Home Office

NON-TECHNICAL SUMMARY

Mechanisms of cardiometabolic disease

Project duration

5 years 0 months

Project purpose

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

Key words

Obesity, Diabetes, Lipids, Lipotoxicity, Cardiometabolic

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

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

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

What's the aim of this project?

To determine how obesity causes metabolic complications.

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

Why is it important to undertake this work?

Obesity is one of, if not the leading cause of multiple major diseases. These include diseases traditionally classed as "metabolic" such as diabetes, and cardiovascular diseases such as heart attacks, stroke, and heart failure. Increasingly, however, obesity is also recognised to be a significant risk factor for diseases such as cancer, asthma, kidney failure and arthritis. Obesity can also increase the risk of severe complications and death from infectious diseases such as Covid 19.

However, we know that what links obesity to diseases is not the weight itself. If you walk around carrying an 80 kg backpack all day, you do not get diabetes - you become physically fit. We believe that what connects obesity to metabolic diseases is primarily a failure in fat tissue itself. Each individual has a genetically and environmentally determined limit on their ability to store fat safely. When that limit is reached, fat begins to accumulate in other organs and cell types. Scientists have now found that this accumulation happens in almost every organ and cell in the body they have studied, from the liver and muscle where it causes insulin resistance which leads to diabetes, to the immune system, where it can increase our risk of asthma and arthritis. We aim to study how fat tissue stops working and when it does, how to either prevent lipid accumulation in other organs, or make that lipid accumulation less damaging.

In an ideal world we would combat the diseases caused by obesity by treating obesity itself. However, despite decades of work by academics and the pharmaceutical industry, it has turned out to be nearly impossible to design drugs to reduce food intake and body weight. Given that we can't make people lose weight, trying to reduce the damage obesity does to our bodies is an essential approach to reducing the disease burden (as of 2016 the WHO estimated that 1.9 billion adults are overweight worldwide). It is estimated that obesity and its associated cardiometabolic diseases will cost the UK economy 50 billion GBP per year by 2050 (www.gov.uk). Furthermore, obesity has a tremendous quality of life cost, increasing the likelihood of developing diabetes 5 fold, some cancers by 3 fold, and high blood pressure, a major risk factor for heart disease by 2.5 fold. Overall, obesity was estimated to account for 23.1% of all deaths in the UK as of 2017.

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

We have **six objectives**. Our first three focus on how fat tissue (also known as adipose tissue) stops working. Healthy fat tissue **a)** buffers the amount of fat (known as lipid) in our blood to allow us to safely cope with eating different types of food; **b)** stores nutrients for the long term; **c)** secretes a mixture of proteins that regulate all aspects of our body's function. When fat tissue stops working correctly, these functions break down and the lipid that should go to fat tissue ends up in other organs like the liver and muscle where it stops insulin working, leading to diabetes. It can also cause heart failure by poisoning the heart, and it can also cause inflammatory diseases like arthritis by poisoning the immune cells.

In our first three objectives, we will determine **a)** the mechanisms and genes responsible for causing a breakdown in lipid buffering; **b)** determine how our bodies wound healing systems, which lead to scarring, get inappropriately activated in fat (and other tissues) and stop it working; **c)** validate how three genes that have been identified as possibly limiting adipose tissue function in humans actually stop fat tissue working.

These three objectives are essential as all three represent understudied or new processes/genes that may link obesity to metabolic complications and thus provide new drug targets.

Objective 4 focuses on how to combat the metabolic defects of failed fat tissue. As mentioned above, when fat fails, lipid goes to other organs. If we can reroute this fat to organs which can burn it (muscle and a specialised thermogenic organ called brown fat), we can stop it from accumulating in other tissues that cannot burn it. However, if we are to send fat to organs that can burn it, it is essential to make sure we can also actually activate their fat burning systems, otherwise they will simply fill with fat and stop working themselves.

Turning on fat burning systems is an attractive approach, as it potentially allows treatment for a collection of "*cardiometabolic diseases*" including diabetes and heart failure. In addition to treating these diseases, it may also cause weight loss through increasing energy expenditure.

Objective 5 focuses on how lipid accumulation in other organs causes toxicity. Under this license we will look at how the toxic accumulation of lipids in organs and cell types, including liver, heart, muscle, pancreas, and the immune system, leads to a wide range of obesity-associated metabolic diseases.

