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NON-TECHNICAL SUMMARY

Epithelial stem cell behaviour away from homeostasis; translational relevance.

Project duration

5 years 0 months

Project purpose

- (a) Basic research

Key words

Squamous epithelium, Development, Tissue injury, Aging, Cancer

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

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 investigate how the cells of the oesophagus (the gullet; the tube that connects the mouth to the stomach) and other related tissues (like the skin) adapt to perturbations such as aging, wounding and tumour formation.

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?

Diseases of the oesophagus are very aggressive in nature, challenging to treat, and with poor overall outcomes. Oesophageal cancer, for example, represents the eighth most common cancer and the sixth most deadly worldwide, with a 10-25% five-year survival. Other oesophageal complications, such as injury, also have a poor survival rate of less than fifty percent. Hence, medical conditions of the oesophagus represent an important social and economic burden, with a significant proportion of our public health budget spent in treatments that are inefficient and, in most cases, unsuccessful. Thus, it is critical to better understand this organ in order to identify clinical strategies that may help us improve the quality of life of patients suffering from these appalling diseases.

More specifically, in the proposed research we will use mice to investigate the way in which the cells of the oesophagus and other related tissues (such as the skin) function under normal conditions, and evaluate how their behaviour changes in response to one or a combination of tissue perturbations (situations driving the tissue away from normal health). These include:

1. Aging
2. Injury
3. Early cancer formation

By addressing these questions, we expect to understand how cells behave in different contexts, and whether this information can be used to change their behaviour in our benefit and improve health. We ultimately aim to improve wound closure, minimizing scarring, and to treat cancer more effectively. Additionally, by investigating cancer formation from its early stages, we expect to identify markers that can assist in the early detection of oesophageal cancer, improving the long-term prospect of those patients.

We anticipate that our work will identify targets of potential medical relevance that will contribute towards solving the challenges being currently faced in the clinic.

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

The main output of our work will be in the form of scientific publications that will be of relevance to other scientists in our or other related research fields. These publications will provide novel information on the processes regulating how the cells that line the oesophagus (epithelial cells) respond when not in normal health. Relevant findings will be compared with other related parts of the body (tissues), such as that of the skin, to identify whether our observations have a wider applicability, beyond the oesophagus.

In our publications, we aim to cover a number of relevant aspects:

1. Identify changes in the wound healing ability of cells during aging.

2. Define how changes in the immediate environment that surrounds a tumour affect cancer formation.

3. Reveal whether cells return to an early developmental stage when perturbed.

4. Uncover the impact of aging and wounding on tumour formation.

As standard practice, the data generated in our work will be made publicly available in free repositories to ensure that it can be used by other researchers and facilitate scientific discovery beyond our own studies. This should reduce repetition of the same work by other laboratories, decreasing the number of animals needed for related research purposes.

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

Our overall challenge is to identify the processes controlling the ability of a tissue to heal (tissue regeneration) and to develop cancer, and translate this knowledge into therapies that benefit the patients and healthcare providers. For this, the immediate aim is to understand how the cells deviate from normal behaviour when they face an injury/damage or develop tumours. Then we need to determine which molecular mechanisms regulate the cellular response. Our ultimate aim is to determine whether we can intervene in these processes, using drugs, in order to take cells back to their normal healthy state.

In this programme of work we will address these important questions. In the short term, new information that comes from this study will be presented through publications and conference presentations, or shared with organizations such as the Wellcome Trust and The Royal Society. This will mostly benefit the scientific community, particularly those with interests in tissue regeneration and cancer. In the long-term, our findings could be used to develop therapies to improve wound healing and treat oesophageal cancer. This will benefit patients suffering from cancer and/or injuries to the oesophagus and/or skin, professionals in the healthcare community, as well as companies with an interest either in i) experimental systems to study disease or in ii) in the potential identification of novel drug targets.

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

We maximise the outputs of our work by doing the following:

- To increase the outputs of the work conducted within this programme, the **management** of individual projects is closely supervised by me. This way I avoid effort duplication and ensure that the resources are maximised and shared between members of my laboratory, where feasible.

