Home Office

NON-TECHNICAL SUMMARY

Pathogenesis and treatment of mitochondrial carrier diseases.

Project duration

5 years 0 months

Project purpose

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

Key words

mitochondria, carrier proteins, citrin deficiency, treatments

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

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?

The aim of this project is to study, and where necessary create, rodent models of mitochondrial diseases, specifically those involving dysfunctional proteins involved in moving small molecules into or out of the mitochondria. The goal is to understand how these diseases develop and to find and test new treatments.

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?

As the mitochondria are so-called "powerhouses of the cell", problems in these proteins can cause a multitude of different diseases, which are currently poorly understood. One disease of particular interest, citrin deficiency, shows 3 distinct disease presentations at different stages of the patient's life characterised by liver problems in young babies, food aversions and slow growth in children and young adults and liver and neurological symptoms in adults. Due to the fact that these diseases affect multiple organ systems across the body, we need appropriate animal models both to understand further how they develop, and to test potential treatments. The goal would be to help find more appropriate treatments for these conditions, and ultimately a cure.

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

At the end of this project the direct outputs we foresee are as follows:

1. **A rodent model of citrin deficiency**. This will either be through confirming the appropriateness of a current commercially available citrin knock-out rodent (rodents lacking the gene encoding the citrin protein) for use in studying the condition, or through generating a new rodent model in house. In particular, this will allow future studies to have a suitable rodent model of the condition on which to work of.

2. A greater understanding of the **molecular and cellular basis** underlying citrin deficiency. Whilst cell culture models (cells used in the lab that have been generated to lack citrin) have provided some insight into some of the underlying mechanisms in certain cell types, they are unsuitable for determining what is happening across different organ systems and across the different stages of the disease. By having an appropriate animal model for the condition, we will gain greater insight into how the condition develops and progresses.

3. Greater understanding of how **treatments** currently used for citrin deficiency are working. Whilst some tools have been developed to treat citrin deficiency, there is little understanding as to how they are working in a therapeutic manner, and at what stages of the condition they are working. By applying potential therapies to an appropriate animal model of the condition, we will be able to discover how they are treating the condition, and provide insight to help develop more targeted therapies in the future.

The primary form these outputs will take will be in publications, in peer-reviewed scientific journals. We do not foresee any products being made from the outputs of this project, but aim to gain a greater understanding of both citrin deficiency and potential treatments for the condition, in order to benefit future therapies.

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

In the short term, the beneficiaries of this research will be other scientists researching citrin deficiency. This is because we will (as publications arise) be providing validation of an appropriate animal model, providing new insight into the underlying mechanisms leading to the condition and how potential treatments work. This research may also be of benefit to other researchers interested in mitochondrial biology and metabolic physiology, as we will uncover further information about the roles mitochondrial carriers play in maintaining healthy functioning mitochondria.

In the longer term, this work will be of benefit to clinicians and clinical researchers involved in treating citrin deficiency. By investigating the molecular and cellular basis to the condition and generating an understanding of how the current treatment options work we will provide an improved foundation from which clinicians can treat the condition. Likewise, this work may benefit researchers in (or interested in) the pharmaceutical industry, targeting citrin deficiency or other mitochondrial carrier (proteins involved in transporting small molecules into an out of the mitochondria, including citrin) diseases. By understanding how the current therapies exert their action, this may pave the way to generate new treatments with greater success in treating the condition and others like it. Ultimately, this will be of benefit to patients and patient families.

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

All new knowledge will be disseminated via open-access publications. We will target journals to ensure that our findings reach the appropriate target audience including both scientists and clinicians working within the field. This will include all findings, regardless of whether they are positive or negative results. We will also present our work at appropriate academic conferences both within the UK and abroad.

Through our continued involvement in the Citrin Foundation, we are part of a global consortium of scientists, researchers and patients all working towards the goal of curing citrin deficiency. As such, we regularly present our work internally within the Foundation, enabling our work to be rapidly disseminated to peers working within the same field. This also enables us to share preliminary results, and gain insight from a network of collaborators to ensure maximal outputs from each experiment.

Species and numbers of animals expected to be used

- Rats: 1060

- Mice: 1620

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 rats and mice from the neonatal stage up to 18 months of age (mice) or 24 months of age (rats).