Objective 6 is a more specific objective to validate a better model of human liver disease progression. Liver disease (known as NAFLD) goes through several stages. The mildest is called "non-alcoholic fatty liver" (NAFL). The next stage is called "nonalcoholic steatohepatitis" (NASH). NAFL is characterised by inappropriate fat accumulation, whereas NASH has fat accumulation, inflammation and liver scarring. We currently have no good models to study how NAFL turns into NASH. Three dietary models of fatty liver disease have been selected to best mimic human NAFLD, and in particular to look at how NAFL turns into NASH. These models have been selected based on historical human and animal data from thousands of experiments. However, the data has been collected over the years and is patchy and non-standardised. We will test the three best candidate models to determine which is the best.

Objectives 1-5 will primarily produce scientific papers as their main output. In addition, we have links with industry and will look to move the results from these objectives into early-stage clinical trials and biomarker discovery. Biomarkers and things we can measure in the blood that tell us about the stage of disease help decide when and how to treat people, and provide information on whether drugs work. We disseminate our data at conferences and via web based media outlets predominantly targeted at the scientific community. In addition to scientific outputs, we also engage with the public and produce lay science pieces.

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

In the short term, the primary beneficiaries will be the scientific community. The immediate beneficiaries will be those working in the field of metabolism. However, given the range of diseases obesity is a risk

factor for, we expect our research to be of benefit to the fields of immunology, cardiovascular disease, hepatology (the study of the liver) and cancer.

Our work will help to inform human clinical trials in both academia and industry. In the longer term, we expect our research to benefit the pharmaceutical industry, where the genes and processes we identify will provide new targets for creating drugs to combat obesity-associated diseases. We have links with the university technology transfer office to maximise the value of our work by patenting it where appropriate.

In particular, **objective 1** may also benefit nutritionists and dieticians. This objective strongly focuses on the impact of the timing of nutrient usage and how it can worsen or improve metabolic disease. As such, it may lead to personalised diets for people with different macronutrients at different times of day (e.g. fat in the morning for some people, carbs for others and vice versa).

Finally, if our work leads to new treatments for the metabolic complications of obesity it will benefit the general public.

For **objective 6,** the overall benefits will be the same. However, the benefit to the pharmaceutical industry will be short-term rather than longer term as we expect an immediate adoption of the chosen optimised model of fatty liver diseases for the development of new drugs.

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

We have many national and international collaborators with whom we share our data and ideas.

We will publish our work in open-access publications. In addition to traditional dissemination routes, we have a website where we post videos regarding our work, in particular technical approaches we have developed for studying metabolism.

Furthermore, we actively engage with the public via lay science pieces and specific web-based media and podcasts.

Concerning the publication of unsuccessful approaches, this perhaps misses the point of science. One of our most successful publications in terms of citations, describes a paradoxical lack of phenotype in a mouse - sometimes the absence of "success," i.e. finding negative results - can be more important than finding many significant changes. Indeed we are currently preparing a manuscript comprised of exclusively non-significant (“unsuccessful”) results which we think will be of great importance to the field.

Species and numbers of animals expected to be used

- Mice: 22500

Predicted harms

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

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

We are using weanling and sexually-mature adult mice up to 15 months of age. This allows us to study cardiometabolic diseases, which are now recognised to impact all ages but become particularly prevalent in middle age. These are the least sentient species suitable for studying metabolism, which involves the complex interplay between many organs. Moreover, there is a large amount of data regarding specific strains of mice and how they develop metabolic diseases, making them suitable to study the function of genes that affect specific aspects of human cardiometabolic diseases.

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

We will be studying mice that develop metabolic disease. These will typically be induced by feeding a modified diet (e.g. high-fat or high-fat/high-sugar diets). Animals may remain on these diets for up to 14 months (from weaning at 3-4 weeks) but typically for a much shorter duration, and this would be expected to lead to obesity, insulin resistance and other obesity-associated metabolic complications, including liver disease, reduced heart function, and inflammation.

We will use genetically modified animals to work out the mechanisms that connect obesity to metabolic diseases. This will enable us to tell which genes/processes predispose us to develop metabolic disease or alternatively protect us against it. We know that temperature is a critical variable for controlling mouse metabolism and we may house mice at different environmental temperatures to control for this. Equally, we will use altered temperatures to investigate how mice make heat.