- By disseminating our observations from early stages, for example via conferences, we create new **collaborations** with experts in research fields beyond our own. They positively contribute to the progress of ongoing projects and ensure their completion to the highest standards. This has previously allowed us to establish collaborations with researchers interested in samples created as a side-product of our research. For example, samples are currently being shared with other

groups examining the formation of cancer in other organs such as the tongue and the cheek, among others.

- Moreover, in our effort to understand the behaviour of cells, we conduct the analysis of large-scale datasets. To maximize the outputs obtained from these complex datasets, we collaborate with experts in theoretical physics, mathematical modelling, and bioinformatics, who have the skills to develop **theories** explaining the results obtained in our experiments. Methods and datasets generated in these types of analyses are made publicly available as part of the relevant research articles enabling new collaborative projects.
- Our work will be published in free, public repositories online and in international scientific journals. In our **publications**, we do not only include positive data, but also experimental results that fail to support our hypotheses. This also prevents duplication by ensuring that other laboratories do not spend resources in ideas that have already been tested.
- We will share our work with the public via public engagement activities. This will allow people to learn about novel aspects of regenerative medicine and early cancer, and will provide the opportunity for a productive exchange of ideas between patients and scientists.

Species and numbers of animals expected to be used

- Mice: 38150

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.

Traditionally, **mice** are used in our research field because they allow relatively easy genetic manipulation, something that facilitates

i) injecting substances that enable us **to track individual cells** over time and follow their behaviour,

ii) manipulating cells **genetically** in order to understand the processes regulating their behaviour.

Our studies particularly focus on the **oesophagus** because of its structural simplicity. This facilitates the visualization and tracking of the cells we are interested in. Our results are then compared with other epithelial tissues, such as the skin, to identify whether our observations are relevant beyond the oesophagus.

This project will expand on our previous work by making use of all the methods that we have developed to study oesophageal/skin cell behaviour using animal models.

Mice will be studied at **different stages of their lifetimes** (from birth to aged stages) because we are interested in understanding why we exhibit a different wound healing response and cancer incidence as we age.

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

Typical experiments

Most of our experiments will use genetically modified animals that allow us to follow the individual cells in which we are interested. This will facilitate the tracking of individual cells to study their behaviour in response to perturbations (such as aging, wounding or tumour formation). To address our research questions, animals will typically undergo one of three main types of experiments:

1-Aging: Animals will be administered various substances to label their cells, by injecting them into their abdomen or under their skin. This will help us track the behaviour of cells at different time points throughout the lifetime of an animal. Samples will be collected after humanely killing mice at different ages from birth and up to 24 months of age. The labelled cells will be analysed to investigate the impact of aging on cells.

2-Tumour formation: Animals will be treated with low doses of carcinogens (cancer-causing agents), typically via drinking water supplemented with sweeteners (such as Ribena) to alleviate any bitter taste. Animals may also be injected with various substances to label their cells (as above). Animals will then be allowed to grow old and tumours to form. Since we are interested in early tumour formation, animals are humanely killed before showing any signs of distress or suffering. Tumour samples will be collected at different times to capture the point at which tumours form. The labelled cells will be analysed to investigate how tumours emerge. Additionally, blood samples may be collected in order to identify markers of potential use to improve cancer detection in the clinic.

3-Wound healing: Animals will have a surgical procedure. Surgery will be completed under general anaesthesia so that animals remain in a state of sleep/unconsciousness throughout the procedure. During this procedure we will wound the mouse skin (up to two wounds of no more than 1 centimetre in size, separated by at least 1 centimetre). Wounds may or may not receive a graft (a piece of living tissue that is transplanted surgically). Grafted and non-grafted wounds will help us understand different regenerative/healing processes. A small number of animals may be treated with various substances to label the cells and track their regenerative response. Subsequently, samples will be collected after humanely killing mice at different ages from birth and up to 15 months of age. These experiments will shed light on how wound healing changes as animals age.