The disease we are primarily interested in, citrin deficiency, shows a characteristic development of symptoms in patients, that occurs in an age-dependent manner, with newborns and young infants displaying a very different set of symptoms compared with adults and older teenagers. We therefore need to utilise animals at multiple life stages, from neonatal animals to aged rats/mice, to both allow us to understand how the disease develops these distinct stages, and to allow us to test interventional therapies that may be useful in the clinic at the relevant disease stages.

Currently, there is no good model for citrin deficiency. Different commercial models have been developed, but have been incompletely studied to determine whether they are a useful model for the disease. We will comprehensively assess these models, making careful consideration of genetic background and baseline physiological measurements, to allow us to determine the most appropriate model for citrin deficiency. Once we have established the appropriate model, we will enable the total number of animals used to be reduced as experiments will be more reproducible and applicable to the clinical condition.

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

We will be studying rodent models of citrin deficiency, a mitochondrial carrier disease caused by mutations in a gene encoding a protein called citrin. All rats and mice used will be genetically-altered to remove the citrin gene, or contain a mutated version of the citrin gene seen in human citrin deficiency. This citrin knockout (animal with the gene removed or non-functioning) may be across the whole of the rat/mouse or only in a particular organ. Some mice/rats may have a genetic alteration, which allows the citrin gene to be removed at a specific time during the mouse/rats lifetime (a so called "inducible model"). Additionally, some mice/rats may have other genes removed. These are genes that provide a complementary function to citrin, and therefore can compensate for the removal of citrin in genetically-altered animals. We will utilise littermates that haven't been genetically altered, or only contain one mutated gene copy in our experiments to help us understand the differences between healthy animals and those that develop the disease.

Animals may be bred in-house, or may be purchased from a commercial supplier. Animals being purchased will therefore be transported from a supplier (predominantly UK based, but on occasion this may be from abroad) to the animal facility. This can be a stressful procedure for the animals, becoming introduced to a new facility and undergoing transit which can be noisy with less than idealised environmental conditions. Animals bought into the facility will undergo an acclimatisation period (a

minimum of 7 days) to allow them to settle into their new environment, prior to any procedure being initiated.

In addition to using genetically-altered animals, the mice/rats may undergo one or more of the following procedures:

- Female animals may receive drugs, delivered under the skin or into the abdomen, to cause them to increase the number of eggs they produce. This will allow us to harvest the eggs, to either store them for future use or generate new genetically-altered models as required. In the latter case, some female animals may undergo embryo transfer, where embryos produced by in vitro fertilisation are re-implanted (non-surgically or surgically under anaesthetic) into a female rat/mouse generating new genetically-altered offspring.

- Animals may be fed a modified diet. This may be both to generate the observable characteristic and clinical signs of the disease (e.g. by using an altered carbohydrate/protein diet) or to test dietary interventional treatments (e.g. altered fat content). Animals may also be exposed to different types of food, or different dietary components (e.g. sugar), including via their drinking water, to determine if they show any preferences for certain food groups, which is a known symptom of citrin deficiency in humans.

- Animals may receive drugs or other compounds known to alter their metabolism. These may be administered as an injection (under the skin, or into a vein, muscle or the abdomen), or orally (by incorporating the compound in their food and water, or directly to the stomach by a technique called oral gavage) . Again, these may be used to generate or challenge the disease phenotype, or to test drugs/compounds that may have a therapeutic use in treating citrin deficiency.

- Some animals may receive, either via injection (into a vein or the abdomen), oral gavage or inclusion in food/water, molecules called tracers. These allow us to follow the particular metabolic processes, and therefore understand more about what is actually happening in a given tissue. Typically, an animal would only receive one of such of these compounds across their lifetime, and would never receive more than five. These compounds are well studied and tolerated well by the rats/mice.

- For short periods of time (usually less than 5 days, but on occasion up to 4 weeks), animals may be single housed. This allows measurements of the individual amounts of oxygen each animal is consuming, their level of activity, accurate food and water intakes as well as urine and faeces production. Additionally, it may be required to single-house animals in order to generate control animals with a matched food intake to another animal in a particular study. In all cases, animals will be rehoused into group housing following the period of single housing, unless this is more stressful for the animals to do so (e.g. causes fighting for particularly aggressive males).