In a small number of animals under this license (less than 5 percent), we will perform a bone marrow transplant to study how the immune system affects metabolic disease. We will need to irradiate mice and give them bone marrow from another mouse.

In addition, an animal may undergo one or more of the following procedures, but a typical animal will experience no more than five of these:

Mice may receive drugs to alter their metabolism or to delete genes selectively at specific times or in particular organs, either through injections (these can be into a vein, into the body cavity, under the skin or directly into an organ), or orally (by mouth via a tube inserted into the stomach or in drinking water). Not all animals will receive such drugs and the volume and number of injections used to administer drugs will be the minimum needed.

Mice may be given substances known as tracers which allow us to understand more about their metabolism. Tracers are substances that allow us to study a metabolic process but do not affect it. For example, we can give a mouse a glucose tracer to determine if it has insulin resistance in its liver, muscle, or both. Not all animals will receive such substances. We give tracers by injection into their body cavity, into their veins or under their skin, and/or orally (in their food, drinking water, or via a tube into their stomach). Typically a single tracer injection is sufficient with/without administration of the tracer in drinking water, but some mice may receive up to 4 injections.

We may house mice alone for periods of time (typically no more than 3-4 days, but on occasion up to 4 weeks). This is necessary to measure the amount of energy they are expending, their activity and food and water intake, and to determine what nutrients they are burning and when. We will also singly house mice for food intake assessment. This can take as much as 2-3 weeks to get a reliable measure of food intake as mice can fluctuate their body weight by +/- 5% in 24 hours under free living conditions (equivalent to 4 kg in an average human!). As such their food intake is very variable over the short term but becomes more stable in the longer term.

Mice may be scanned using a non-invasive imaging method (e.g. TD-NMR) to examine their body fat percentage. Animals are briefly restrained (usually less than 3 minutes) in a plastic tube. This is both painless and harmless.

Some animals will be anaesthetised for less than 1 hour so that we can image their organs (usually heart or liver) using either ultrasound or MRI. We need to anaesthetise the mice in order to obtain clear images without the animal moving around. No animal would be expected to undergo this more than 6 times in their lifetime with at least 1 week between each imaging procedure.

At the end of the experiment, each animal will be killed using the most humane method that is possible, that does not prevent us from obtaining good scientific data.

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

Animals developing features of metabolic disease will show a range of adverse effects, including: obesity and insulin resistance, inflammation, tissue fibrosis and reduced heart function. They may experience some general discomfort associated with their increased body weight and become lethargic. Conversely, some specific diets (particularly to cause fibrosis in the liver) can cause mice to lose a large amount of weight. This weight loss happens in all mice and is not in itself a sign of illness. As weight loss is not a good sign of ill health when mice are on these diets we have to carefully monitor animals in other ways to ensure that they are not unwell. None of our interventions are expected to cause pain. Some genetically-modified animals might be expected to show similar adverse effects, whilst others would be protected from the impact of metabolic disease.

Animals undergoing bone marrow transplants may be unwell for several days after the irradiation, eat less, and thus lose weight. They are expected to start to recover body weight after 14 days and fully recover by 4 weeks.

Mice are not expected to experience any lasting harm from injections per se. Tracers would not be expected to cause any adverse effects. Drugs which alter metabolism may lessen or worsen the adverse effects associated with metabolic disease, but we will test them to make sure they do not cause unwanted side effects that are harmful to themice.

Single-housing can be stressful for mice, but we will minimise the time each animal is housed alone. Where possible, animals will be re-housed in groups with their original cage-mates following a period of single-housing.

Animals anaesthetised for imaging/implantation of microchips (e.g. for sending body temperature to a receiver) will feel groggy as the anaesthetic wears off but will experience no pain or lasting harm. The imaging processes themselves are painless and harmless.

Non-invasive imaging (e.g. TD-NMR) for body composition analysis requires restraint, which can be stressful to mice, but causes no other pain or harm to mice.

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

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

97% mild

3% moderate

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

- Killed
- Kept alive
- Used in other projects

Replacement

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

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

Cardiometabolic diseases involve the interplay between many different organs. For example, the development of diabetes as a result of obesity mostly involves dysregulation of food intake control by the brain, a loss of fat storage by the fat tissue, the accumulation of toxic fat (known as lipid) species in the liver, muscle and the immune system that prevent insulin working. In turn, the beta cells, the cells in our pancreas that produce insulin, over-secrete insulin to counteract this "insulin resistance". The beta cells can only produce so much insulin and as insulin resistance progresses they ultimately fail. Such a system cannot be modelled in a dish in the lab (in vitro). Instead, it has to be studied in a mammalian system such as the mouse.

