achieve its aims and if not, why not?**

A variety of antisera were raised successfully against many targets for diagnostic, research and therapeutic applications.

Plasma was produced for diagnostic kits to aid the detection of a clotting factor in human blood.

**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.**

### **What are the potential benefits that will derive from this project?**

Research projects for advancement of science, improved reagents for diagnosis and potential treatment.

Early stage development of novel therapeutic treatments for diseases such as Clostridia, Leishmania, snake bites, Ebola, colchicine poisoning, CPG (cancer marker for novel cancer therapy - DNA oligonucleotides containing unmethylated deoxycytidylyl-deoxyguanosine dinucleotides are commonly referred to as CpGs

Identification of improved antibody reagents for human, animal and plant diseases, e.g. malaria, drugs of abuse, legionella, ricin, and botulinum.

There is a great unmet demand for antivenoms, especially in the third world, where every year many thousands of the poorest people and their children die from snake bite or suffer serious consequences such as amputations. Antivenoms are the only effective treatment for snake bites or many other envenomations. However, there is currently a crisis in supply, particularly for Africa. The challenge is to provide affordable treatments for the populations at most risk.

### **Species and numbers of animals expected to be used**

#### **What types and approximate numbers of animals will you use over the course of this project?**

Up to about 250 sheep, 10 goats, 2 donkeys and 20 hens will be used each year.

### **Predicted harms**

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

#### **In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?**

Only mild procedures are used for injection of the animals. The collection of their blood is comparable to blood donations by humans. Antibodies from hens may be obtained by collecting their eggs, without need for bleeding, because hens' antibodies are found in their egg yolks as well as their blood. Sheep and goats, with their contractile spleens can



adapt to regular bleeding, with no effect on their health. Unlike many other species used for antibody production sheep and goats are genetically programmed for “affinity maturation” of the antibody response; leading to much higher antibody affinity than other species.

Donkeys pose a problem because, like horses, a vigorous local immune and inflammatory response may lead to swelling and a sterile abscess at the immunisation site. Donkeys may also show wide individual variability and some may show substantial reaction to injections. Only Freund’s Incomplete Adjuvant will be used in donkeys.

Less than 1% incidence of systemic reactions, e.g. anaphylactic shock, is expected or anaemia.

During the previous 5 years of the licence no systemic reactions have been observed.

A mild severity level is expected in animals used in short projects and this can become moderate severity in animals used for longer projects.

## **Retrospective assessment**

Published: 15 September 2022

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

No animals exceeded the moderate severity stated in the project licence. No animals were reused on the antibody production protocols. The experience of the sheep on the antibody production protocols were mild transient pain from needle insertion, lump formation around immunisation sites which in some cases reached moderate severity due to their size, mainly animals on longer projects. Ulcers and inflammation at these areas was observed in some cases but were treated and healed and no animals were required to be culled as a result of this. No anaemia or anaphylactic shock was observed on these protocols.

No animals on the normal blood products protocols experienced more than a mild severity. The experience for animals on this procedure consisted of only mild transient pain from needle insertion. Only sheep were used for normal blood products during the lifetime of the licence, no donkeys or goats were used for this objective. Sheep were reused on this protocol.

## **Replacement**

### **State why you need to use animals and why you cannot use non-animal alternatives.**

There are still applications for which animal derived antibodies are essential, e.g. antivenom production, where antibodies to all of the epitopes of the toxins and peptides are required: 10s to 100s. It has been proven that using large farm animals, e.g. sheep and horses, commercial production for human therapy of life threatening diseases is reliable and cost effective: products should be, safe, effective and affordable at their point of use.



Donkeys need to be used to produce the secondary reagents, i.e. donkey anti-sheep IgG, Fc, / Fab. These reagents cannot be produced in sheep as they would not be recognised as foreign and hence not produce an antibody response. Donkey secondary reagents are used in assays which employ a sheep antibody as the primary reagent.

### **Retrospective assessment**

Published: 15 September 2022

#### **What, if any, non-animal alternatives were used or explored after the project started, how effective were they and are there any lessons worth sharing with others?**

There was an increase in the number of RNA extractions from white blood cells of immunised sheep. While this didn't completely replace the use of animals, it shortened the length of the procedures for the animals and reduced the number of animals required on protocols.

Big steps were made in the development of in-house phage display technology which is expected to be implemented during the lifetime of the current Home Office licence.

### **Reduction**

#### **Explain how you will assure the use of minimum numbers of animals.**

Re-use, providing there are no scientific or welfare prohibitions, reduces the total number of animals used in regulated procedures. Intelligent up front design and selection of the antigen can have a large positive effect on the desired antibody response and thus limit the numbers of animals required in order to obtain the required results.

Ig has an on-going research programme with partners to develop methods for sheep monoclonals and phage display antibodies through purification of peripheral blood lymphocytes from hyper-immunised sheep. These two projects have the potential to reduce the future number of animals as once immortalised antibodies can be produced in-vitro and the use of further animals is reduced or eliminated.

### **Retrospective assessment**

Published: 15 September 2022

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

There was an increased requirement for white blood cell isolation procedures for animals on antibody production protocols. The white cell isolation preparation from a blood donation enabled the projects to finish sooner, reducing the length of time the animals were subjected to immunisation and bleeding. This procedure also allows for a reduction in the number of animals required on a project.

Numbers of sheep were reduced on the normal blood products protocol by the reuse of sheep. Sheep for this protocol were sourced from the antibody production protocols once they had completed their projects, and after the NVS had inspected the animals to determine that they had not suffered or were likely to suffer from their previous use. All



animals reused on a normal blood products protocol had a high condition score and heavy weight.

## Refinement

**Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.**

The phylogenetic distance of hens from mammals may lead to raising of superior antibodies against some mammalian proteins, which may have advantages for certain diagnostic applications.

Donkeys are employed for the production of second antibodies only. Second antibodies must be produced in a species distant from the host used to produce the primary antibody.

Antibodies produced in animals ("polyclonal" antibodies) are the most effective because of the power and exquisite specificity of the immune system. "Monoclonal" antibodies may be produced in the laboratory but they are not as effective as polyclonals for these purposes, and are more expensive.

Sheep and goats provide the most effective and economical source of polyclonal antibodies for antivenoms and other therapeutic uses. They may be bled regularly with no ill effects. Polyclonal antibody production must be initiated in order to provide the B-cells or RNA required for in-vitro techniques such as monoclonal or phage display antibody production.

The use of animals such as sheep, goats, donkeys and hens permits the husbandry of animals under high standards of farming conditions; with grazing in fields in the summer and indoor housing with feed supplements in winter. Their useful life is generally longer than commercially farmed animals. All animals are under the day-to-day care of an animal welfare officer and are visited frequently by an external veterinary surgeon. Refined techniques include optimised emulsion preparation, targeted immunisation sites, breed selection (hybrids not pure breeds, larger breeds).

## Retrospective assessment

Published: 15 September 2022

**With the knowledge you have now, could the choice of animals or models used have been improved at all? How did you minimise harm to animals during the project?**

Sheep were chosen as the main species for antibody production due to their relative ease in handling reducing stress on the animal, and their contractile spleen which allows faster recovery from blood donations. Sheep used during the life of the project licence produced high affinity antibodies and larger volumes of blood donations than would be possible with many other species.

Alpacas will be added to our current licence as alpacas (camelids) naturally produce quantities of small antibodies with a simpler structure that can be turned into nanobodies which can be of greater advantage in many applications.



The use of Freund's adjuvant has been the choice of adjuvant for many years and continues to be the best choice for raising antibodies in animals. The use of Freund's adjuvant has not resulted in any more than moderate reactions and not resulted in the termination of any animals.



## 12. Modulation of murine lung injury by hormones, cells and growth factors

### Project duration

5 years 0 months

### Project purpose

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

### Key words

Lung injury, sepsis, vitamin D, steroid

## 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
- Required at inspector's discretion

## Objectives and benefits

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

### What's the aim of this project?

Broadly speaking the animal studies proposed aim to define if altering of hormone levels influences animal models of lung infection (pneumonia), abdominal infection (sepsis) and severe lung damage that can occur following these infections known as ARDS.

Using genetically modified or dietary adjusted mice we will determine if severity and duration of lung damage can be improved using cells and or vitamin d replacement. We will determine whether these approaches improve the models to be more reflective of human disease.



We have genetically engineered cells known as mesenchymal stem cells (MSCs) to over express an enzyme known as HSD-1. We believe these cells will help protect and promote recovery from the lung damage and inflammation induced in the different mouse models proposed.

Clinical applications would be possible following these experiments since we could generate human MSCs that over-express HSD-1 and undertake phase I-II studies once we have animal proof of concept that recovery is enhanced with tMSCs

To inform clinical trial design, we will also study the effects of replacing vitamin D in mice that have low levels of vitamin induced by a special diet. We will study how long after the start of the injury we can give vitamin D to try and determine how to treat patients with the drug,

Vitamin D replacement could be rapidly studied in humans as a phase II trial if vitamin D treatment improves the resolution of infection. Key unanswered questions are how long after the injury is vitamin D replacement effective and is there a threshold effect for the effect of vitamin D –ie what blood level of vitamin D provides the maximum benefit

### **Retrospective assessment**

Published: 26 June 2023

**Is there a plan for this work to continue under another licence? Yes**

**Did the project achieve its aims and if not, why not?**

We have achieved objectives 1 and 2 which were to establish two models one of acute respiratory distress syndrome and early sepsis. Use of these models has resulted in successful publications. Aims 3 and 4 were the most complex and was unfortunately only partially achieved. The programme of work designed to use an intranasal model of pneumonia and secondary infection (aim 3) was successfully delivered however our aim to investigate age related differences (aim 4) in immune response pneumonia and a secondary model of infection were not fully completed as we have yet to fully define the mechanisms driving our observed immunoparesis, typified by neutrophil dysfunction. This may be related to project delays. It took longer than anticipated to characterise this model both in terms of logistics (wait time for aged mice) and by necessity (COVID-19 pandemic and subsequent lockdown).

We now have a well-established secondary model of pneumonia, that we can use to demonstrate the same kind of age-related impairment of bacterial elimination accompanied by neutrophilic inflammation seen in aged humans. This model will form the basis of our subsequent licence which will focus on using this model to understand the mechanism of this impairment. Before a new licence application is submitted we wish to examine this defect in a wider cohort of human disease before returning to this model for further exploration of the mechanism.

**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.**



### **What are the potential benefits that will derive from this project?**

We will potentially identify new ways of treating patients with sepsis / pneumonia and ARDS.

### **Species and numbers of animals expected to be used**

### **What types and approximate numbers of animals will you use over the course of this project?**

- Mice

Approximately 8200 over the 5 years – most will be colony for breeding.

### **Predicted harms**

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

### **In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?**

Intratracheal administration of bacterial Lipopolysaccharide.

This model we have used to generate our preliminary data in mice.

LPS is a part of the cell wall of many bacteria. When injected into the lungs of mice it causes inflammation – with cells called neutrophils being recruited to the lung.