- Some animals may have access to food restricted either completely for a short period of time or partially for a longer length of time. In the first case, this would be to enable us to carry out measurements in fasted animals, similar to how some blood tests require fasting before the blood to be drawn. Whilst in the short-term removing access to food can be distressing to the animal, it causes no lasting harm, and food will always be immediately returned following the experiment (usually withdrawal of blood). In the case of partial food restriction, this will be to ensure we can

match food intake between animals in a particular study. Whilst the animals may prefer to have unlimited access to food, the level of food restriction will not be harmful for the animals involved.

- Some animals may be challenged to behavioural and/or exercise tests. This allows us to examine how their fitness level changes during disease progression (exercise) and mental acuity (behavioural testing). Animals would be acclimatised and trained to utilise the test equipment minimising the stress caused. The initial training period may cause some transient stress (as the animals are introduced to a new environment and new equipment), and exercise testing requires the use of a motivational air puff placed behind the animal. However, the most refined methods will be utilised to minimise the stress the animals experience during the testing, and animals will undergo testing as few times as possible across their lifetime, with exercise testing (arguably the most stressful test) likely occurring only once in the vast majority of cases.

- Some animals may be scanned by non-invasive imaging techniques (such as nuclear magnetic resonance or ultrasound) to look at how their bodies or organs are changing during the disease progressing. This may involve brief restraint of the animals, without anaesthetic, in a painless and harmless manner, or alternatively a brief (less than 1 hr) anaesthetic to allow clearer scans to be obtained, without the animals moving around. No animal would be expected to go through more than 6 scans of any type in their life time, and if an anaesthetic was involved, there would be at least 1 week between each imaging procedure.

- Some animals may have blood periodically withdrawn. This would be from a peripheral vein (such as in the tail or leg), with minimal distress or pain to the animal. Animals are briefly restrained in a tube, whilst the blood sample is drawn. Animals may be warmed prior to the procedure, to encourage the blood vessels to dilate, which makes the finding the veins and withdrawing the blood faster, and thus less stressful for the animals. Multiple blood samples may be required across the lifetime of the animal, but a minimum of a two-week period will be left between samples, with no more 15% total blood volume sampled within a 28-day period.

Whilst a typical animal will experience no more than 5 of these procedures, there may be a cumulative effect of these procedures. The most extreme case would involve the following: a genetically altered animal plus: (a) feeding an altered diet for the entire experiment, (b) single housing the animal for 5-days (twice, with several months in between), (c) anaesthetic administration for non-invasive imaging (three times, with several months in between each administration), (d) an exercise test (with associated training) occurring once and (e) periodic blood withdrawal, approximately once a month. Despite their clearly being a cumulative effect, we will space out the procedures across the experimental timeline to prevent multiple stressful procedures occurring in quick succession. In this way, we will aim to minimise the cumulative effect and distress associated with the experimental model.

At the end of the experiment, each animal will be killed using the most humane method possible, whilst still allowing collection of blood and tissue samples to not be detrimentally affected for scientific analyses.

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

We are aiming to generate and validate a rodent model of citrin deficiency. Rats or mice, if they display a similar disease progression to that of the human condition would be expected to experience three distinct phases of the condition:

1. Newborn animals would be expected to show some signs of liver dysfunction. This may include slight yellowing of the skin (jaundice) and production of a fattier faeces. This phase, if left untreated, would be expected to last until the rats or mice were 3-6 weeks old. Monitoring of the condition would be undertaken, and where necessary, animals would be treated in line with the best practice for treating human citrin deficiency.

2. Animals would then be expected to go through an 'adaptation' period. Here, most animals as they continue to grow would be expected to have very few adverse effects as a result of their genetic mutations. It is possible that some rats and mice may grow slower than if they were not carrying the genetic alteration for citrin deficiency, and they may show some voluntary food aversions, resulting in needing to feed the animals a slightly different diet. A small proportion of these animals may develop some fatigue symptoms, and may have difficulty processing fat, which may result in them becoming lethargic.

3. In older, aged animals, liver dysfunction may again develop. Here, we expect the major change to be in the way the animals are able to handle protein, resulting in the build-up of a waste product called ammonia in the blood. This may result in the animals experiencing some confusion or disorientation alongside fatigue and lethargy. Urine and faecal output may be altered in these animals.

Some animals will experience just one of these phases, some more than one and some none and all. We expect the minority of animals to experience the final phase, both because it is less frequent to develop in human patients (and we therefore expect it to develop less frequently in any animal model), and because fewer animals will be raised to this age.