For any of these typical experiments we may sporadically treat a small number of animals with drugs that will help us discover the processes that regulate cellular behaviour.

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

Please note the adverse effects presented here are based on our previous experience using the proposed techniques. We anticipate that our work will have a minimal impact on the health and welfare of the animals, and will not lead to long-lasting pain, suffering, or distress. The genetically modified mice used are not expected to show deviations from normal health.

1. **Substances administered** during the course of this project are not expected to cause any harms that affect the animal's day-to-day life. In certain instances, treated animals may experience transient episodes of weight loss up to 15%, from which they usually recover after 2-7 days.

2. **Carcinogens** (cancer-causing agents) used in this project (such as DEN, derived from tobacco) have not been observed to affect the normal behaviour of treated animals. However, as animals bearing tumours age, they may sporadically present transient weight loss of up to 15%. In very rare instances, these mice become sick in which case they are humanely killed.

3. **Wounding/grafting surgeries** to the skin. These will be limited to wounds of up to 1 centimetre in diameter, in order to ensure that the injury does not cause distress to the animal. All surgeries are carried out under anaesthesia and animals receive pain relief medication that will remove any discomfort during the procedure and in the recovery period. Mice normally recover from surgeries within 10 - 15 minutes and show normal behaviour. Sporadically mice may display a small amount of swelling in the wounded/grafted area. This naturally disappears 7 days after the procedure. This swelling has not been observed to cause distress to the animals.

4. When **baby mice** (mice that have not been weaned) undergo a procedure (typical duration of 5-20 minutes), they are returned with their brothers and sisters to their mother immediately afterwards. The mother has always been observed to welcome her babies back.

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)?

This project will use **mice**. 38% of the protocols have been categorised as mild, and 62% of the protocols are classified as moderate, due to their nature (including carcinogen treatment and surgical protocols). However, from our previous experience, we foresee that the **actual severity** of our mice will exceed:

-Mild: 90% (approx.)

-Moderate: 10% (approx.)

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

- Killed
- Used in other projects
- Kept alive

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?

Animal experimentation is required to obtain relevant information on cellular behaviour within the living organism. In order to identify treatments of relevance to improve wound healing and reduce tumour growth, we need to understand the way in which different cell types interact with each other. The oesophagus and skin are complex organs formed by different layers and tissue compartments that contain different types of cells. In response to an injury or genetic alterations leading to cancer, all these cells need to communicate in order to elicit a coordinated response that results in the healing of the wound or the formation of a tumour, respectively. Unfortunately, this complex communication network is something that so far cannot be fully explored merely by growing cells outside the animal. Additionally, most techniques that work with cells outside of living animals do not fully reflect their normal behaviour over long periods. Hence, animal work is necessary to disentangle how different cells communicate and cooperate in health and disease; an aspect of critical importance to understand the basis of tissue regeneration and cancer.

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

In my laboratory, we have developed methods to grow tissues outside the animal. We can currently grow epithelial tissues/cells (e.g. from the skin/oesophagus), as well as underlying supporting tissues (called stromal cells). This approach represents an outstanding tool to replace the use of animals for short-term experiments studying wound healing and tumour formation. At the moment, all our projects make use of this technique, wherever possible.

Why were they not suitable?

Tissues are formed by many different types of cells. We are interested in understanding how those different types of cells (such as epithelial, mesenchymal, and immune cells) interact with each other and contribute to aging, wound healing and tumour formation over long periods of time. Unfortunately, to date, by growing cells/tissues outside the animals, we are not able to fully mirror the complex cellular interactions and long-term changes in cell behaviour that take place in the animal. Until then, experimentation with animals will still represent the gold standard to unveil these intricate processes.

Reduction

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

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

The predicted number of animals needed for this project has been based on the following:

1. Animal numbers have been worked out with the advice of expert **statisticians**. This ensures that our experiments are designed properly and that the results obtained have enough statistical power to draw

meaningful biological conclusions.