The effect on the mice is moderate but they become unwell at 24-48 hours with increased breathing rate and a degree of weight loss. We ameliorate this by giving pain killers and fluids.

The animals are closely monitored and any that show significant suffering are euthanased..

Cecal ligation and puncture (CLP, severe severity).

Many of the procedures undertaken in this model are similar to as outlined above.

The injury model is a surgical model where anaesthetised animals undergo an abdominal incision, followed by tying off (ligation) of the caecum of the bowel. The caecum is then punctured with a needle to release faecal contents into the abdomen that causes infection and sepsis.

As a result of CLP, there is a potential for pain if the anaesthetic depth is inappropriate, mice lose weight and may become unwell – lethargy starry coat, reduced activity. Animals are monitored postCLP using a clinical scoring system that means that the severity of the model can be kept to moderate. Animals undergo humane killing if they reach a severity of 4 on that scale.



There is a small risk of the sutures used to close the incision opening up post-operatively. Animals are given fluids and analgesia prior to the incision being closed to reduce the adverse effects,

Pneumococcal Pneumonia model (moderate severity).

Many of the procedures undertaken in this model are similar to as outlined above in the IT LPS model. This model involves the intranasal loading of the pneumococcus bacteria. Putting the bacteria into the nose makes the mice sniff the bacteria into the lungs. The intranasal injection may cause stress due to restraint and transient discomfort from the tubing insertion and or anaesthetic injection. The animals do develop some respiratory distress and are assessed clinically using a scoring system outlined in to minimise the severity of the model which is moderate in severity. Analgesia and fluids may be given to reduce the adverse effects.

### **Retrospective assessment**

Published: 26 June 2023

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

Mice undergoing Caecal ligation and puncture on this licence were scored carefully to ensure no animals were exposed to undue suffering however this is a severe procedure as it is required to model polymicrobial sepsis. These animals therefore demonstrate significantly reduced movement, and weight loss. A small number of animals used in sham surgery or those with tristetraprolin (TTP) knock in modification had a retrospective moderate severity. This is reflective of the fact that they have undergone a surgical procedure and so despite use of peri- and post- surgical analgesia, there is still the potential pain and a systemic response to the surgery. The presence of TTP means that the animals are more tolerant to the effects of infection hence these were of a moderate severity.

In our intranasal pneumococcal model, approximately 15% had a retrospective severe outcome. All these severe mice were humanely killed within the first 6 hours of intranasal inoculation with live bacteria. During the first 6 hours of infection, animals experience reduced movement and activity, and piloerection due to the fever and inflammation induced by the bacteria. However, after this period, animals improve and make a recovery. The animals that were humanely killed within the first 6 hours were those that had a high clinical score and were deemed unable to continue the procedure or were deemed unlikely to recover. We used a careful dosing strategy in collaboration with our animal facility to ensure any harm to the animals was minimised. The remainder of the mice used in this model experienced a moderate severity of suffering during the experimental timecourse. Only 78 mice were used under this licence. Of these, 26 animals experienced a retrospective severity of severe, and 52 experienced a retrospective severity of moderate. One of the animals recorded as severe was unfortunately found dead. Upon investigation it was determined that the animal died due to overwhelming infection. This was reported to the Home Office as a Standard Condition 18. The issue was addressed and no further incidences occurred.

### **Replacement**



## **State why you need to use animals and why you cannot use non-animal alternatives.**

The animal experiments are necessary to allow us to ascertain whether our proposed novel treatments for pneumonia, sepsis and ARDS have efficacy as a proof of concept in mice. We build upon our previous work in mice using these models. We cannot use fish for this work as they do not have lungs and there are no validated sepsis models.

We have already used other translational methods such as primary human alveolar macrophage and alveolar epithelial cell culture in order to minimise and reduce the number of animals required but we need the animal model to study the mechanistic implications of this project in more detail.

### **Retrospective assessment**

Published: 26 June 2023

## **What, if any, non-animal alternatives were used or explored after the project started, how effective were they and are there any lessons worth sharing with others?**

We are a translational research group and we routinely used primary cells from humans such as neutrophils and peripheral blood mononuclear cells as non-animal models to support our animal experiments. We have established a precision cut lung slice model (PCLS), using lung tissue from the healthy margin from patient resections. PCLS can be maintained in culture for 5 days where they can accurately model the cell-cell interactions of the lung and resident immune cells in their native architecture. We have taken what we have learned during this licence to apply this model an variety of infectious challenges and we are working with researchers across our college to apply this in settings such as tuberculosis which would otherwise require an animal model.

## **Reduction**

### **Explain how you will assure the use of minimum numbers of animals.**

The proposed experimental designs and methods of analysis of the results have been discussed with Dr Peter Nightingale statistician. Based upon preliminary data we have performed power calculations for the inflammatory mediator measurements. For most of the experiments, sample sizes will be set generally using a significance level of 5%, a power of 80%. The effect sizes for the inflammatory mediators and indices of permeability that we have calculated our samples sizes for are 25%. Otherwise, we will use the least number of animals to provide an adequate numbers of cells for the purposes of culture, generally on the basis of previous experience (ours, or from the literature). In terms of the numbers of animals required, we expect that 8-12 animals per treatment group should be sufficient to obtain the required results.

### **Retrospective assessment**

Published: 26 June 2023

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



We reduced the number of animals on this licence based on carefully designed dose response experiments in conjunction with our animal facility using very small sample sizes. This approach ensured that when the full experiments were undertaken using larger numbers of animals, we had already established the minimum dose needed to elicit the required response (especially in aged mice). This avoided needing to remove animals from the study earlier than expected, which would have necessitated a repeat of the experiments and further animal use.

The use of shams was unavoidable as we needed to evidence that the surgery itself did not cause a significant prolonged immune response. However we were able to use a smaller number of animals for this in comparison to our other groups. Once this was demonstrated, no further shams were used.

We have also investigated using bone marrow derived cells from naïve mice, as an ex vivo model to complement our human ex vivo human model of precision cut lung slices which we have developed to reduce animals used in an experimental procedure. These cells were obtained from wild type animals available as unavoidable surplus stock generated from inhouse breeding colonies, rather than additional animals being bred specifically for this purpose.

We intend to more extensively employ these models in future as exploratory models.

Unfortunately, some mechanistic problems cannot be addressed in these models but this has helped us reduce the number of mice used in this licence and we will continue this in the future.

## Refinement

**Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.**

### Why use Mouse lung injury models?

Mouse models have provided extensive insights into the pathophysiology of sepsis / ARDS and represent the most commonly used animals for this purpose worldwide.

The proposed murine models have been defined in the c57 black strain by ourselves / collaborators -providing data for appropriate power calculations to minimise the numbers of animals needed.

The ability to study genetically modified mice specifically bred e.g with HSD-1 deficiency allows us to address one of the central objectives of this project.

### Justification of Animal Models Chosen:

We believe that it is necessary to look at a range of animal models as no single injury to mice reproduces the complexity and extent of human sepsis and ARDS.

Direct Lung injury- (IT) LPS challenge: The LPS responsiveness of the C57BL/6 mouse strain has previously been defined, with cellular inflammation peaking at 48 hours post inhalation. Mice receive IT 50 µg/mouse lose weight, develop pulmonary neutrophil influx, show impaired respiratory system compliance and have increased BAL total protein, all peaking at days 1-2. Appropriate controls are IT PBS.



Direct Lung Injury- Pneumococcal pneumonia models a clinically important direct infective lung injury. Utilising different strains of the bacteria allows for a variation in the severity and duration of infection / inflammation. Strains INV1041 causes an acute lung inflammation over a few days and bacteraemia, whereas strain 03-3038 yields a less severe, longer term infection which is ideal to study the resolution phase of the infection/ inflammation. Remote Lung injury: CLP causes mild pulmonary damage characterized by neutrophil infiltration, increased levels of CXC chemokines, and oedema formation in 24 hours. This model causes abdominal infection with systemic septicaemia. To use appropriate controls we will use sham operated controls, who undergo laparotomy but do not undergo CLP.

We have refined the CLP model in our previous licence to ensure there are no deaths by only using a single pass of the needle to puncture the ligated caecum and to limit the post-CLP time points to within 24 hours to minimise the risk of mortality in these animals – this allows us to study early but not late sepsis.

**Minimising Animal Suffering:** For all models, we will resuscitate the animals at the end of the operation by injecting pre-warmed normal saline subcutaneously/ intraperitoneal/ intravenously. Postoperative analgesia and fluids (e.g. saline) may be given if clinical signs indicate the necessity, and any animals showing unexpected distress/problems related to the procedure will be terminated.

### Retrospective assessment

Published: 26 June 2023

**With the knowledge you have now, could the choice of animals or models used have been improved at all? How did you minimise harm to animals during the project?**

In our CLP models we reduced the experimental timecourse to minimise the time in which animals experienced a severe level of suffering. Surgery was performed aseptically according to the current best practices as per LASA guidelines. Animals that had undergone surgery received peri and post-surgical analgesia, were recovered in a warm environment, were provided with additional fluids and given wet mash or supplementary gels on the cage floor to aid in recovery.

In our intranasal model of pneumonia we designed small scale pilot experiments to fully characterise the model in younger mice before moving to older mice where a more severe level of suffering was anticipated. During this time animals were closely monitored to ensure harm was minimised wherever possible, mice were anaesthetised during administration and analgesia given as a cautionary measure. The close monitoring of animals by our animal facility staff ensured animal welfare was a priority during this project.

Model choice: Based on the learning obtained in this licence I believe Intranasal inoculation is a refinement over intratracheal instillation reducing potential harm from ketamine usage whilst achieving similar delivery to the airways. Intranasal administration also mimics natural infection so will increase translation of findings. Further to this, the approach used to induce inflammation associated with infection will be reconsidered as a more clinically relevant approach using a wider range of bacterial insult may be more appropriate



## 13. Interactions between mast cells and helminths in inflammatory disease

### Project duration

5 years 0 months

### Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants.
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes.

### Key words

mast cells, helminths, diabetes, arthritis, cardiovascular disease

### 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
- Required at inspector's discretion

### Objectives and benefits

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

### What's the aim of this project?

The purpose of this licence is to identify the role that mast cells play in the induction, perpetuation and amplification of immune responses in inflammatory, infectious and allergic disease and thus lead to the development of new medicines. We will carry out research into the fundamental mechanisms by which mast cells trigger or control inflammation so as to reveal novel targets for therapeutic intervention. We will then identify biological and chemical reagents with the potential to control and prevent inflammatory diseases. We also aim to evaluate novel compounds for anthelmintic activity.



## Retrospective assessment

Published: 4 October 2022

**Is there a plan for this work to continue under another licence? Yes**

**Did the project achieve its aims and if not, why not?**

We were able to address a number of aims as follows:

AIM 1: Preliminary studies demonstrated that mast cells could be activated by helminth products both directly and indirectly via IgE and that this resulted in the production of key cytokines required for the polarisation of allergic responses.

AIM2: We showed that mast cells played a role in cardiovascular disease with respect to the regulation of vein graft remodelling. We also showed that mast cells influenced arthritis through activation of inflammatory pathways.