In general, the experimental procedures we will be carrying out will either be to (a) test treatments for the condition, and therefore are expected to show less adverse effects or (b) to allow us to track and monitor the changes occurring in the animals as a result of the condition. In this second case, any adverse effects, as outlined below, will be transient and lead to no lasting harm:

- Feeding the animals a modified diet may be required in order to initiate a distinct phase of the disease, and thus may result in an adverse effect outlined above. However, in some cases, modified diets will be used to test potential treatments for the condition, or to ameliorate certain aspects of the disease, and therefore will result in reduced harm to the animals.

- In this project, drugs or compounds known to alter metabolism will typically be utilised to improve the disease condition and therefore would be expected to lessen the adverse effects. Tracers would not be expected to cause any adverse effects. Administering the compound, drug or tracer would not cause any lasting harm from the injection or oral route *per se*, although the animal may experience a small amount of discomfort during the actual administration.

- Single-housing can be stressful for the animals, but causes no pain or lasting harm. However, times spent in single-housing will be minimised and only carried out when absolutely necessary. Animals will also be kept in close proximity to their original cage mates as we have found this

helps to reduce the stress associated with the single-housing in the past. Wherever possible, animals will be rehoused with their original cage mates following the single-housing period. If this is not possible, e.g. for a group of particularly aggressive males which begin to fight upon rehousing, the reintroduction process itself may be distressing, and they may have to remain single-housed, which would also cause some distress. However, all attempts will be made to prevent this occurring and minimising the number of animals that may experience this.

- Behavioural and exercise testing can cause some transient stress to the animals as they are removed from the home cage for testing. However, animals will be acclimatised to all equipment to minimise stress. For some tests (e.g. exercise testing), the training process is associated with some distress, as animals have to explore the equipment which is foreign and potentially uncomfortable. For example, for exercise testing, animals must experience the air puff at the end of a treadmill so they are motivated to run during the testing, and although this is the most refined method od motivating running, it is still an averse stimuli and so it may be distressing to the animals. In all cases, there is no lasting harm from carrying out these tests.

- Non-invasive imaging is harmless and pain-free. Mice and rats that are anaesthetised may feel slightly disorientated and groggy as they recover from the anaesthetic. However, the effects of the anaesthetic are transient and the animals will not suffer lasting harm as a result of the procedure.

- Blood sampling may cause a small amount of discomfort during the actual procedure. However, the animals will not experience any lasting harm as a result of having blood drawn.

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

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

Mice:

Subthreshold: 20%

Mild: 50%

Moderate: 30%

Rats:

Subthreshold: 20%

Mild: 50%

Moderate: 30%

What will happen to animals used in this project?

- Killed

Replacement

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

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

The conditions associated with mitochondrial carrier diseases are multi-system, and therefore to fully understand these conditions and how to treat them, we need to understand how they progress across organ systems, and which treatments are effective, at which points. In order to do this, we need good animal models for the conditions, which need validation prior to use. Our initial disease of interest, citrin deficiency, is poorly understood and has no good rodent model of the condition. We therefore first need to validate rodent models of citrin deficiency, before we can use it to study the underlying mechanisms of the disease development and progression, and then use these models to develop and test new treatments for the condition.

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

We have actively been generating cell culture models for citrin deficiency, using established cell lines that we have genetically altered to contain disease carrying mutations. Thus far, we have generated the first and only clonally derived cell line for use in studying citrin deficiency. Using gene editing technology, we have created both complete and partial knock-outs of the citrin gene in liver cells, which has allowed us to comprehensively characterise the changes occurring in their growth and energy generating pathways as a result of loss of citrin function. We have presented this work at conferences both within the Citrin Foundation (Global Symposium 2023) and internationally (European Bioenergetics Conference 2024). We are also in the process of using newer gene editing techniques to generate cell models of common specific variants of the citrin gene that are seen in patients, so we can understand the differences between complete loss of function of citrin, and changes that also occur clinically.

We have also considered using clinical samples, collected from the cohort of patients connected via the Citrin Foundation.

Why were they not suitable?

We have currently been utilising established liver cell lines and using gene editing approaches to generate cell culture models for citrin deficiency. However, there are limitations with the cell culture models available, even for just studying the changes in the liver cells, which is the primary organ affected by the disease:

- Established cell lines have multiple copies of genetic material (more than 2 copies of each gene). This means that the genetic editing we introduce does not accurately reflect what is happening in a human patient. Furthermore, the different numbers of genes (resulting from multiple copies of DNA) means that the cell line metabolism is very different to that of a liver cell, and thus does not give us a "true" picture of what is going on in the disease.