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

We look to use animal alternatives where possible. We use cell culture models (cells in a dish in the lab that allow us to study aspects of how specific organs work) to look at specific mechanisms that occur in isolated cell populations. Results from these experiments can both be informed by our animal work and help us to design better animal experiments.

In addition, we have developed two sophisticated culture systems to help us to study fat and liver.

Firstly, over the past 5 years, our laboratory has been developing a human stem-cell based adipocyte culture system. This protocol takes us in a stepwise manner from stem cells through to fat precursors,

and then ultimately mature fat cells. Importantly it captures all the known developmental stages that the stem cells go through before ultimately becoming fat cells. The stepwise manner of our protocol makes it a powerful tool to probe genes regulating fat cell formation.

In addition to the work on stem cells, we have also developed an improved in vitro (in petri dishes) model of human hepatocytes (the primary cell found in the liver that performs its metabolic functions like controlling blood glucose levels) culture. A long standing issue in the field of liver disease has been the lack of good in vitro models, with even primary isolated hepatocytes losing many of the features they show when still in the mouse. In culture, hepatocytes rapidly regress from being mature hepatocytes and instead come to resemble fetal-like (similar to the immature hepatocytes found in developing babies while still in utero) cells within hours.

We have collaborated with a company that uses special liquid handling systems and complex three-dimensional biological “scaffolds” to produce synthetic organs. We have worked with them to create a model of human liver that includes both the hepatocytes and the other liver cells (Kupffer cells, the primary immune cell of the liver and another liver support cell, called the stellate cell). The results from this system were remarkable – producing a set of conditions which kept hepatocytes in almost the same state as we see in vivo.

Why were they not suitable?

While cellular models, even sophisticated ones such as the “*organ on a chip*” liver model (a tool that aims to reproduce the biology of an organ in silico), can address specific questions, they cannot allow the study of the complex interplay between multiple organs that leads to metabolic disease.

Reduction

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

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

These numbers are primarily based on the level of work over the previous two licenses and the amount of funding we have in place and expect to use to fulfil our aims and objectives.

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

Metabolic disease has a complicated and lengthy disease progression. When we try to study it using diet-induced obesity, early stage events will occur in days, if not hours, of diets being changed, later stage events manifesting over weeks, and long term events that take months to appear. Given that many events happen at many times, perhaps the most critical question for reducing animal

usage is to determine the correct length of time a mouse should be fed a high-fat diet for before we begin studying it.

For example, one specific lipid in the liver of mice called arachidonic acid will reset its levels within 24 hours of a change in diet and then remain constant for 3 months. This lipid is a key regulator of inflammation. As such many genes use it to make signalling molecules and there is a sophisticated system is in place to defend its levels. If we work on genes that regulate arachidonic acid levels, then the most suitable length of time to feed a diet to a mouse may be as little as 24 hours. Conversely, fibrosis is the cumulative build-up of scar tissue due to inappropriate activation of wound healing processes. It may take a period of as much as 9 months of high-fat feeding to develop measurable fibrosis in some organs.

To address these questions we have set about modelling the development of obesity associated metabolic complications to allow us to select the optimum length of interventions for studying specific disease processes. We have built several time courses of responses to different diets, which gives us much better information for selecting the length of our intervention.

In addition to optimising the conditions, we carefully consider which control groups are necessary in order to address the scientific question as we design individual experiments, and we use power calculations to ensure that we are using an appropriate number of animals.

We consider carefully how to get the most out of each animal we use. For instance, many of our animal studies involve the generation of models of metabolic disease (often reflecting a human condition such as diabetes, obesity or fatty liver disease). The nature of these diseases is such that they affect many organs of the body. As such, if we plan an animal study designed to probe the effects of diabetes on the liver, for instance, we think carefully about which other organs researchers in the laboratory can work on, such as heart and adipose tissue, and harvest these from the same animals. This reduces the number of animals used overall, increases the amount of data obtained from a single animal, and allows us to examine links between different tissues/organs by combining data from the same animals, thereby enhancing the quality of the information produced.