AIM 3: We developed a novel *in vitro* assay for anthelmintics and used these to identify new compounds with anthelmintic activity. We further demonstrated that these were effective *in vivo*.

Overall these findings demonstrate that mast cells play a key role in infection, allergy and inflammatory disease. Further we have developed a novel assay for the identification of new anthelmintics

**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.**

**What are the potential benefits that will derive from this project?**

The project will lead to greater understanding of the immune processes that lead to a range of inflammatory diseases. It will help to identify novel therapeutic reagents for more effective treatment against some of the most important inflammatory and infectious diseases that impact on the health and economy.

**Species and numbers of animals expected to be used**

**What types and approximate numbers of animals will you use over the course of this project?**

- Mice, 3,400 (680 per year)

**Predicted harms**

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



**In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?**

The most likely adverse effects for all procedures are moderate as assessed by weight loss of less than 10% in comparison to age matched controls. Where appropriate analgesia will be used. All animals will be euthanised at the end of the experiment. Any animals displaying deviation from normal health, other than due to the inevitable effects of the procedure, will be promptly euthanised or referred for veterinary attention, the former being the more likely course of action.

Parasite infection may result in mild gastrointestinal discomfort for a maximum of three weeks (weight loss, hunched posture and piloerection). Moderate muscle discomfort may result (altered gait, reduced movement and abnormal posture) however these effects should be rare. Very rarely, animals may also show respiratory distress (shallow, fast breathing) from migration of larval stages of the parasite this effect should be transient (< 48hours). The dose of parasites to be used will be adjusted so as to limit the effects of the infection, although the gastrointestinal and muscular effects are an inevitable consequence of the infection.

Animals developing arthritis may show signs of ill health, e.g. listlessness, hunching, weight loss. Food and water will be placed with easy reach. Extra bedding and nesting material will be added for comfort. At the peak of the inflammatory response animals may have severe loss of limb movement for a maximum of 7 days. In a typical experiment, less than 10% of the animals are euthanised because of adverse effects.

The development of diabetes is characterised by polyuria, polyphagia, and polydipsia, disease will be confirmed by measurement of blood and urine glucose levels. Mice in the later stages of overt diabetes may exhibit weight loss, hunching and immobility. Once diabetes is confirmed mice will be sacrificed by a Schedule 1 method.

For the study of cardiovascular disease ApoE knockout used have a high circulating cholesterol level, however, this rarely causes adverse effects. Dietary manipulation will not cause weight loss but could cause weight gain which should not affect the health of the animal. If any health problems arise the appropriate treatment will be given.

**Retrospective assessment**

Published: 4 October 2022

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

The majority of animals were unaffected by the procedures.

The arthritis model we used showed moderate adverse effects for less than 10% of animals. No animals were euthanised as a result of these adverse effects.

For animals infected with the parasite less than 5% suffered transient moderate weight loss.

We did not carry out any studies on mice with diabetes due to lack of funding.



For the study of cardiovascular disease animals were monitored and did not display any adverse effects

## Replacement

### **State why you need to use animals and why you cannot use non-animal alternatives.**

Our study will begin with the investigation of the fundamental role of mast cells in the development of inflammation. Extensive *in vitro* studies will be conducted initially using established cell lines or isolated murine white blood cells to identify the immunological pathways that may lead to inflammation. It is essential to conduct these studies in animal models because although preliminary experiments can be done *in vitro*, this does not reflect the complex interactions which take place in a whole animal during disease development. All the animals used in this project will be mice as they are the lowest species which have an immune system similar to that of humans. Furthermore, a range of reagents and genetically modified strains of mice are available to provide more definitive answers to the questions addressed

### **Retrospective assessment**

Published: 4 October 2022

### **What, if any, non-animal alternatives were used or explored after the project started, how effective were they and are there any lessons worth sharing with others?**

We conducted a number of *in vitro* studies of mast cell activity. We were able to use these to explore the role of helminth in shaping the immune response however they are somewhat limited as they do not explore the spatial and temporal relationship of mast cells with other elements of the immune response

Furthermore, we initially evaluated anthelmintic activity using an *in vitro* screening method which we developed. This enabled us to identify lead compounds before testing their efficacy in animal models. This assay would be of benefit to others interested in developing new anthelmintics since it is a more objective and measurable assay than those currently in use.

## Reduction

### **Explain how you will assure the use of minimum numbers of animals.**

Statistical advice has been sought and will be used to ensure that the minimum number of mice used will be consistent with the aims of the protocols while achieving statistically valid results. Techniques such as *in vivo* imaging permit multiple analysis on single animals thus reducing the numbers required.

### **Retrospective assessment**

Published: 4 October 2022



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

We reduced the number of mice that were used in arthritis models by the use of mixed cage models. In these experiments, controls and treated mice are kept in the same cage and identified by ear marking. By using mixed cage models, a single repeat of the experiment is sufficient to confirm reproducibility. This has reduced the number of animals in arthritis models by 1/3.

Further we also reduced the number of animals required in the arthritis studies by incorporating *in vivo* imaging of neutrophil influx into the joints. By injecting luminol, it is possible to detect and quantify neutrophil influx into paws and joints. This allowed us to generate more data from the same number of animals. Each animal also acted as its own internal control and so fewer control mice were necessary.

## **Refinement**

**Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.**

Animals will be group housed in cages which permit free movement and contain environmental enrichment appropriate to their species. Husbandry and care procedures are based on best practice, and regular monitoring will be conducted by highly trained staff. In all cases, the endpoints of the experiments will be measurements acquired from tests which are considered minimally traumatic to the animals and are of short duration. Pilot studies are planned wherever necessary.

## **Retrospective assessment**

Published: 4 October 2022

**With the knowledge you have now, could the choice of animals or models used have been improved at all? How did you minimise harm to animals during the project?**

The mouse is the chosen species for testing the novel compounds as there are well characterised, gold standard, industry-employed, models of human diseases that can be utilised with an unparalleled array of reagents and resources to allow elucidation of mechanism of action. Further we had already refined the models that we use in previous project licences and as a result none of the animals used experienced severe adverse effects. Animals undergoing protocols involving mast cell depletion and arthritis were supplied with softened diet to enable them to eat without standing on potentially sore feet.

Injections into the joint and into the dermal layers of the skin were conducted under general anaesthesia to minimise potential suffering due to pain/misplacement of needles.

Removal of tail tips for genotyping (assessing genetic status) was replaced with blood sampling from a needle prick or collecting of tissue removed during ear marking for identification. We also implemented the single needle use and “cupping” animal handling.



## 14. Mechanisms of cardiovascular regeneration

### Project duration

5 years 0 months

### Project purpose

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

### Key words

Regeneration, Epicardium, Neovascularisation, Vascular protection

### 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
- Required at inspector's discretion

### Objectives and benefits

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

### What's the aim of this project?

While cardiovascular diseases remain the primary cause of mortality and morbidity worldwide, there is an urgent need to tackle the clinical and economic burden of heart failure. Our project aims to understand how to protect blood vessels against disease, such as heart attack and stroke and, since this is not currently possible and heart attacks are prevalent, to encourage the heart's own repair processes to replace damaged muscle in heart failure patients. Our previous research led us to identify a powerful growth factor that functions in the body to repair wounds. We found that Thymosin  $\beta$ 4 is required in the embryo for development of stable, muscle-coated blood vessels and for migration of cells from the outer layer of the heart (epicardium-derived cells; EPDCs) to build coronary vessels. Significantly, we found that, if we treat adult mice, in which we surgically induce a heart attack, with this factor, these "dormant" embryonic processes can be reactivated to replenish muscle and restore blood supply, leading to an improvement in heart function. Although very promising, the proportion of new muscle that can be created from EPDCs is currently inadequate. We will build on these important findings and i) investigate the role of



growth factors in vascular disease and potential to repair diseased vessels; ii) explore strategies to enhance EPDC-based heart regeneration.

## **Retrospective assessment**

Published: 7 April 2022

### **Is there a plan for this work to continue under another licence?**

Yes

### **Did the project achieve its aims and if not, why not?**

Significant progress was made towards understanding how new coronary vessels are formed in the heart after a heart attack. While these were found to partially replicate the processes used in the embryonic heart during development (the two main sources of vessels reactivated to contribute in disease), the molecular pathways controlling their activation were profoundly altered between embryonic development - neonatal injury (regenerative) - adult injury (nonregenerative), which may help us to understand why the regenerative response in adult mammals is inadequate and guide us in developing therapies to augment repair.

We have gained important insights into factors that control the proliferation, differentiation and migration of outer layer epicardial cells in the developing heart and have identified fundamental differences between the active embryonic epicardium and relatively dormant adult epicardium. From these comparisons, we have identified promising targets to promote a more effective activation of the adult epicardium to augment regeneration of the adult mammalian heart. We also performed a detailed characterisation of the mouse epicardium, profiling the main changes over the course of development and evaluating the contribution of these cells to the forming heart. This study importantly clarified a number of misconceptions in the field, that have been hindering progress in this area.

We delineated a novel mechanism through which smooth muscle cells of the coronary arteries and aorta are preserved to protect against arterial diseases (atherosclerosis) and abdominal aortic aneurysm. This is relevant for human disease as the pathway involved has been implicated through human genomic studies as having a key role in determining the progression and outcome of these diseases.

These important areas of research will be developed further under another licence.

**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.**

### **What are the potential benefits that will derive from this project?**

Damage to blood vessels causes accumulation of cholesterol and leads to destruction of muscle and elastic layers, to weaken the vessel. Rupture of weakened vessels causes life-threatening events (heart attack, stroke or aneurysm – balloon-like swelling of the body's main artery). Our studies to date have given us some insight into the signalling that occurs on the surface of vascular smooth muscle cells to protect them from destruction. We hope



to increase our understanding of these pathways and tests key growth factors that could be used therapeutically to protect against these major lifethreatening diseases.

After a heart attack, a significant portion of the heart's muscle is irreversibly damaged, leading to heart failure in an increasing number of patients. By showing that dormant cells in the outer layer of the heart (called EPDCs) can be re-activated to make new muscle and blood vessels, we have already identified a promising new target to treat heart failure.

However, we believe the process can be made far more effective, if we understand more precisely the signals that control EPDCs. Encouraging the heart to rebuild itself by making new muscle may provide a powerful treatment for the 900,000 heart failure patients in the UK and millions worldwide.

## **Species and numbers of animals expected to be used**

### **What types and approximate numbers of animals will you use over the course of this project?**

Our work will be performed in mice. We expect to use a total of 20,000 mice over the 5 year term of the project. 16,000 of these will be used for breeding and collection of tissue samples; only 4,000 will undergo more invasive procedures as part of our experiments.

## **Predicted harms**

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

### **In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?**

Most animals will be used for breeding and will experience mild or moderate procedures. We expect the genetically altered mice to display no/minimal adverse consequences during their normal life course. We will study the process of heart and blood vessel development in the embryo during pregnancy. Embryos will normally be studied at an early stage of development, before pain sensations have been acquired. These embryos will die shortly after they are collected, so there is minimal potential for suffering. Understanding how the heart is "built" in the embryo will provide clues as to how we may "re-build" the adult heart.