- We cannot "see" the different stages of the disease in cell culture models. We can measure how metabolism, cell stress and cell growth change over time, and how potential treatments work directly onto genetically altered liver cells, but cannot relate this back to the different stages of the human disease.

- Whilst the liver is the primarily affected tissue, the disease affects the whole body, and we need to understand the interplay between what is happening in different organ compartments, and systemically (i.e. in the blood). We cannot do this using cell culture as we only have one cell type, in a dish, not communicating with the other tissues of the body. Moreover, there are likely to be regional variations within any given organ, such as the liver, in how it is affected by the disease as it is progresses. These regional differences are not able to be observed, or studied without the whole organ.

We will continue to utilise our cell culture models, alongside the animals in this project, to further our understanding of the cellular mechanisms at play in the disease. They will allow us to interrogate possible metabolic pathways that may be altered in detail, following on from the changes we detect in the animal models, but we need to observe the changes first in an animal model of the condition.

Where possible we will utilise clinical samples in our work. However, given the wide range of mutations that cause citrin deficiency (more than 100), and the relatively small number of identified patients worldwide (approximately 1000 known to the Citrin Foundation, although the prevalence is relatively high in East Asia), there is expected to be a fair amount of variation in samples collected which would make establishing a how the mutations cause the clinical symptoms shown very challenging. We would need to carry out carefully controlled experiments in animal models to understand the causal relationship before using clinical samples for validation of our findings.

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?

Our aims fall in two parts, (a) validation of a rodent model of citrin deficiency and (b) utilising a valid model of citrin deficiency to understand the molecular mechanisms causing the disease and testing potential treatments.

For the validation aspect of the project, we anticipate we will need to generate 4-8 cohorts of different ages of each proposed model of citrin deficiency. There are currently four commercially available models of citrin deficiency, and breeding colonies will need to be established to ensure we can have age-matched controls (animals without the mutations in the citrin gene, that have been bred and raised in the same way so provide a comparative baseline group to compare the citrin deficiency model animals to). Previous work has indicated that between 6-10 animals per group is sufficient to indicate if

there are differences in groups. We may need to generate a new model, e.g. an inducible model or liver-specific knock out, and thus also require animals for generating these new animals. We therefore anticipate utilising 310 rats and 420 mice for validation and generation of new models.

For the second aspect of the project, we estimate we will run around 10-15 individual experiments, each with between 2 and 8 groups. With our previous work indicating that 6-10 animals would be sufficient per group, this indicated that we would anticipate utilising 800 animals' total.

We will also need to establish breeding colonies for the mice and rats. As only half of each litter will initially be utilised for experiments (those animals carrying the mutation in the citrin gene [the 'knock-outs'] and those without the mutations ['wild types']), we anticipate needing breeding colonies of 900 rats and 1400 mice across the two breeding protocols, of which half the animals will be maintained on these protocols only.

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

During the design of individual experiments, we ensure that we include appropriate controls to address our particular scientific question and utilise statistical analysis to ensure an appropriate number of animals are used. In order to minimise animal numbers and ensure randomisation and blinding where possible, we will utilise the NC3Rs Experimental Design Assistant to help design individual experimental set ups.

Whilst much of our work will focus on the liver, the primary disease affected by citrin deficiency, we will take a systems level approach (looking across the whole body rather than one tissue) when establishing individual experiments, involving either studying or banking tissue across organ systems for later analysis. This ensures that, not only do we get a more complete understanding of how the disease impacts the whole body, we have 'pre-collected' tissue from previous studies for follow up analysis, and thus don't need to use more animals for future work.

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

Whilst we appreciate that we are likely to need to set up our own breeding colonies in order to study the animals throughout their lives, we will utilise the resources on efficient breeding available from the NC3Rs to ensure that animals are not wasted e.g. by using intermittent breeding methods. Where possible, we will buy in animals from a commercial supplier instead of setting up a breeding colony, to ensure breeding is only established when absolutely necessary. We will also follow best practice with regards to archiving strains, to ensure that constant breeding is not required.