2. Animal numbers have been calculated based on the **animals used in my laboratory** in similar work over the past 5 years. I have also accounted for an increase in the number of members that form my laboratory, something anticipated for the next five years.

3. We have carefully considered the best way to make sure we keep the **lowest number of different types of mice (strains)** for breeding, while ensuring we have enough mice to use for experiments.

4. Test experiments, called **pilot experiments (**which use a smaller number of animals; typically 3) will be used to calculate the amount of a substance that we can safely inject into the animal. It is important to find the smallest amount of the substance that has an effect but that will not harm the animal in any way. This will then allow us to perform the intended experiment using only enough mice to be sure that the results we obtain are statistically significant.

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

We have taken advice received from a local, qualified expert in statistics. This will make sure that each experiment produces statistically meaningful data. Where possible, all experiments are designed to get the most information using the least number of animals possible. We will also take into consideration the NC3Rs guidance and experimental design assistant tool (https://www.nc3rs.org.uk/experimental-design-assistant-eda; https://nc3rs.org.uk/3rs-advice-project-licence-applicants-reduction). Additionally, all experiments will be designed taking into account the PREPARE guidelines (a document that gives scientists advice on how to plan animal research and experiments).

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

We implement different measures to reduce the number of animals used. Among them:

- Efficient breeding techniques: We will breed our genetically modified mice in a way to minimize waste. Any types of genetically modified mice (strains) that are not being used for scientific studies will be frozen as embryos for future use.

- Experimental design: Critical experiments are designed in collaboration with experts on statistical physics. With their assistance, we determine the number of experimental animals required to answer each of our scientific questions.

-Mouse strain management: animals are bred only to fulfil our experimental needs. However, any unavoidable excess of animals is used for in vitro experiments (culturing cells outside the animal), validation or pilot studies (as long as they are compatible). The latter represents a critical consideration in our laboratory, and allows us to ensure that mouse waste is minimised as much as possible. While this approach requires a significant amount of coordination and team effort, it is essential to reduce the number of animals we breed or purchase.

- Animal and tissue sharing: I have a network of collaborators within the UK who work in my or a similar research field. We exchange particular types of mice (strains) between us to reduce animal imports, the need for additional breeding and its associated excess. Similarly, to increase the output of our work, we share tissues with members of other research groups and collaborators who examine different areas of the body (tissues), such as the tongue and the cheek. Additionally, we are registered in the animal tissue sharing list of our institution.

- Growth of tissues outside the animal (3D tissue cultures): Using this method, one single oesophagus can produce sufficient cells to perform 3 experiments instead of one. Thereby, this system effectively reduces the number of animals used by two thirds. However, as indicated above, studying interactions between different types of cells remains a limitation when using this technique.

- Mathematical modelling: By working with our collaborators, we use our data to develop mathematical models that explain how cells behave and interact with each other. The created hypotheses are then tested in the laboratory. This makes our science more targeted, which allows us to significantly reduce the number of animals needed to understand how cells work.

- Advanced imaging and molecular methods: Dissected samples are analysed using state-of-the-art whole-tissue imaging and molecular techniques that require minimal tissue material to obtain meaningful results.

- Optimization of skin transplantation technique (grafting): We have implemented diverse adjustments in skin transplantation experiments, which increase the quality and statistical value of our data while substantially reducing the number of animals required.

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 genetically modified (GM) mice to carry out our plan of work. Most of our GM mice bear genetic alterations that allow us to label the cells allowing us to track their behaviour in the tissue. Hence, the genetic alterations we propose are not expected to lead to any adverse effects.

The animal procedures proposed in this study will inflict minimal pain, suffering or distress to mice:

- We **administer substances** to mice to label and track cells over time. These methods are based on previous studies and our long-term expertise using them. We use the lowest dose needed to observe an effect. Hence, we expect our treatments to cause no harm to animals.