A smaller number (maximum 20%) will undergo more invasive procedures to model human cardiovascular diseases:

In some mice (maximum 10%), we will simulate a heart attack by tying a suture around a major coronary artery. Over 90% of mice will survive the procedure and show no clinical symptoms. Whilst some discomfort is associated with surgical procedures (minimised with the use of anaesthetics and analgesics), the mice generally recover well; they will be closely monitored post-operatively and further analgesia provided, if required. Any animals that show signs of distress will be humanely killed.

Up to 5% will be fed a "western diet" to induce atheroma (cholesterol-rich plaques similar to those that form in human arteries). Suffering will be minimal as, over the duration of the experiment, plaques are highly unlikely to rupture and cause a heart attack or stroke.



Up to 5% will be used to model abdominal aortic aneurysms by infusion of the naturally occurring hormone Angiotensin II. As with humans, the majority of cases will be asymptomatic; however, in rare cases (<5% of those treated with Angiotensin II), aneurysm rupture may occur and may cause considerable pain, albeit short-lived as death will rapidly ensue (within 1 minute).

At the end of the experiments, animals will be humanely killed and tissues collected for biochemical and histological analysis.

## **Retrospective assessment**

Published: 7 April 2022

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

87% of mice (10,194) were used for breeding only and displayed minimal adverse consequences during their normal life course.

A further 6% (720) additionally experienced moderate procedures, such as the administration of substances during pregnancy to induce genetic alterations in the embryos. Most embryos were studied at an early stage of development before pain sensations are acquired. Moreover, the embryos died shortly after they were collected, thus there is minimal potential for suffering, even in the <5% of embryos studied with abnormal development of the cardiovascular system.

31 genetically altered mice were fed a high fat diet to induce formation of cholesterol-rich plaques similar to those that form in human arteries. The only discomfort experienced by these mice was greasy fur, causing mild skin irritation and scratching in some cases. None of the plaques were allowed to develop long enough to risk causing heart attack or stroke. 159 mice (1.4%) were infused with a hormone, Angiotensin II, to model abdominal aortic aneurysm. The majority of mice experienced only minor to moderate suffering, as they underwent surgery, with anaesthesia and analgesia, to implant the mini pump under the skin for sustained release of Angiotensin II. For 22 mice, the experience was classed as severe, with 15 humanely killed due to showing signs of discomfort or weight loss (large aneurysm confirmed post mortem) and 7 died following aneurysm rupture. Pain in these animals was short-lived, as rupture occurs rapidly and our frequent monitoring ensured no prolonged suffering beforehand.

In 405 adult and 158 newborn mice (4.8% of all animals), a heart attack was simulated by tying a suture around one of the major coronary arteries. 64% of these mice showed no clinical symptoms, therefore the suffering was moderate (surgery with anaesthesia and analgesia). A further 23% of mice displayed symptoms (lethargy, weight loss) but survived, with additional pain relief, to the end of the experiment. 8% displayed severe symptoms (typically breathing difficulties) and were humanely killed. 5% died due to cardiac rupture or heart failure.

## **Replacement**

**State why you need to use animals and why you cannot use non-animal alternatives.**



Cardiovascular diseases, injury and repair are brought about by a complex interplay between cells of the heart, vessels and the immune system. Thus, despite advances in *in vitro* cell based systems, computer modelling and the benefits of using clinically relevant patient biopsies, none of these methods faithfully models events such as atherosclerotic plaque progression, aortic aneurysm, myocardial infarction or heart failure. Hence, the use of animals is unavoidable if we are to answer important questions about causes of, and identify effective treatments for, cardiovascular diseases.

That said, we have developed and validated a range of cell-based systems that allow us to model some aspects of cardiovascular cell behaviour (in particular, the EPDCs that are central to our project). As one example, we can test the efficacy of small molecule drugs on EPDCs grown in a dish and, only once promising compounds with potential for therapeutic use in humans have been identified, would we test the drugs in live mice.

### **Retrospective assessment**

Published: 7 April 2022

**What, if any, non-animal alternatives were used or explored after the project started, how effective were they and are there any lessons worth sharing with others?**

We are working to develop a protocol to model the process of blood vessel cell formation from a type of stem cell derived from human skin (iPSCs). We aim to optimise a protocol to produce endocardium (one of the two sources of coronary vessels in the heart), such that we can identify and test compounds that drive the formation of new blood vessels. If this is successful, it will not only minimise the use of severe procedures in mice but testing in a human cell system may increase the likelihood of any new therapies translating successfully to the clinic. We have applied for NC3Rs funding to develop this model.

### **Reduction**

**Explain how you will assure the use of minimum numbers of animals.**

We will manage animal breeding carefully to reduce animal numbers to the minimum required for our experiments and colony maintenance. Power calculations will be performed prior to the start of an experiment to establish appropriate sample sizes. We will use the minimum number to achieve the required statistical power (using too few animals is wasteful if it leaves us inadequately powered to draw meaningful conclusions). We work carefully to make sure that we can derive as much useful information as possible from each mouse. For example, we may take the aorta, heart and blood from a single animal which, depending on the treatments given, can be used in multiple projects. From one heart, we can determine expression (changes) of approximately 35 genes or we can collect around 100 sections of aorta or heart to localise protein expression within the tissues, using antibody staining

### **Retrospective assessment**

Published: 7 April 2022

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



As proposed, careful planning of experiments and power calculations were the primary means to minimise animal usage. Moreover, we were able to obtain a number of different measurements from each animal. For example, assessing heart and vascular function in the living animal, followed by collection of histological sections, protein and RNA samples from each heart and/or aorta post mortem. After completion and publication of some studies, we were able to make good use of any remaining tissues (spare sections/ protein lysates) for related studies, thereby avoiding the need to use additional animals. Careful management of animal colony breeding was particularly important during the pandemic-enforced laboratory closures, after many planned experiments had to be postponed and rescheduled.

## Refinement

**Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.**

Mice represent an accurate model organism for the study of cardiovascular development and they are essentially the only mammalian model amenable to genetics to assess individual loss or gain of gene function in the context of cardiac growth and regeneration. Our project requires the use of cardiac-specific reporter lines for tracking cell fate and behaviour in development and following injury.

We have much experience in the disease models proposed and this has enabled us to develop the most refined protocols, frequently through collaborating with groups that have prior extensive experience. We work hard to optimise animal welfare pre- and post-operatively. Based on our experience, that of our collaborators and on published studies, we constantly look for refinements to our protocols, both to minimise harm to our animals and to ensure the highest quality of data to underpin our scientific research.

## Retrospective assessment

Published: 7 April 2022

**With the knowledge you have now, could the choice of animals or models used have been improved at all? How did you minimise harm to animals during the project?**

The choice of animals and models are the gold standard and there is little scope to refine these as we implemented the most refined approaches from the outset. Small changes were made to improve either the scientific value of our animal research (point 1) or animal welfare (points 2-3):

with improved understanding of gene expression in the developing heart, we determined a narrower developmental window in which to induce genetic targeting selectively in the cell type of choice (epicardium) and limit off-targeting of other cell types (notably coronary endothelium), compared with what had previously been used for this model. This refinement was important to ensure robust conclusions from our studies; we, therefore, highlighted this refined approach in a publication.

For surgical procedures that required insertion of a tube to provide mechanical ventilation (intubation), we introduced the use of a specialised platform to enable optimal positioning



of the head and which includes a fibre optic cable to improve illumination and visibility of the airways. This substantially reduced the time needed for intubation (and thereby the total time under anaesthesia) and also limits the chance of injuring the airway.

As tamoxifen was increasingly recognised to cause irritation to the abdominal cavity over recent years, we adopted oral gavage, the most refined method, as the exclusive route for administration of tamoxifen towards the latter period of the licence.



## 15. Metabolism and Pharmacokinetic Studies

### Project duration

5 years 0 months

### Project purpose

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

### Key words

Regulatory, Metabolism, Pharmacokinetic, Animal

### 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.

- Required at inspector's discretion

### Objectives and benefits

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

### What's the aim of this project?

Investigations of metabolism and pharmacokinetics of xenobiotics (foreign substances) are carried out in animals (*in vivo*) as part of broader safety testing programmes in order to generate information to assess risk of adverse effects compared to beneficial effects resulting from exposure to a new drug and its metabolites. The data acquired enables national and international regulators to decide if a new drug or chemical should be sanctioned for use in the public domain. These investigations are conducted with pharmaceuticals, agrochemicals, animal health and biotechnology products, biocides, food additives and industrial chemicals.

### Retrospective assessment

Published: 27 May 2022

### Is there a plan for this work to continue under another licence?



Yes

### **Did the project achieve its aims and if not, why not?**

Investigations of metabolism and pharmacokinetics of xenobiotics (foreign substances) are carried out in animals (in vivo) as part of broader safety testing programmes in order to generate information to assess risk of adverse effects compared to beneficial effects resulting from exposure to a new drug and its metabolites. The data acquired enables national and international regulators to decide if a new drug or chemical should be sanctioned for use in the public domain. These investigations are conducted with pharmaceuticals, agrochemicals, animal health and biotechnology products, biocides, food additives and industrial chemicals. The aims of the programme of work described in this licence were achieved during the term of the Project Licence.

The aims and objectives of this programme of work were:

- 1) *To generate and report data from in vivo (animal) metabolism and pharmacokinetic investigations in order to satisfy the legal requirements of international regulatory and government agencies.*

The data produced from these experiments provided information on the extent and duration of systemic exposure to test materials, and identified the organ systems involved together with the efficiency of absorption, metabolic changes, and retention or elimination of the drug from the systemic circulation. The different study protocols presented in this licence addressed the different aspects of Absorption, Distribution, Metabolism and

Excretion (ADME) studies, according to specific regulatory and scientific needs during differing stages of development and evaluation of a new pharmaceutical, agrochemical or veterinary medicines.

Data generated from the program of work supporting the successful ADME characterisation of compounds, and progression to further drug development studies or safety studies (or clinical trials) is held by the Project Licence Holder, and has been shared with the site Animal Welfare and Ethical Review Body (AWERB) and PEL holder as part of the retrospective assessment of the licence. However, it is not always possible to establish if or how many of the compounds tested during a program of work at a Contract Research

Organisation (CRO) complete development and make it to market. With drug development typically taking ca 12 years from candidate screening to market authorization, it is rare to establish success of candidates assessed until much later following the program of work.

*To generate and report data from in vivo metabolism and pharmacokinetic screening studies to aid the selection of compounds for further development.*

In vivo screening or candidate selection studies were performed, typically in rodents using a small number of animals per dose group. These studies were generally designed to investigate blood and/or plasma exposure and pharmacokinetics, although organs and tissues were also taken at termination for investigation too.

Pharmacokinetic screening studies usually occurred in larger animals (minipigs, dogs and NHPs) prior to initiation of a full drug development programme to ensure that the most suitable species had been selected as the non-rodent model.