The initial phase of our project aims to validate a rodent model of citrin deficiency. Whilst various approaches to generate animal models of the disease have been taken in the past, there has been no systemic, organised study of whether these models do, in fact, model the condition. Through the initial phase of the project, we aim to have a valid rodent model for the condition, which will enable us to reduce the number of animals required in the later stage (understanding the mechanism and testing therapies) as only the validated model of the condition will be required. This will also help reduce

numbers of animals utilised more generally beyond this project, as results obtained will be more reproducible once in a validated animal model of the condition.

Pilot studies will be used in order to minimise the number of total experimental groups needed to answer particular experimental questions. This will also enable us to also optimise experimental conditions and refine the methodology we use in our experiments. For example, we may need to intervene with dietary modification in these animals, as in the human condition, to minimise their carbohydrate intake, and a small pilot study will determine whether this is necessary, before we move on to studying a full cohort of animals throughout their lifetime.

At post mortem, all tissues are routinely stored to ensure that the minimum number of animals are used across the programme of work. Whilst the disease of interest primarily affects the liver, we will routinely collect tissues across the body, both to enable a more complete understanding of the disease, and to reduce the number of animals that may be required in future studies. This strategy has been of great success in the license holders previous laboratory, where six peer-reviewed papers (thus far) have been published from banked tissue from a single-mouse study, initially carried out in 2012.

We also happily take part in the sharing of tissue, both for optimisation purposes and with collaborators for experimental purposes. When we need to optimise a particular experimental procedure *ex vivo,* we work to utilise tissue that has already been collected for another experiment but is in excess, so no animals need to be killed specifically for optimisation purposes. We likewise extend this courtesy to others. In order to do so we are already part of internal tissue sharing and colony management resources, utilised for this purpose. We have also utilised tissue collection from animals held within a commercial supplier, to enable us to begin validation of the animal model, in concert with another lab in Switzerland, to enable us to establish maximal data about the animal models prior to having any animal experiments take place.

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 be utilising genetically altered rats and mice which will not express the mitochondrial protein citrin. Whilst we will be aiming to model and characterise an animal model of the human disease citrin deficiency, for most of these animals' lives, we expect them to experience no more than perhaps some dietary changes, and thus no lasting harm. If some of the animals experience more symptoms associated with citrin deficiency, this may cause some fatigue and some changes in behaviour, which may be accompanied with some signs of liver dysfunction (jaundice) but will not be severe.

In some cases, we may feed animals a different diet or administer a compound via the animal's diet or drinking water. This may be to develop a particular clinical symptom, or to test a potential treatment. In

all cases, the food/water will remain palatable to the animals, so they will continue to eat and drink. Providing an intervention in this way allows us to non-invasively administer our desired compound (or altered diet) to the animals, and therefore reduces the distress associated with alternative methods, such as direct feeding into the stomach (oral gavage) or injections. However, occasionally, in order to give a specific dose of a compound (e.g. a potential therapeutic drug), we may need to provide it via the oral gavage or injection route. When this is required, we will utilise the route of delivery that is both optimal for the efficacy of the compound, but also allows the compound to be delivered in the most pain-free way. To ensure this, we will carefully way-up the volume required, viscosity of the injection and how many injections would be needed, to enable compounds to be delivered in a manner that causes the least pain, suffering and distress. For all injections, single use needles will be used, and sterility would be ensured to prevent infection.

On occasion, animals will need to be single housed. This may be for behavioural testing (see below) or for measuring whole body oxygen consumption in “metabolic cages”. Single housing the animals can be distressing for them, but we will aim to minimise this by (a) only carrying out these tests when it is the only possible technique to achieve the scientific aims (therefore minimising the number of animals that experience this); (b) keeping the time for single housing as short as possible; (c) housing single-housed cage-mates near each other, so they can still smell each other, as we have found previously this helps minimise the distress associated with single housing, and; (d) wherever possible, rehousing animals into group cages following the period in single housing. By following these optimisations, we are able to minimise the distress associated with any period of single housing.

Much of our work relies on monitoring changes to normal body functions that occur as a result of the genetic mutation in the animals. Some animals may undergo non-invasive imaging, which require temporary restraining of animals or brief anaesthetic. The scans themselves are painless and cause no lasting harm, and allow us the measure and follow many changes in how the animals are functioning without having to undertake much more invasive procedures such as tissue biopsies. Whilst restraining the animals can cause transient distress, the scans on conscious animals are very quick (less than 2 minutes) minimising this stress. Anaesthetic is only used when we need the animals to be still for longer periods of time for imaging (less than 1 hour) and although animals will feel groggy on recovering from anaesthetic, it is well tolerated in rats and mice and causes no lasting harm.