For several specific techniques we have made changes that enable us to reduce the number of animals we need in experiments.

For body composition we have performed a series of extensive trials to optimise the conditions under which body composition is performed. These allow a low variance in data to be obtained. Variance is how much the data is spread. To be confident a result is accurate, we need either data with low variability or to use lots of mice. By reducing variance we improve data quality, decrease the need for repeat measurements, and reduce the number of animals needed per group. Additionally for euglycaemic-hyperinsulinaemic clamping (the best method for measuring insulin sensitivity), we have comprehensively reworked our protocol to use smaller infusion volumes by using smaller tubing and better mixing taps. This has reduced the time the procedure takes, improved data quality, and reduced animal usage.

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

We have increasingly begun to use pilot studies. These were initially used under our previous license to check the safety of drugs in a small number of animals before using them in a larger cohort. However, researchers rapidly realised the benefit in terms of being able to assess the efficacy of these drugs from the pilot animals.

We employ a designated member of staff who oversees our animal program to ensure the breeding of colonies and experimental design is optimal, and animal wastage is minimised. We routinely cryopreserve all our lines to avoid "tick over colonies" wasting mice and also prevent the phenotype of the mice from changing due to being bred for many generations.

We collaborate widely with other groups across the UK, Europe and the rest of the world and regularly ship samples we have banked to these collaborators for their own purposes, maximising the benefit from previously conducted studies.

We are actively engaged in pursuing data science and artificial intelligence based approaches to the study of metabolism. We regularly reanalyse our old data sets with new collaborators who are experts in the field of data science. As approaches move on, more sophisticated or new ways of interpreting gene expression, lipid and protein data are developed. As such, our existing data may lead to exciting new discoveries and new publications with no need for extra mice.

Refinement

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

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

We will use mice.

We will typically use a mixture of genetically altered (we have deliberately introduced some DNA into them), spontaneous genetic mutant animals (e.g. the ob/ob mouse was found in laboratory colonies as a mouse that was enormously fat - it has a single DNA base pair that has gone wrong) and "wild-type" animals (we have not genetically altered them, nor do they have any known mutations). The majority of our genetically modified animals are congenic, possessing the DNA we have introduced into them in all cells from conception. We also work with a number of mice with pieces of DNA called “loxP sites”. These loxP sites allow deletion of the gene in one or more organs by expressing a gene called a recombinase (known as Cre) that deletes anything between the loxP sites. The deletion of the gene is usually caused by making the Cre have the same expression as a gene found in a specific tissue (e.g. albumin, the most common protein in the blood, is produced only in the liver). We can make the Cre inducible. This means it is normally not active, but if we give the mouse a drug, it turns on the production of the Cre recombinase and deletes our gene of interest. In some cases we will use viruses to express the Cre. The genes that will be altered will be chosen to address our objectives. They will largely fall into four categories: Those affecting adipose tissue function, those affecting energy expenditure

(thermogenesis), those affecting lipid quality or quantity in organs, and finally those are leading to alterations in fibrosis. Importantly, all of the major areas we are researching are involved in human diseases which cause minimal pain. Even end-stage liver and heart failure are characterised by signs such as weight loss (liver failure) or shortness of breath (heart failure). Therefore, we do not expect our genetic mutations in and of themselves to cause any pain, suffering or distress. The development of metabolic disease is slow, so we will be able to monitor for and kill mice before more serious harms manifest.

We will use two primary routes to induce obesity and study its complications. The first is the use of modified diets that make mice obese and insulin resistant, as well as causing diseases such as liver and heart disease. These are usually high in fat, contain refined carbohydrates, and we may also add sugar to the drinking water. It is notable that human fast food tends to be high in fat and refined carbohydrates, and is often accompanied by sugary drinks, making this a depressingly accurate model of the "western" food intake patterns driving the global obesity epidemic. High-fat diets are preferred by mice when given a choice between them and chow. They can lead to a greasy coat but generally cause no pain, suffering or distress.

In addition to obesity we may use diets to study specific aspects of lipid metabolism, where they contain different types and/or quantities of fat. Equally we may use diets to induce fibrosis that lack specific micronutrients (such as amino acids or vitamins). These may cause weight loss, but the weight loss is not caused by the fibrosis, but due to the fact the diet is not as tasty to the mice (the opposite of the high-fat diets).