- We also use drugs to understand how cells work and explore the earliest stages of **tumour formation** (carcinogens). Given our interest in early cancer, these treatments use low doses of these drugs. Our experiments have been optimized for over a decade, which allow most mice to live without complications for over a year. Carcinogen administration is typically performed non-invasively via drinking water.

- For **injury and tissue transplantation** (grafting) experiments. These methods have been significantly refined in my laboratory over the last five years. For skin wounding, we use instruments known as “punch” biopsies or biopsy forceps, which are specially designed to collect tissue specimens in the clinic. These instruments allow us to perform small skin wounds that favour rapid healing and diminish animal discomfort. Over the years, we have also optimised the size of the grafts, reducing it when compared to standard practice. We currently perform grafts of a maximum of one centimetre in diameter with optimal results. The most significant improvement of our refined method resides in the bandaging used to protect the wounded area. For this, we use transparent Tegaderm medical dressing, which constitutes a very thin transpirable barrier that protects the wound and keeps it aseptic, while allowing animals to move freely. This significantly reduces the stress experienced by the mice while recovering from surgery. When compared to classical bandaging, Tegaderm has the added advantage of allowing constant monitoring of the wounded area after surgery. Animals typically remove the Tegaderm by themselves on day 4-5 after surgery; by then, the exposed wound/graft allows for closer monitoring and mice are completely freed from any dressing.

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

In our studies, we investigate how different cell types function under normal circumstances, and how they change their behaviour in response to injury and early cancer. Given the differences observed in wound repair and cancer incidence as we age, it is important to investigate cell behaviour over long periods of time and at different stages throughout the life of animals (from birth to aging). Unfortunately, these long-term experiments require living organisms, limiting the use of other less sentient animals such as anaesthetised mice or tissues grown outside the animal.

Another critical aspect of our work focuses on investigating how different cell types interact and impact on each other's behaviour. For these studies to be clinically relevant, they need to be performed in mammalian animals, closer to humans. Results in lower species such as fish, worms or fruit flies, would be of very limited medical relevance.

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

All methods in this project will use techniques that reduce animal stress and make sure the animal does not suffer as detailed below:

- Surgery procedures:

- Surgery will be carried out in a clean manner (using aseptic technique). We will make sure to meet the level set out in the Home Office Minimum Standards for Aseptic Surgery and the LASA Guidance on Preparing for and Undertaking Aseptic Surgery (2017).

- We will ensure that animals suffer as little as possible during surgical procedures by giving the animals medication to manage their pain under the advice of the Named Veterinarian Surgeon.

- Animals will be monitored before and after surgery to ensure that any deviation from normal health is picked up. Observations will be monitored on a chart. Animals will receive additional pain relief medication as needed, and advice from the Named Veterinarian Surgeon will be sought if animals show any deviation from normal behaviour.

- Wounding of the skin is performed using tools called "punch biopsies", instead of scissors. This allows to produce clean skin biopsies, which favours rapid healing and diminishes animal discomfort. The size of the grafts has been optimised to increase grafting success. The refinement of this technique also allows us to reduce the number of animals used.

- Skin wounds will be on body sites excluding face and paws to minimise discomfort.

- We have refined the skin wounding technique to use minimal bandaging/dressing, in order to avoid any restriction of animal movement while protecting the implant until grafted.

- General:

- We will make sure to use the best care methods to improve the quality of life for the animals. Mice will be placed in cages and will be provided with nesting and bedding material to stimulate mouse activity and provide them with shelter if needed.

- Side effects from procedures will be monitored by regularly weighing animals, daily health checks, and the use of scoring sheets, as required. This will prevent animal suffering. Animals showing any signs of suffering will be immediately killed. This is called the Humane Endpoint. We are not expecting animals undergoing procedures under this licence to experience suffering. If an animal does begin to look unhealthy, we will monitor it more frequently and provide pain relief if needed. If it does not ameliorate the animal will be killed by a humane method.

- Some substances have to be given to animals by injecting them in specific places. We have therefore asked to be allowed to use different administration routes (e.g. intraperitoneal [injection into the abdomen], oral [via the mouth] and subcutaneous [injection under the skin]) in this project. We will always use the least harmful route possible to give an animal a substance. This is to make sure we cause the smallest amount of discomfort or pain to the animal.