We will sometimes need to take blood samples from animals. Whilst the act of having small amounts of blood taking may cause a small amount of distress or pain, there is no lasting suffering or harm. We will utilise the most up-to-date, refined procedures for blood sampling, including taking blood from the leg or tail vein and “micro-sampling” to take minimal blood samples from each animal. In this way, we can ensure that we are causing the minimal distress possible during the procedure itself.

Some animals may need to undergo tests to assess their exercise capacity. We are utilising a methodology that is optimised to minimise distress for the animals which undergo these tests. Usually, animals undergoing exercise testing are required to be exposed to averse stimuli (shock grid, air puffs) throughout the testing, with testing only ceasing when animals remain stationary for a prolonged period of time despite continuous exposure to the stimulus. Whilst we are using the more refined air puff stimulus, the continuous exposure can remain distressing for animals. Our applied method instead requires animals to only be exposed to air puffs during training on the equipment. The test instead stops when the animal spends time within a set distance of the end of the treadmill and cannot be encouraged (by the researcher or an air puff) to continue. Animals therefore do not experience the

continuous air puffs that normally end a classic test, therby reducing the distress. Animals participating will recover fully from the training and actual testing procedure and therefore not experience any lasting harm.

Likewise, some animals may undergo behavioural testing. Where possible, these will be “home cage tests” where we can assess behaviour either directly in the animal’s home cage, or in an identical environment to the animal’s home cage (but with a single animal). In these cases, the animal has very little idea that any testing is occurring, and thus there is little if any distress associated with the experiments. In other tests, we will undertake behavioural tests that are well established in psychology and neurobiology. In order for us to get usable data out of these tests, we need the animal’s performance in the test to reflect their actual behaviour, and not any distress associated with the testing equipment. We therefore will utilise well established protocols for acclimatising and training animals to all the testing equipment prior to any testing. This enables us to ensure that any distress is minimised. The tests themselves are not painful and cause no lasting distress or harm.

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

Mice and rats are the least sentient animals that are suitable for studies of mammalian metabolism. Most non-mammalian organism that possesses a citrin gene, e.g. Zebrafish, have copies of the gene that is very different to the human version, and resembles a protein that is not found in the organs affected by citrin deficiency. Less sentient animals, such as flies and worms, actually do not have a citrin gene at all and therefore are unsuitable models for us to utilise for trying to model the condition. We are therefore unable to use species that are of a lower neurophysiological sensitivity to study the metabolic physiological alterations occurring in a disease such as citrin deficiency.

Given the complex way citrin deficiency presents, with separate clinical conditions occurring in infants and older people, we need to study animals across their lifespans. Metabolism also alters over development, so the interaction that mutations in the citrin protein have with these developmental metabolic changes are important for us to measure, in order to gain a better understanding of the disease progression and how new therapies could be developed. Whilst some experiments will involve animals at an immature life stage, we will need to follow animals to adulthood and as they age, in order to gain a greater understanding of the disease progression.

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

In all studies, animals will be monitored frequently. Our work relies on us detecting potentially very small changes in the animals with different genes, so animals will be very closely monitored throughout their lives. More often than not this will include weekly measurement of body weight, with cage food and water intake likely measured more frequently (e.g. 2-3 times a week). This ensures that animals become habituated to the researchers handling them, reducing any stress associated with this husbandry task. We also employ the use of welfare scoring systems to ensure that there is a standardised measure of picking up subtle changes in animal welfare across different researchers that may be checking the animals.

We utilise the most refined methods for handling animals, such as cupping and tunnel handling, which minimises any stress associated with handling procedures. Animal home cages also will contain

environmental enrichment (to the extent at which they will not interfere with scientific studies) including, but not limited to, comfortable nesting material, shelters, play tunnels and gnawing blocks, to further optimise welfare. Any animal purchased from a commercial supplier (as opposed to being bred in the animal facility) will undergo an acclimatisation period of at least 7 days following arrival in the facility before they are used for any breeding or experimental protocol, to allow them to adjust to the new environment and recover from any distress associated with the transit.