The second approach for causing obesity will be to use genetically altered animals that model specific aspects of cardiometabolic disease. The most commonly used animals in our laboratory are the spontaneous mutant ob/ob and db/db animals that lack leptin hormone or its receptor respectively. Leptin tells our bodies we have enough fat. When mice lack leptin, they will keep eating as they think they need to store fat, even though they have large amounts already. A loss of leptin causes massive obesity due to a high food intake. The ob/ob model is useful as it causes obesity without the need for the feeding of altered diets. This can make the ob/ob particularly suited for studies where manipulating dietary lipid composition and content would confuse the interpretation of our results. The ob/ob mice are extremely obese and need some special care. They need to have larger mouse houses. Furthermore, they may develop diabetes and drink and urinate more, requiring more frequent cage changes to stop them from developing sores on their body.

In terms of phenotyping, we use several major types of analyses. Firstly, we use "*tolerance tests*" to determine the response of mice to different nutrients and/or hormones. This involves an injection or use of a tube that goes into the mouse's stomach to deliver a fixed amount (known as a bolus) of fat, carbohydrate, protein or insulin, followed by the collection of serial blood samples, usually from the tail vein of the mouse. We take serial samples to see how different metabolites in the blood of the mouse change in response to the bolus. We have a dedicated team of trained technicians who conduct these tests routinely and are able to take the minimal blood volumes that are scientifically necessary. As they perform these tests for multiple groups they are highly experienced and are able to conduct these bleeds without restraining the mouse.

We also assess energy expenditure using a device called an indirect calorimeter. Calorimeters measure heat - which is proportional to how much energy the mouse is expending. We conduct calorimetry in home cages and it has a minimal welfare burden.

We house animals at altered environmental temperatures. Mice are normally housed between 20 and 24ºC. We have specialised cabinets to control the temperature we house mice at. Almost all mice can tolerate temperatures as low as 4 degrees and as high as 30 degrees well. Considerable evidence suggests higher environmental temperatures make the metabolism of mice for many parameters we are interested in (development of fatty liver, cardiac function, energy expenditure) more comparable to humans. Equally, lower temperatures can allow us to study processes such as heat production in more detail. The cabinets we have are able to keep the temperature within 1ºC of the target temperature preventing dangerously high or low temperatures from occurring. Rarely some genetically altered mice will not be able to survive in the cold. We carefully monitor animals from strains we have not studied before when we place them in cold cabinets for the first week to make sure they are able to tolerate low temperatures.

Finally, we have a number of specialised techniques including clamping (a sophisticated technique for assessing insulin sensitivity that provides better and more detailed information than tolerance tests) and thermogenic capacity (a technique for measuring the most energy a mouse can expend) measurements. In the literature these are both routinely conducted on conscious or unconscious mice. We perform them at the end of the experiment on unconscious mice that have been given anaesthetic. The mice are killed before they wake up meaning they suffer as little pain and distress as possible.

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

We use less sentient animals where possible and have published using flies to study specific research questions. However, two of our major focusses in this license are on adipose tissue function and thermogenesis (the process of heat production). Flies do not possess adipose tissue. Furthermore only mammals and birds possess the major thermogenic organ brown fat. As humans are mammals, then a mammalian system is more suitable for translation to humans than an avian system.

We do use terminally anaesthetised mice for some procedures, however metabolic disease takes years to develop in humans and months in mice. As such we need to conduct tests in either conscious mice, or using recovery anaesthesia, to track the development of metabolic diseases over time.

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

We have made multiple refinements to our techniques over the past few years. We undergo a continual practice of reviewing our procedures in order to improve them from both welfare and scientific perspectives. Indeed, these are not separate endeavours. The researchers under the license are all aware that the higher the standard of welfare, the better the quality of the data, making them committed to all aspects of refinement.

With respect to glucose tolerance tests (a test where we administer a single large dose of glucose and then see how well the animal can return its blood glucose to normal) we have implemented various stable isotopic forms of glucose. Stable isotopic forms of glucose are types of glucose that are biologically identical to normal glucose, but have atomic "labels" which we can detect using a device called a mass spectrometer. We can use stable isotopic glucose to determine changes in the mouse's own glucose production (from the liver) during a GTT and separate that from the glucose we have given the mouse. As such we can see if our mouse has changes in liver function, which can occur in insulin

resistance, as well as more general problems dealing with a large amount of glucose. Stable isotopes are biologically identical to normal glucose and therefore provide more data from the same experiment for no more pain suffering or distress.