Data generated from the program of work supporting the successful characterisation of compounds, and progression to further drug development studies (or clinical trials) is held by the Project Licence Holder, and has been shared with the site AWERB and PEL holder as part of the retrospective assessment of the licence.

*To use the data obtained to help refine the design of subsequent nonclinical safety and clinical studies, especially during large programmes of work.*

Data on the Absorption, Distribution, Metabolism and Excretion of the test material in a rodent and non-rodent species are required as part of the regulatory submission process for pharmaceuticals, agrochemicals and veterinary medicines. The pharmacokinetic profile of the test compounds evaluated under this licence were fully defined in order to understand dose to exposure responses. These data were typically utilised to assist in extrapolating and predicting exposures in humans and to assist in defining dose administration regimes in drug safety tests. By collecting excreta, bile and expired air, the rates and routes of excretion were determined and extrapolated to man. Excreta, plasma and tissues were also assessed for the presence of test material or associated metabolites to assist in the understanding of the compounds method of action and elimination, in compliance with international regulatory guidelines. The distribution of test material or radiolabelled metabolites into tissues and subsequent elimination were also investigated. The transfer of radiolabeled drug-related material across the placenta or its excretion into milk from a nursing dam were also investigated.

Metabolism studies in support of veterinarian health or agrochemical drugs were performed in the target species (i.e. dog, goat or chicken).

Data generated from the program of work supporting the successful ADME characterisation of compounds, and progression to further drug development studies or safety studies (or clinical trials) is held by the Project Licence Holder, and has been shared with the site AWERB and PEL holder as part of the retrospective assessment of the licence.

*To develop and validate new animal welfare friendly models to support regulatory studies, as indicated in the 3Rs section of this project licence.*

The positive 3Rs developments achieved through the program of work are detailed in the subsequent 3Rs sections of this retrospective assessment.

**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.**

### **What are the potential benefits that will derive from this project?**

The principal benefit of this project is the provision of data to facilitate sound regulatory decisions regarding selection of species for toxicology studies, and extrapolation to humans (for pharmaceuticals); setting of exposure limits (agrochemicals) and animals (veterinary products).

### **Species and numbers of animals expected to be used**



## **What types and approximate numbers of animals will you use over the course of this project?**

Over the 5 year life of this Project Licence, it is estimated that 16500 mice (plus 1300 transgenic mice), 16500 rats (plus 300 transgenic rats), 150 hamsters, 60 guinea pigs, 300 rabbits, 512 dogs, 500 minipigs, 500 cynomolgus monkeys, 20 goats and 200 chickens may be used. Adult animals will be used in all protocols. Neonate and juvenile rats may also be used to assess how the metabolism of a novel test material differs from that in an adult animal. Data generated from these studies is used to support human dose level selection.

## **Predicted harms**

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

**In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?**

The majority of animals on these studies are expected to have little, or no adverse effects. A very small number of animals, may show more significant adverse effects. Humane endpoints will be adopted if animals show excessive effects. Animals will either be humanely killed at the end of a study and the carcass (or individual tissue) investigated for test compound residues. Alternatively, animals may be retained for re-use on a different study or, where appropriate, rehoming may be considered.

## **Retrospective assessment**

Published: 27 May 2022

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

The overwhelming majority (>99%) of the animals utilized during the programme of work fell within the mild and moderate severity categories. In general, moderate severity was a result of the cumulative procedural effects, such as multiple needle sticks used to obtain blood samples for pharmacokinetic evaluation of test compounds, or as a result of confinement to metabolism cages in excretion balance (ADME) and pharmacokinetic studies, or as a result of surgical cannulation to obtain biological samples.

Due to the nature of the dose levels investigated in PK and ADME studies being below any targeted adverse effect level, the investigations were designed to minimize any adverse effects as a result of the test compounds administered. Therefore 'side effects' from the test compounds were overwhelmingly absent, or mild (transient) in nature.

Of the animals utilized, approximately 45% experienced mild and 55% experienced moderate procedural related harms.

The procedural harms experienced by the animals can be detailed as follows:



A typical study would see animals dosed either singly or on multiple occasions with a test material, prior to repeated sampling for blood or other bodily fluids. This may or may not involve a surgical procedure to enable dosing or sampling.

Some studies would require confinement in a metabolism cage, usually for a maximum of 8 days, but on some occasions for a maximum of 15 days continuously (with appropriate permissions and scientific justifications).

At the end of the study, the animals were humanely euthanised to preserve the integrity of tissues being collected to fulfil the scientific objectives of the study.

For bile duct cannulated animals, collections took place for up to 5 days post-dose. Where collections were intermittent, tethering and un-tethering of animals occurred to allow greater freedom of movement of the animals.

Where approved by the Named Veterinary Surgeon, animals were retained for re-use on further investigations. This was common for dogs and non-human primates (NHPs), especially where a simple blood sampling investigation was conducted after administration of a drug.

All animals were suitably acclimatized to both their home environment (after receipt) and to experimental conditions prior to initiation (eg metabolism cages, or slings and chairs to help support blood sampling or dose administration).

Where animals were used as companion animals (typically for studies involving goats or chickens) they were not subjected to any regulated procedure, and were suitably re-homed at the end of the study. During the course of the program of work we approved the re-homing of naïve, and subsequently non-naïve, animals that had been utilised on a small number of studies.

## Replacement

### **State why you need to use animals and why you cannot use non-animal alternatives.**

At present there are no scientific and legally acceptable evaluations for the absorption, distribution, metabolism and excretion (ADME) of xenobiotics that will satisfy regulatory requirements other than the use of animals. However validated *in-vitro* tests are used to support selection or replace these, wherever possible.

### **Retrospective assessment**

Published: 27 May 2022

### **What, if any, non-animal alternatives were used or explored after the project started, how effective were they and are there any lessons worth sharing with others?**

In the case of new pharmaceuticals, the regulatory authorities are obliged to protect human volunteers for clinical trials by requiring use of proven test systems and accepting use of animals until an *in-vitro* alternative has been demonstrated to be reliable and reproducible. The EU Commission Directive 2003/63/EC stipulates Standardised Marketing Authorisation Dossier Requirements that include pharmacokinetics, i.e. the study of the fate of the active substance and its metabolites within the organism. This



includes the study of absorption, distribution, metabolism and excretion of these substances. Information on distribution and elimination is necessary in all cases where such data are indispensable to determine the dosage for humans. Investigations are continually evolving into pharmacokinetic modelling from in vitro data, thereby allowing better prediction of human responses and reducing the number of animals needed to generate the required data, but currently these remain limited in their reliability to go beyond indicating trends, due to the nature of complex metabolic processes in vivo. Similar international guidelines and directives are in place regarding agrochemicals and their ability to contaminate the food chain and industrial chemicals and their effect on the health and welfare of humans.

However, whilst other non-animal studies are carried out at the establishment in support of in vivo work, as recommended by the international regulators, no in-vitro tests can yet model the complex and integrated homeostatic mechanisms governing ADME of new drugs and hence, in vivo testing is still a requirement. Work is on-going to refine the prediction of human pharmacokinetics from in vitro data, and also to establish better in vitro systems to generate data without the use of animals.

In minipigs, investigatory work continues to provide better characterisation, which may allow the replacement of dogs and possibly non-human primates by this species. Concurrently with this, alternative bleeding techniques (ear or saphenous vein sampling) continue to be refined & developed that will provide a less stressful method of blood sampling from this species.

## Reduction

### **Explain how you will assure the use of minimum numbers of animals.**

Studies are designed to provide maximal scientific value from the minimum number of animals, whilst using 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.

All studies are subjected to ethical review of the objectives and procedures, prior to commencement of the work. Without approval of these, the study would not be carried out.

Where possible, animals may be re-used on a number of non-related studies.

Wherever practicable, a combination of sample collections (eg excreta collections, blood sampling, terminal tissue sampling) from the same animal will be used, to reduce overall animal usage.

Due to increased sensitivity in analytical methods, techniques have been developed that allow smaller sample volumes to be used. This has led to the reduction of the animals used by allowing more samples to be collected from each animal.

### **Retrospective assessment**

Published: 27 May 2022

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



The number of animals used during the course of the program of work reflected the nature of the objectives in that more rodents were used in first in vivo studies to study pharmacokinetics and ADME properties of drugs.

Studies were always designed under this programme of work such that the minimum number of animals were used in order to obtain the maximum information, whilst the scientific objectives of each study are met, in accordance with regulatory requirements and agreed standard practices.

The vast majority of the studies conducted used the minimum number of animals to demonstrate the ADME characteristics of a drug (n=3). However, the establishment also had available professionally trained statisticians to help design studies, especially where the test compound was a biological and use of statistics could provide a guide as to the best number of animals required to determine if a novel new compound is a statistically acceptable biosimilar to the originator molecule. All study protocols were reviewed by the AWERB at the establishment against known guidelines and the Company's ethical compliance policies.

In order to fulfil the requirements of the regulatory agencies, animal group sizes for metabolism studies are variable, depending on the test substance being used (e.g. pharmaceutical or agrochemical), the specific type of study being undertaken and the strain of animals being used. For instance, for most studies involving pharmaceuticals, group sizes did not generally exceed 3, unless a sound scientific rationale was provided. When dealing with agrochemicals, group sizes for rodents are similar to those used for pharmaceutical studies. However, only a single goat was used for each study group and hen studies consisted of group sizes of 10 or more.

Reduction in animal use was achieved during this program of work where a number of candidate compounds (each in trace amounts) were co-administered in a single (cassette) dose with consideration to avoid drug-drug interactions. Also, in the case of certain PK screening studies, colonies of animals (dogs and primates for instance) were used to allow comparative data generation. These approaches minimised the total number of animals required for pharmacokinetic studies. Notwithstanding the above, studies were subject to AWERB review and approval by the Project Licence Holder.

Alternative blood sampling techniques (eg saphenous vein sampling) allowed a reduction in the total number of animals required to generate the necessary data. Also, increased sensitivity of analytical techniques has allowed the establishment to investigate a number of procedures that have allowed micro-sampling of blood in several species. These changes allowed a more comprehensive profile to be generated from single animals (hence reducing the need for "composite" type studies, where multiple animals were needed to generate the required data), or by allowing a cross-over design to be used on smaller animals (typically rodents).

The tissue distribution studies conducted have been standardised across the industry for regulatory submission for many years. During the course of this program of work the study designs were revised further to reduce the number of animals that were utilised for these studies (typically n=3 per timepoint reduced to n=1 per timepoint) and the number of timepoints were reduced (8 to 7 typically) which resulted in a large reduction in the number of animals used on study.



## Refinement

**Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.**

The majority of animals used during the course of this licence will be rodents. Adult animals will be used in all protocols; additionally, neonate and juvenile rats may also be used. Scientific opinion, including that of the regulatory agencies, indicates the use of one rodent and one non rodent species for many of the metabolism studies that are required. The most appropriate non-rodent species will be selected with reference to studies including, but not limited to in-vitro cross-species metabolism, in-vitro toxicity studies, and pilot pharmacokinetic and safety assessment studies. The species selected based upon this information is the one which is predicted to align most closely with human in terms of sensitivity, receptor homology and metabolism. Where scientifically justified the minipig will be used in preference to dog or primate, however, there are limitations in the metabolic characterisation of this model. For example the liver enzymes and transporter mechanisms for xenobiotics are yet to be established.