Animals that may need to be restrained, e.g. for imaging or for blood taking, can also be introduced to the restraining tubes by the researchers during their frequent interactions with the animals during a study, prior to any procedure being carried out. This ensures that animal is familiar with the restraining devices, and observes minimal stress when the procedures (e.g. non-invasive imaging or blood sampling) are ultimately carried out. Where possible, restraining tubes will also be made of a red plastic to further minimise the distress to the animals, as it minimises the light they detect, and is more comforting for them. Any experiment that involves needle use (blood sampling, drug delivery, etc.) will utilise single use needle protocols, and needles will be the appropriate size (gauge and length) for the animal in question and route of delivery.

In the animal facility, animals will be maintained on regular light/dark cycles (12h/12h) in a temperature and humidity-controlled environment. If for some reason, the animals need to change location (e.g. for monitoring their whole-body oxygen consumption rate for measuring metabolic rate), the environmental conditions will be as closely matched to that of their home cage environment as possible. The exception to this may be the temperature, as for some metabolic rate measurements, the animals may be maintained at a temperature closer to that at which they don't have to use any energy to maintain core body temperature (the thermoneutral temperature).

Whilst animals may have to be single-housed, for example as in the metabolic rate experiments above, this will be undertaken as infrequently as possible, and for the shortest possible time. Animals will also be kept in proximity to their cage-mates, as previous experience has shown that this reduces the stress they experience during the period of single-housing, and often allows us to return the animals to group housing afterwards.

Any experiment that requires more advance equipment e.g. behavioural testing or exercise testing, will always be preceded by either a training period or, if this is not possible due to the specific scientific question, an acclimatisation period, where the animal is able to experience the equipment and get used to the new environment without any data being collected. This is also important to us, as it ensures that the results we collect are valid and reflect an actual change in the animal's physiology, rather than a change resulting from generic acute stress due to a new situation.

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

Experiments will be planned in line with PREPARE[1] guidelines and ARRIVE[2] guidelines (v2) will be used when reporting studies.

Guidance of the most refined techniques for, for example, handling, blood sampling and enrichment, from the NC3Rs resource library[3] will be followed, and regularly reviewed to ensure it is up to date.

Guiding principles from the Laboratory Animal Science Associate (LASA) will be followed with respect to record keeping[4], mortality reduction[5] and best practice in behavioural aspects[6] of our work. We also will carry out all genetic alteration experiments in line with the LASA position paper on transgenics[7].

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[1] Smith AJ et al. PREPARE: guidelines for planning animal research and testing. Laboratory Animals. 2018;52(2):135-141. doi:10.1177/0023677217724823

[2] Percie du Sert N et al. (2020) Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0. PLoS Biol 18(7): e3000411. https://doi.org/10.1371/journal.pbio.3000411

[3] NC3Rs Resource Library. Accessible at: https://nc3rs.org.uk/3rs-resources/

[4] LASA Guiding Principles on Record Keeping for Personal License Holders. Accessible at: https://www.lasa.co.uk/wp-content/uploads/2018/05/Record-Keeping.pdf%20

[5] LASA Resource on Avoiding Mortality in Animal Research and Testing. Accessible at: https://view.pagetiger.com/RSPCAAvoidingMortalityResearchReport/RSPCA

[6] LASA Guiding Principles for Behavioural Laboratory Animal Science. Accessible at: https://www.lasa.co.uk/wp-content/uploads/2018/05/LASA_BAP_BNA_ESSWAP_GP_Behavioural_LAS_Nov13.pdf

[7] LASA Position Paper: Transgenics. Accessible at: https://www.lasa.co.uk/wp-content/uploads/2018/05/Position_Transgenics.pdf

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

The license holder receives monthly updates from the NC3Rs to remain up-to-date with current advances in 3Rs research. The license holder also subscribes the Tech3Rs specialist bulletin to receive current information relevant to 3Rs practice in husbandry and care e.g. improved handling, blood sampling etc. We also receive information on relevant courses from the Named Information Officer of the Establishment. We work closely with an experienced laboratory animal models manager, which enables a large wealth of experience to be disseminated effectively to our group.

The small number of researchers working under the project enable these advances, which are often easy to implement, can quickly be passed from one member of the team to the whole group. Discussion and feedback within the team is welcome allowing new techniques and advances in the 3Rs to be implemented effectively throughout the project for the positive benefit of the animals.