- All animals that are brought into the animal facility will be allowed at least 7 days to get used to their surroundings. This process is called acclimatisation. We will also allow them to get used to the animal technicians prior to use. This will reduce the amount of stress the animal experiences and will improve their well-being.

- To improve the quality of life of our animals, we always house them in groups, unless strictly necessary due to experimental reasons. Animals have enrichment in their cages (such as wood sticks, bedding and nesting material) for extra comfort and enhanced mental and physical health.

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

- We follow the guiding principles on good practice for Animal Welfare. Our experiments are planned following the "Planning Research and Experimental Procedures on Animals: Recommendations for Excellence" (PREPARE) and "Animal Research: Reporting of In Vivo Experiments" (ARRIVE) guidelines. We attain to the LASA (Laboratory Animal Science Association) guidelines, as well as the NC3Rs published strategy for improving animal welfare (see publications details below).

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    1. -Prescott MJ, Lidster K (2017) Improving quality of science through better animal welfare: the NC3Rs strategy. Lab Animal 46(4):152-156. doi:10.1038/laban.1217

    2. - LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery. A report by the LASA Education, Training and Ethics section. (E Lilley and M. Berdoy eds.). http://www.lasa.co.uk/publications/

    3. - Smith D, Anderson D , Degryse A, Bol C, Criado A, Ferrara A, Franco NH, Gyertyan I, Orellana JM, Ostergaard G, Varga O, Voipio H (2018) Classification and reporting of severity experienced by animals used in scientific procedures: FELASA/ECLAM/ESLAV Working Group report. Lab Animal 51(1S): 5-57. doi: 10.1177/0023677217744587

    4. - Smith AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T (2018) PREPARE: guidelines for planning animal research and testing. Lab Animal 52(2): 135-141. doi: 10.1177/0023677217724823.

- For surgical procedures we follow the Laboratory Animal Science Association (LASA) Guidance on Preparing for and Undertaking Aseptic Surgery (2017) and the Home Office Minimum Standards of Aseptic Surgery.

- For the breeding of genetically altered mice, we will follow the guidelines provided by the Home Office and the NC3Rs Resources on 'Genetically altered mice' detailed in:

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    1. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/773553/GAA_Framework_Oct_18.pdf

    2. https://www.nc3rs.org.uk/GAmice

- Additionally, for cancer studies, we will refer to:

- Workman P, Aboagye EO, Balkwill F, Balmain A, Bruder G, Chaplin DJ, Double AJ, Everitt J, Farningham DAH, Glennie MJ, Kelland LR, Robinson V, Stratford IJ, Tozer GM, Watson S, Wedge SR, Eccles SA, Committee of the National Cancer Research Institute. Guidelines for the welfare and use of animals in cancer research (2010). Br J Cancer 102(11): 1555-1577. doi: 10.1038/sj.bjc.6605642.

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

-At our institution, we count on with the outstanding support of our Biofacility Service. They keep us informed (through their central team) about new developments on 3Rs and offer us expert advice on how to implement them in our ongoing studies (via our very experienced team of animal technicians, Named Animal Care & Welfare Officer (NACWO), named veterinarian surgeon (NVS) and Named Information Officer (NIO) all of them experts in animal experimentation).

-We follow the website of the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs, available at https://nc3rs.org.uk/resource-hubs). This allows us to stay up-to-date on the most relevant information.

-We also use our Institutional 3Rs search tool. This contains an up-to-date database with information on the best ways to reduce or replace animals in our experiments. It also contains advice on how to refine methods in order to reduce animal stress.

Additional guidance and information on the most appropriate and refined techniques for our studies may be obtained from external sources, including:

- Laboratory Animal Science Association (LASA)
- Institute of Animal Technology (IAT)
- Norecopa (https://norecopa.no/databases-guidelines)
- Relevant literature