The most widely used characterised second species for metabolism studies is the dog. When considering veterinary products, the drug may be specifically aimed at dogs, in which case it is obligatory to study the target species. Dogs are only used where the purpose of the programme of work can only be achieved by their use. Under this project licence the use of dogs will be limited to the support of human or veterinarian healthcare and will not be used in industrial chemical or food additive testing.

Whilst studies with non-pharmaceuticals will be mainly carried out in rodents, rodents are not always suitable models for the assessment of the kinetics/disposition or metabolism of any test material so there may be the need, on occasions to assess certain non-pharmaceuticals (specifically agrochemicals) in another species such as a dog due to the required physiology in rodents or other species not providing a suitable comparison to man (as mentioned in OECD TG 417). The use of dogs in these cases would be for regulatory purposes, and should another species not be a suitable model, full scientific justification would be requested as to why a rodent species is not suitable.

Studies involving non-human primates are only initiated where no scientific or feasible non-human primate alternative exists. Primates will only be used in the testing of pharmaceuticals for use in life threatening or debilitating clinical conditions in humans. It should be noted that in vivo and in-vitro testing will sometimes indicate that primates are the only practical animal model that can be used in metabolic studies to give an indication of what may be expected in man, in order to fulfil the requirements of international regulatory agencies for the purpose of investigating life-threatening or debilitating clinical conditions in humans.

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.

Individual studies will be 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.

## Retrospective assessment



Published: 27 May 2022

**With the knowledge you have now, could the choice of animals or models used have been improved at all? How did you minimise harm to animals during the project?**

The ADME study designs used are regulatory driven and the establishment has been submitting data and reports to all of the major regulators for more than 30 years. Scientific opinion, including that of the regulatory agencies, indicates the use of one rodent and one non rodent species for many of the metabolism studies that are required. Options are often limited for non-rodent species due to supply constraints, need for species with achievable husbandry requirements and need for background knowledge of the metabolic performance characteristics of the animal. The most widely used second species for metabolism studies is the dog. When considering veterinary products, the drug may be specifically aimed at dogs, in which case it is obligatory to study the target species. The choice of animal model, whether rodent, or non-rodent was based upon both scientific justification, and welfare considerations, to enable the fulfilment of the programme of work and provide data on the test compounds investigated. These were appropriately chosen based upon set criteria, and reviewed and approved by the Project Licence Holder and AWERB.

In order to minimise animal harms, all dose levels used under this licence were expected to be sub-toxic or result in only mild toxicological or pharmacological effects. Trained animal practitioners observed all animals (after surgery and/or post-treatment) at least twice daily and at pre-set post-dosing intervals. Named Animal Care and Welfare Officers (NACWOs) and vets were available to assist the Personal Licence Holder in documenting reactions to treatment and deciding when and how to intervene to prevent any suffering. In the case of surgically prepared animals, these were also observed at least once a day during the postsurgical period, and received both analgesics and anti-inflammatory drugs post-surgery during recovery.

Dog and primate metabolism cages have been modified significantly to allow dual or triple housing (where appropriate), thereby introducing companion animals within the experimental space, and reducing the stress of restricting social interactions of animals. The increased sensitivity of analytical techniques (as indicated in the previous section) led to micro-sampling of blood (in all species) and reduced the volume of blood required to be sampled from animals and allowed better data to be generated.

Limits on blood sampling, restraint and preparation of animals for blood sampling procedures were imposed in the interests of animal welfare and are documented in the Project Licence.

Further examples of refinements implemented during the lifetime of the licence included: Introduction of dual cannulated rat model, to study elimination of drugs in bile, whereby the re-infusion of artificial bile salts into the duodenum enabled a physiological more stable and robust model to be used for these investigations. Use of a tail cuff allowed animals to be housed as a group, rather than singly, during recovery periods post-surgery. The better success of this animal model allowed a reduction in the number of animals used in these investigations.

Modification of rat metabolism cages to ensure easier access to diet and water.



Environmental enrichment for rodents in metabolism cages (glass rings and a shelf/hide). Metabolism cages have also been tinted to reduce light levels to animals.

Combining two cages in pregnant rabbit studies, to ensure a secluded area for nesting  
Purchase of new, larger cages for housing hens. The introduction of nest boxes, slow increase/decrease of lighting (mimicking dawn/dusk) at switch over and increased acclimatisation periods

Redesign of floor grids for dogs and mini-pigs when housed in metabolism cages for greater comfort.

Introduction of jugular bleeding in rats with manual restraint.

Replacement of grid floor with solid floors for rodent studies (excluding excretion balance studies)

Introduction of transient saphenous cannulae to allow re use of minipigs without the need for invasive surgical procedures

Introduction of vascular cannulae in rats so that excretion and pharmacokinetic studies may be combined.

Development of multi-housing metabolism caging for primates (up to three) and dogs (up to three), allowing a companion animal(s) during collection of excreta.

Refinements for blood sampling included:

Jugular vein of conscious rats (no pre warming or tube restraint is required for this method).

In conscious mice, by superficial transection of a lateral caudal tail vein (also by dislodging the consequent clot or scab - no pre warming is required for this method).



## 16. Autophagy in the hematopoietic system

### Project duration

5 years 0 months

### Project purpose

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

### Key words

Autophagy (self-eating), leukemia, autoimmunity, ageing immunity, vaccination

### 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
- Required at inspector's discretion Objectives and benefits

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

### What's the aim of this project?

Autophagy is a cellular process by which cells dispose of and recycle unwanted material.

This process has only been discovered about a decade ago, and most research has been performed on cells in culture. We now know that too little autophagy in blood cells can lead to cancer (leukaemia), failure to generate functional blood cells (differentiation), and immune deficiencies (inherited disease that makes patients susceptible to infections). Too much autophagy is also inappropriate as it leads to autoimmunity. Finally declining levels of autophagy with age result in poor immune and vaccination responses. It is unknown what exactly goes wrong when autophagy levels are altered. In this project we are firstly addressing the mechanism by which autophagy affects immunity and haematological malignancies. To date there are no adequate drugs with acceptable side effects which provide a defined mode of action to modulate autophagy in blood cells. Finding novel drug targets and drugs is the second objective of this project.

### Retrospective assessment



Published: 18 January 2022

## Is there a plan for this work to continue under another licence?

Yes

## Did the project achieve its aims and if not, why not?

Autophagy is the main degradation pathway for bulk material in the cell.

The last project licence was instrumental in elucidating the role of in immune cell fate and function, either in healthy cells or in the context of disease.

We investigated (1) autophagy's role in shaping differentiation in hematopoietic cells intending to dissect the mechanism, (2) novel drug targets along the autophagy pathway to improve vaccination in the elderly, (3) genetic diseases based on mutations in the autophagy pathway and attempted to treat mice with the same mutation on the basis of our findings.

This programme is to be continued under the authority of a further project licence.

### 1) Basic mechanisms

Autophagy shapes differentiation

- Autophagy provides free fatty acids for neutrophil differentiation (published in Immunity, 2017)
- Autophagy is key to long lived, self-renewing cells. The hematopoietic stem cell (HSC) is compromised without autophagy, a finding subsequently confirmed in other stem cells with high impact on regenerative medicine. We have been able to rescue the severe phenotype of mice without autophagy in their HSCs (anemia, bone marrow failure) with Rapamycin (manuscript in preparation).
- Both memory T cells and tissue-resident B1 cells require autophagy for self-renewal.

### 2) Translation opportunities

- Rejuvenation via autophagy We improved flu vaccination efficacy in old mice by restoring autophagy levels with spermidine. We patented this finding, and subsequently uncovered the mode of action of spermidine and provided proof of concept in humans. On the basis of this work, we are conducting a clinical trial to improve Covid and flu vaccination in the elderly with spermidine.

### 2) Genetic diseases

With our collaborators we found that deficient autophagy is the underlying cause for several inherited immune deficiencies. Our recent study identified a third protein, called GIMAP6 which we found inhibits autophagosomal delivery to the lysosome. This is now a therapeutic target for these patients (manuscript in preparation).

**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.**



## **What are the potential benefits that will derive from this project?**

This project will result in a better understanding of the cell biology of immunity (vaccines including in the elderly), of immune deficiencies, autoimmune diseases (lupus, IBD, arthritis) and haematological malignancies (leukemia). We hope to discover novel drugs to modulate autophagy in blood cells. We have already found that the naturally occurring drug spermidine induces autophagy and can rejuvenate immunity in the elderly. We will elucidate the mode of action of spermidine in blood cells, as well as determine the minimal dose to be given to have an effect, its distribution in the body and its side effects. We will investigate if spermidine is provided by the bacteria growing in the gut. We also aim to trial therapies for a new autoimmune mouse model, which is translational for recently identified patient cohort. These are all necessary steps for a clinical trials in humans in the future.

## **Species and numbers of animals expected to be used**

### **What types and approximate numbers of animals will you use over the course of this project?**

We will be using an absolute maximum of 21,470 of inbred mice over 5 years. Most of them will have a genetic modification.

## **Predicted harms**

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

### **In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?**

10000 mice are undergoing procedures on the mild protocol and will not experience any adverse effects. For the disease models we will be using (lupus, arthritis, skin inflammation, inflammatory bowel disease, and response to a pathogen) the adverse effects are expected to be of moderate severity. The main adverse effects induced by pathogens will be respiratory distress and weight loss linked to the induction of viral challenges, the main adverse effect of arthritis is swelling of the joints with pain and reduced mobility, adverse effect of inflammatory bowel disease is weight loss, diarrhoea and some pain, adverse effect of lupus is swollen abdomen, skin rash around the nose, accumulation of fluid under the skin and in the abdomen leading to a flabby swollen appearance, accompanied by pain and lessening mobility. Moderate severity is also in place for some of the proposed protocols to allow for the apparition of moderate adverse effects that cannot be predicted. Such effects may for example be due to the administration of substances that have not yet been tested/titrated in vivo and need to be used as a replacement/complement to the substances typically used or to genetic alterations leading to a particular susceptibility to the injected substances. Animals will be monitored throughout the course of the disease for the development of potential adverse effects and humane end points are in place based on the expected adverse effects. Animals will be killed if they approach the severity limit. At the end of the experiment, all animals will be killed and tissues will be collected for further investigation. Severe severity is in place for a very limited number of mice (100) to test treatments and biomarkers for a



specific autoimmune deficiency. We will monitor mice very closely when we treat them as we aim to use therapies to stop the genetic defect which currently causes sudden death.

## Retrospective assessment

Published: 18 January 2022

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

The vast majority of our mice (>90%) underwent no procedures or procedures of subthreshold/ mild severity.