For clamping we have added stable isotopes allowing the harvesting of tissue (as the samples are non-radioactive). This allows molecular analyses and histology to be connected to gold-standard measures of insulin resistance (for example we can see if mice with bigger adipocytes, or more inflammatory molecules in their blood are more insulin resistant).

For calorimetry we have improved our data analysis by regressing the variable RER against change in body-weight, reducing the variance ("variance" is how noisy the data is – the noisier the data the more mice you need to be confident an effect is real) and improving the biological meaning obtainable from the data. We also singly house mice ahead of calorimetry experiments to acclimate them to the new environment before conducting the tests. Furthermore our new calorimeter is an open circuit system, with smaller sensors for things such as movement. This means we can house mice that were formally group housed close to their cage mates, with better vision of them and, because it is not a fully sealed chamber, they can also hear each other.

For altered temperature housing we use temperature chips which allow us to monitor the mice for drops in body temperature without using more invasive approaches like rectal thermometers.

For feeding of modified diets we have reduced the amount of diet added to the cage and increased diet changes to improve palatability of the food for the mice and as a by-product data consistency. High fat diets go "off" more quickly than chow ones as the fats in them can become rancid and have an unpleasant odour and taste. Furthermore, through highly precise food intake measurements we have been able to identify the importance of consistency of when animals are culled after a diet change. The replacement of old high-fat diets with new leads to a spike in food intake. This is important as spikes in food intake may change the expression of genes or cause increases in hormones like insulin in blood. By controlling when mice are culled after a diet change we reduce unnecessary noise, (and decrease variance) and reduce the number of mice we need to be sure a result is real.

Feeding of modified diets. Certain modified diets (particularly those that cause fibrosis in liver) can cause mice to lose weight. This weight loss is not a sign the mice are sick - all the mice lose similar amounts of weight and end up lean. However, this means we cannot use weight loss as a sign mice are not well. To improve our ability to assess mice we have now included body condition scoring. We use a chart that includes pictures for reference as well as clinical signs to make sure our mice are healthy. We conduct such scoring weekly, unless mice are showing lower body condition scoring in which case we may do it more frequently.

We have now included deuterated water (D20) tracing. D20 is water which heavy hydrogen (deuterium) has replaced the normal hydrogen. When our bodies make fat from sugars they incorporate hydrogen or deuterium from water. If we give mice D20 we can detect the new fat and distinguish it from the old fat using a mass spectrometer. Alterations in how much fat our body makes occur in diseases like diabetes and NAFLD. This provides additional important scientific information with no additional welfare burden to the mice.

For administration of agents we implemented an agent safety protocol. This follows a classic 3+3 design used in phase 1 human clinical trials and involves dosing 3 animals with the drug for one week

to determine the agent is safe. We are also able to collect serum and tissues from these samples to provide pilot information for subsequent larger studies.

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

Laboratory Animal Science Association (LASA) guiding principles documents of aseptic technique(https://www.lasa.co.uk/current_publications/)

ARRIVE (Animal Research: Reporting of In Vivo Experiment) guidelines for preparing papers for publication (https://www.nc3rs.org.uk/arrive-guidelines)

PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for planning our experiments (15 topics including formulation of the study, dialogue between scientists and the animal facility, and methods) (https://www.ncbi.nlm.nih.gov/pubmed/28771074).

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

The project license (PPL) holder is subscribed to the National Centre for the Replacement and Reduction of Animals in Research (NC3R) monthly e-mail newsletter and uses the NC3R website (https://www.nc3rs.org.uk) as a resource for guidelines, practical information, links to publications and video and training materials. The PPL holder has attended the international Federation of European Laboratory Animal ScienceAssociations (FELASA) conference. In addition the animal technicians who work for the PPL holder's institute regularly attend both Laboratory Animal Science Association (LASA) and FELASA conferences and disseminate information regarding 3Rs approaches to the institute. The PPL holder is an editorial board member of Laboratory Animals, the official journal of both LASA and FELASA, which published advances in animal welfare and husbandry matters.

Advances in the 3Rs will be disseminated to the laboratory through the weekly laboratory meetings and through meetings with our technician core in order to have a holistic approach incorporating the license holder, researchers and technicians.