The GIMAP6 deficient patient we were investigating, was very sick and presented with severe autoimmunity with no treatment options. We discovered that GIMAP6 KO mice, a model for these patients, die unexpectedly around 20 weeks, probably due to autoimmunity and kidney failure. In order to find a treatment for GIMAP6 deficient patients, we wrote an amendment adding a new severe protocol to the licence. This allowed us to treat animals where death would be an outcome to investigate the mechanism of action and potential targets or therapeutics. Firstly we increased monitoring. Then as we got to know the mouse model better, we learned early signs and terminated the experiments before death occurred so that no severe harm was done to those mice. The investigations filled a gap in our understanding of how to treat the patients (more patients with a mutation in the same gene were found in the meantime). Based on our experimental data, we recommended to the clinicians to treat the patients with rapamycin. There were 22 mice used under this new protocol. 9 had a sudden death and 11 culled as sick for having early signs of ill health hence experiencing perhaps moderate severity.

387 were genotyped by blood sampling so experiencing very transient mild pain. 110 mice were administered Tamoxifen by oral gavage up to 5 times, experiencing transient weight loss and hunched posture and reduced activity. Mice that are gavaged with tamoxifen develop a series of harmful phenotypes including permanently enlarged testis, unexpected weight loss etc. This was a general issue found across the establishment. We changed the protocol to lower and fewer doses of Tamoxifen and tighter monitoring. There were 839 immunised by MCMV and 177 immunised then had up to 3 intraperitoneal injections and two times blood sampling. Pain due to intraperitoneal injection are a mild and transient discomfort to the animals.

124 bone marrow chimera created irradiation followed by reconstitution by intravenous , experiencing weight loss, reduced activity, however, regained weight and reached their original weight within two weeks. Those that didn't regain weight and lost more than the allowed weight loss were killed humanely before any suffering. 24 out of these had modified diet after recovery and the rest had two times blood sampling and up to three times of substances administered hence the cumulative pain and suffering are of moderate severity.

15 mice were aged. We only observed a minor increase in body weight, and for some of them, loss of hair, which is normal for their age. No other harmful phenotypes were observed. To investigate the age-associated phenotypes in the immune system and keep it consistent with previous experiments, we culled the mice before the maximum time allowed. No cumulative suffering was observed for these mice.



102 mice were used under protocol 4, they had oral gavage up to 5 times or substances administered orally (DSS) for colitis model. Tamoxifen treatment itself did not lead to inflammation in the colon. After administration of DSS, mice got inflamed and showed some mild body weight loss which is a sign of inflammation (less than 10%). Mice were assessed by daily checkups (posture etc) and body weight loss and also by examining rectum and faeces consistency, rectal prolapses and diarrhoea. No strong phenotypes are being expected (due to low concentration of DSS, typically 2%).

## Replacement

### **State why you need to use animals and why you cannot use non-animal alternatives.**

We are replacing animal experiments with in vitro cell culture models whenever possible. Examples of this is the use of samples from lupus patients, blood from ageing donors and from vaccinated donors. However, some experiments can only be done in vivo, for example there is no in vitro technique to replace the entire differentiation of blood cells from a stem cell. Secondly, the complexity of the interactions between the different cell types of the immune system cannot be adequately reproduced in a whole organism. Lastly dose, kinetics, side effects and tissue distribution of a novel drug need to be tested in an animal before its development as a drug for humans.

### **Retrospective assessment**

Published: 18 January 2022

### **What, if any, non-animal alternatives were used or explored after the project started, how effective were they and are there any lessons worth sharing with others?**

Many tissues and organs are frequently collected from animals that are killed humanely. Samples of spleen, lymph nodes, lung, bone marrow, and occasionally adipose tissue and blood are used to maximise the information we can obtain from each mouse, particularly when it does not involve additional welfare cost to the animal. The tissues obtained are used in a broad range of downstream investigations, e.g. protein analysis, staining and histology, gene expression, immune cell function to aid our understanding of disease mechanisms taking place in our mice models of infection, inflammation and autoimmunity. To investigate hematopoiesis or immune responses, a whole organism is required as there are many cells and signals involved that we don't know yet and therefore cannot recapitulate fully in vitro.

As Tamoxifen administration in vivo has led to some unexpected adverse effects, we are increasingly moving to inducing the deletion of the gene in vitro whenever possible.

## Reduction

### **Explain how you will assure the use of minimum numbers of animals.**

Here are some of the measures we are taking to reduce the number of animals:



1. Use of pilot experiments in small number of mice, especially for those procedures, for which there is no local or international expertise.
2. Obtain training elsewhere if expertise is not available
3. Taking into account our previous experience and those of our collaborators to determine group size
4. Optimization of extraction methods to provide sufficient numbers of particular cell types with minimum usage of mice
5. Inclusion of colonoscopy will allow individual mice to be followed throughout disease development for inflammatory bowel disease. This will reduce the number of mice required to look at the disease as it progresses
6. Maximising use of harvested cells and tissues: setting up a shared local aged mouse colony.
7. Archiving of frozen tissue samples to permit analyses of novel factors without additional in vivo experiments.
8. Embryo freezing of strains that are not currently in use.

Outsourcing to the expert: Generation of monoclonal and polyclonal antibodies will be outsourced when appropriate project authority exists

### **Retrospective assessment**

Published: 18 January 2022

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

We calculate the number of mice for each experiment using such as the NC3Rs Experimental Design Assistant (EDA) online tool. We conduct and report our experiments for publication according to ARRIVE guidelines.

We limit the number of animals by grouping experiments, i.e test different drugs in one experiment only requiring one control group.

We were breeding mice to obtain littermates and experimental mice in a 50:50 ratio to minimise breeding of mice that are not of the right genotype.

### **Refinement**

**Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.**

Mice and rats are the lowest vertebrate groups on which well-established models of immune and hematopoietic diseases of interest have been developed. Mice are preferable to rats because of the greater availability of reagents (e.g. monoclonal antibodies, tetramers) specific for this species and availability of genetically modified mouse strains. After consultation with our collaborators, we have selected the disease models that best model the human disease with known side effects. We have determined doses and kinetics of immune modulatory chemicals and biologics in past experiments as we have many years of experience working on genetically modified animals.



We will minimise welfare costs to the animals through rigorous model development approach and by continuously refining our protocols: through the use of pilot experiments, shortening the experiments to a minimum duration while still allowing a significant read-out and reducing dose and times of administration of substances.

We have refined the genetically modified animal breeding, as most mouse models will only lack the cellular process of autophagy in a specific subpopulation of the blood system (neutrophils, T cells, B cells), which means that these mice will not develop disease unless their immune system is challenged. Furthermore the immune system challenge models have been carefully selected so that animals undergo the minimum pain. Number of injections will be kept to a minimum, and disease duration kept as short as possible as to still allow meaningful conclusions. Painkillers will be administered where they do not interfere with the disease process.

When testing the treatment options for the autoimmune patient cohort in our mouse model, we will very tightly monitor their welfare and make sure we use the relevant gender and biomarkers that will inform us about response to treatment and enable us to identify early humane end points to limit suffering.

### **Retrospective assessment**

Published: 18 January 2022

#### **With the knowledge you have now, could the choice of animals or models used have been improved at all? How did you minimise harm to animals during the project?**

Mice that are gavaged with tamoxifen develop a series of harmful phenotypes including enlarged testis, unexpected weight loss etc. This was a general issue found across the establishment. We changed the protocol to lower and fewer doses of Tamoxifen and tighter monitoring.

The genetic models for autophagy deletion are necessary as it is the only way to understand the role of autophagy in the immune system. We have mostly chosen to delete autophagy genes in a tissue-specific way as knocking out autophagy ubiquitously (in every tissue) would cause harmful clinical effects outside the cell type of interest (such as we have seen previously it may cause anemia, myeloproliferation, or hepatomegaly which we avoid now). This approach avoids a harmful phenotype in tissues that we are not studying and we address our scientific question with the right tools.

After consultation with our collaborators, we have selected the disease models that best model the human disease with the least side effects. They were chosen to reduce number of mice (the majority of animals develop disease and therefore there is little waste) with minimal clinical signs.

To challenge the immune system we and other have developed tools that measure immune correlates to the named pathogens. Developing these tools can take several years (e.g. TCR and BCR tg mice, tetramers, epitope mapping), and these are now so well established that comparison between different labs has become possible and also



reproducibility within my lab is ensured. They are usually well tolerated as the immune system controls these pathogens.

We have determined doses and kinetics of immune modulatory chemicals and biologics in past experiments over many years of experience. For example, we have stocks of purified antibodies to deplete T cell subsets (e.g to CD4/CD8), and have figured out which LPS concentrations to minimise harmful effects and yet stimulate innate immune responses.

We have many years of experience with bone marrow chimera for hematological projects and they are therefore well established in my lab. They are often the only way to interrogate whether for example a manifestation of disease or cellular ageing phenotype is due to blood cells, minimise breeding of genetically modified mice (as one is enough to generate many BM chimera) and get meaningful data. Single cell transplantation experiments are rare but they are essential to understand the fate of stem cells. Any harmful effects from the irradiation procedure are now well controlled with regular monitoring. When reconstitution is not successful, weight loss is an early sign before any harmful consequences are apparent.

Pilot studies have been undertaken in small numbers of animals to determine the lowest effective dose of tamoxifen to induce genetic alteration in a conditional knock-out affecting hematopoietic cells. Information from this study should enable safer and effective gene 'knock out'.



## 17. Immunopathology of experimental malaria infection

### Project duration

5 years 0 months

### Project purpose

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

### Key words

malaria, immunity, brain, immunopathology

### 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 project's objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.**

#### What's the aim of this project?

The overall objective of this project is to identify the key pathways responsible for the development of immunopathology (i.e. tissue damage caused by the immune system) during malaria infection. It is becoming clear that the most severe complications of malaria infection, including cerebral malaria, respiratory distress and severe anaemia, are due, in part, to over activation of the host's immune response to the parasite. However, in the case of cerebral malaria, we still do not understand the interplay and interactions of the malaria parasite with cells of the immune system and with the endothelial cells that form the cerebral vasculature. Consequently, we still do not know the mechanism of vascular damage that leads to leakage, brain swelling and fatal outcome during the condition.

Moreover, we do not know how anti-malarial drug therapy promotes recovery from the cerebral malaria syndrome in some individuals but not others, or the responses within the brain that resolve tissue damage and neuronal dysfunction following successful treatment



of the condition. Crucially, in general, we do not understand how pro-inflammatory and anti-inflammatory immune responses develop and function during malaria infection, and why immunological homeostasis (i.e the balance between protective and pathogenic immune responses) sometimes fails to be achieved, leading to immunopathology. In particular, we have very limited knowledge of the behaviour of effector and regulatory T cells within tissues during malaria infection and how their interactions with other cells within the tissue determine their protective vs pathogenic activity.

### **Retrospective assessment**

Published: 25 April 2022

#### **Is there a plan for this work to continue under another licence?**

Yes

#### **Did the project achieve its aims and if not, why not?**

The overall aim of the project was to identify the key pathways responsible for the development of immunopathology during experimental malaria infections. Specific objectives were:

##### **Determine the immunological and parasitological processes that lead to Experimental Cerebral Malaria (ECM).**

Achievement: We made substantial progress in understanding the pathogenesis of ECM and we importantly strengthened collaborations with researchers working on human cerebral malaria, so we could improve the translational and clinical impact of our work. For example, we identified how immune cells (such as T cells) act alongside the parasite to cause pathology within the brain during ECM and we identified the importance of blood brain barrier disruption, vasogenic oedema, and brain swelling in undermining the recovery from ECM following antimalarial drug treatment. As fluid accumulation within the brain and brain swelling are major pathological events in human paediatric CM, our results have increased the clinical implications of our animal research. We have also successfully examined some of the immunological pathways that influence the recovery from the ECM syndrome, and which control acquired (infection-induced) resistance to the ECM complication, which provides insight into how natural immunity to malaria is developed by people living within malarial endemic countries.

##### **Define how pro-inflammatory innate, adaptive and memory immune responses develop and function during experimental malaria infection**

Achievement: This objective was inherently linked with objectives 1 and 3 and we made substantial progress in identifying where immune cells position within the brain to cause ECM. Whilst results are not yet published, we also developed a new high-dimensional imaging platform where we can examine in unprecedented detail where specific immune cell populations compartmentalise within a complex physiological tissue environment and which other cell types they interact with. We also developed an ex vivo explant tissue system (we remove intact organs from mice and place directly under a microscope in an imaging chamber to keep the tissue healthy for >4 h) so we can examine the dynamic behaviour of immune cells within the spleen during infection. Using this, we have revealed that T cells with suppressed effector function appear to interact with other cell populations



in a different manner during infection than T cells with strong anti-parasitic and pro-inflammatory capacity. This gives new insight into how to manipulate specific immune responses during malaria infection, for example how to degrade pathogenic immune responses whilst preserving memory immune responses.

### **Define how regulatory innate, adaptive and memory immune responses develop and function during experimental malaria infection**

Achievement: We made significant progress in understanding how immune regulatory pathways (such as the cytokines IL-10 and IL-27 and suppressive cellular receptors [e.g. PD1]) influence the strength of the immune response during malaria, and how alterations in nutrient utilisation by T cells during infection controls their effector function. This has provided new information on why immune responses during malaria are often sub-optimal and shortlived, suggesting methods to modify immunological pathways and improve immunity to malaria.

Overall, the objectives in the previous project were large overarching programmes of work but we made significant progress in all areas, which refined the objectives within our new Project Licence.

**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.**

### **What are the potential benefits that will derive from this project?**

This project may significantly advance our understanding of how protective and pathogenic immune responses form during malaria infection and will help to identify the specific events that lead to development of immunopathology, including cerebral malaria. These results should enable the development of targeted therapeutic and adjunct treatments for severe malarial disease, as well as identifying mechanisms through which to augment protective immune responses.

### **Species and numbers of animals expected to be used**

#### **What types and approximate numbers of animals will you use over the course of this project?**

The general project plan will involve infecting resistant (for example BALB/C) and susceptible (for example C57BL/6) strains of inbred and transgenic (for example IL-27R KO, IL-10-GFP reporter) mice with different species of Plasmodium parasites that cause specific types of immunopathology. We expect to use approximately 8600 mice during the course of this 5-year project, with 500 used on parasite maintenance / establishment; 5000 in different experimental designs; 3000 in GAA colony breeding and 100 for obtaining GAA animal tissue.

### **Predicted harms**

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



**In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?**

Depending upon the species and strain of malaria parasite and the strain of mice utilised, malaria infection may lead to mild, moderate or potentially severe suffering. *P. yoelii* XL, *P. berghei* NK65 and *P. berghei* ANKA parasites have the potential to cause severe suffering due to high parasitaemia and associated anaemia or cerebral pathology (the latter in case of *P. berghei* ANKA) in susceptible mouse strains. However, of the experiments involving infections that have the potential to cause severe suffering in animals, not all infections will be allowed to progress to the stage where severe suffering occurs. For example, animals may be euthanized at time points of infection preceding development of severe suffering to examine immune cell activation, regulation or the early events in pathogenesis of severe malaria infection. We expect that 5% of animals that enter into experiments on protocol 2 of the licence may experience severe suffering.

To help us to definitively address the importance of specific immunological pathways, we will employ different immunomodulatory techniques within the protocols, including localised and systemic administration of blocking and activating compounds, at precise stages of the experiment in relation to malaria infection. This will allow us to delineate the sequence of events that are necessary for the development or prevention of severe malarial disease and those important for parasite control. Most immunomodulatory techniques should not directly promote animal suffering. Irradiation with cell reconstitution will be performed under established protocols with appropriate monitoring to detect acute radiation sickness or reconstitution failure or more chronic radiation-induced suffering, such as dental complications. Anticipated effects due to anti-parasite chemotherapy will be monitored and animals exhibiting non-transient and non-recoverable levels of severe suffering will be quickly identified and euthanized. We will perform behavioural tests (such as novel object recognition or Y maze), to assess cognitive capacity of animals following malaria infection, specifically cerebral malaria. The behavioural tests are non-invasive and will not cause animal distress or suffering.

The final fate of animals on the licence will be either the transfer of animal to other project licences with the authority to receive animals that have undergone the procedures specified in the individual protocol or animals will be killed by exsanguination under terminal anaesthesia, a schedule one method, or following intravital (in animal) imaging under terminal anaesthesia.

**Retrospective assessment**

Published: 25 April 2022

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

Animals were generally infected once or repeatedly with specific species / strains of Plasmodium parasites. Infection with *P. yoelii* NL generally induced a self-resolving infection where the mice experienced mild suffering (evidenced by ruffled fur, abnormal posture and little weight loss [ $<15\%$ ]). Immunomodulation (administration of antibodies to block proinflammatory or regulatory pathways) occasionally modified the severity of infection so a proportion developed transient (resolving) moderate suffering associated with lethargy and unsteady gait. This increase in severity was due to alterations in parasite



burden and / or elevated immune activation which caused recoverable levels of immune-mediated pathology. Animals were closely monitored during these experiments in line with our well-established grading system to ensure they did not experience unnecessary duration or level of moderate suffering (generally any animal experiencing moderate suffering for longer than 24 h was culled).

Infection of mice with *P. berghei* ANKA parasites caused the development of experimental cerebral malaria (ECM), which involved severe levels of suffering. This is a rapid onset complication of malaria with a clear stepwise progression, where animals first exhibit minor suffering before progressing to reduced responsiveness to stimulation, ataxia, respiratory distress, ultimately leading to prostration, paralysis and convulsions. The syndrome is associated with significant pathology in the brain (extensive oedema, brain swelling, neuronal injury and haemorrhage) and is fatal if not treated. 80% of mice will, however, survive the syndrome if treated with anti-malarial drugs at the correct stage of disease, although they will also exhibit substantial brain pathology before recovering. This is the best available model for human cerebral malaria and we establish our experimental protocols to exactly recapitulate the treatment challenge for the human disease (i.e. we need to develop better treatments for the established syndrome as it is currently not possible to identify individuals at risk of developing the syndrome and to provide prophylactic treatments). Whilst a high proportion of mice infected with *P. berghei* ANKA developed moderate – severe suffering, we closely monitored mice under strict guidelines using a well-defined grading system, to ensure that we identified the vast majority of animals at the necessary stage of infection for experimentation, and no animal experienced unnecessary levels of suffering (i.e. our experiments did not require animals to proceed to paralysis and coma, and we stopped experiments or treated mice before these points were reached).

Some animals underwent multiple rounds of infection and anti-malarial drug treatment (up to four rounds of infection) to mimic the development of infection-induced resistance to severe malarial disease that occurs within malarial endemic countries. However, animals were not allowed to experience moderate or severe suffering during each round of infection and were only allowed to experience moderate or severe suffering during one of the infections.

#### **Total numbers of mice used in the project were:**

Sub-threshold:	1067
Mild:	1650
Moderate:	478
Severe:	497

The numbers equate to 13% of animals on the licence experiencing severe suffering.

## **Replacement**

### **State why you need to use animals and why you cannot use non-animal alternatives.**

We can only address the majority of our questions when a complete immune system is present in its normal anatomical and physiological configuration (for example within the spleen, the major site of immune priming and parasite killing during malaria infection), or when parasites and immune cells can interact with the complex architecture of the intact



brain (leading to cerebral malaria); the use of animals is, to a significant extent, unavoidable.

## Retrospective assessment

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**What, if any, non-animal alternatives were used or explored after the project started, how effective were they and are there any lessons worth sharing with others?**

We employed *in vitro* co-culture models between brain endothelial cells and parasites for very specific questions, but this system did not capture the complexity of the physiologically intact brain, or the myriad interacting factors that shape cerebral malaria development, so was unable to replace *in vivo* experimentation.

## Reduction

**Explain how you will assure the use of minimum numbers of animals.**

We calculate the required group size using data from pilot experiments, previous experience, and published work to ensure that we have sufficient power to detect a biologically relevant effect using as few animals as possible. *In vitro* assays, such as co cultures of parasites with endothelial cells or T cells with antigen presenting cells, will be utilised to replace animal experimentation where possible; however, animals will frequently be required to obtain materials for use in these *in vitro* experiments.

## Retrospective assessment

Published: 25 April 2022

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

We tried to incorporate longitudinal assessments of animal suffering and neurological function such as using the non-invasive Rapid Murine Coma and Behavioural Scale (RMCBS) and imaging, which allowed us to obtain high quality data from the same animal, reducing the numbers of animals used. Also, although malaria infection is inherently variable, by improving our ability to identify animals at specific stages of disease, we were able to lessen intra-group heterogeneity and reduce animal usage.

## Refinement

**Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.**

Mice are the most appropriate species for this work as murine malaria infections are the most wellcharacterised of the various animal models and so much is known about their immune systems and all the reagents that we require are available. In terms of cerebral malaria, there is accumulating evidence that the nature of blood brain barrier disruption and the relative importance of the perturbation in driving cerebral pathology, are very similar in mice and in humans, validating the animal model for the study of the human



condition. Animal suffering will be minimised by closely monitoring all animals in relation to a well-defined grading system and providing analgesia, when required. Using our well-defined grading system, of the 5% of animals that may experience severe suffering during the course of our experiments, all of these animals will not experience prolonged suffering for more than a few hours.

### **Retrospective assessment**

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#### **With the knowledge you have now, could the choice of animals or models used have been improved at all? How did you minimise harm to animals during the project?**

I am content that the choice of animals or models could not be altered, and we continue to use these in new current Project Licence. We did make alterations during the course of the project by adopting more refined methods to monitor our animals under protocol. Specifically, we refined how to assess the stage of experimental cerebral malaria development and determine the precise time for each animal to undergo drug-treatment and/or immunomodulation. Our utilisation of the non-invasive Rapid Murine Coma and Behavioural Scale allows us to further prevent unnecessary suffering and to improve the quality of our experiments. We also expanded the number of stages in our infection grading system to provide more clarity to experimentalists and BSF staff on the expected progression of the ECM syndrome, which has improved understanding and communication for animal monitoring and welfare.

