Home Office

NON-TECHNICAL SUMMARY

Cancer and its microenvironment: From mechanisms to translation

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

Cancer, Tumour microenvironment, Cellular damage and senescence, Immune system, Ageing

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 central aim of this project is to investigate how damaged tissues are predisposed to progress to cancer. Evidence is accumulating that the tumour microenvironment (non-cancer cells around the tumour) is a crucial player in cancer initiation and progression, as it fuels tumour growth, and hence we are interested in the contribution of **(i) cellular types** (such as immune system and vascular cells; **(ii) cellular states** (such as a particular type damaged and dysfunctional cells that we know as “senescent” cells); **(iii) pathological process** (such as inflammation and fibrosis) and **(iv) ageing**, to cancer development. We will use this information to identify targetable biomarkers (altered biological molecules or factors) for the design, development and validation of novel detection and therapeutic tools.

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?

Cancer incidence rates rise significantly with age and has been attributed to the accumulation of mutations in particular cellular types, such as the cells of the surface of our organs as tissues, also known as epithelial cells, over time. Emerging evidence, however, suggests that the development of a damaged and inflammatory microenvironment also promotes the outgrowth of cells subjected to the activation of oncogenes (mutated genes that have the potential to cause cancer). Thus, it is likely that the combination of accumulated genetic alterations along with a tumour-promoting niche drives the development and outgrowth of early tumours. However, the environmental changes associated with early tumour development are incompletely understood.

To address the central aim of our project our laboratory largely focuses on preclinical models of lung cancer, while remains open to malignancies in other tissues to validate the universality of our findings. Lung cancer is the most common cause of cancer-related deaths worldwide. Although the efforts devoted to lung cancer research over the last decades have been formidable we have not yet deciphered the underlying mechanisms or processes promoting malignant transformation of some cells into cancer cells, and the specific microenvironmental conditions driving lung cancer initiation remain largely unknown. Consequently, the available tools to achieve lung cancer early diagnosis, as well as the existing therapeutic approaches, are not good enough to tackle this disease. Unfortunately, most of the cases are still diagnosed at advanced stages and ~85% of the patients die within a 5-year window from the first diagnosis.

In the proposed project, we will use a variety of mouse models to gain insight into how damaged and/or cancer-predisposed tissues influence tumour onset and cancer progression. More specifically, we will investigate the niche that fuels lung cancer onset and development, as well as its interplay with different tissue stressors and pathological conditions, including:

1. **The tumour microenvironment (TME).** E.g. immune system and vascular cells.

2. **Cellular states.** E.g. Damaged cells (senescent cells).

3. **Chronic and inflammatory conditions upon injury/damage**. E.g. lung fibrosis

4. **Ageing.**

We will address how these processes and cellular states collectively, or independently, relate to cancer initiation and progression. This information will allow us to gain insight into the fundamental processes and drivers leading to cancer development, and also to identify more specific targetable signalling pathways and altered molecules (biomarkers). Ultimately, we will use this information to design and validate novel tools for cancer early detection and more efficient therapeutic interventions. These may include new tracers and detection probes, and pharmacologically active compounds consisting in small molecules/inhibitors, activatable drugs (known as prodrugs), and nanotechnologies for cancer-targeted and tumour microenvironment-targeted therapies. This is therefore a high return translational project with commercialisation potential and we envisage that precision-targeted medicine is key to improving outcomes for patients with cancer.

Our ultimate goal following successful completion of this project is to immediately move and prioritise our most promising innovative therapies into early-phase clinical trials to establish their safety and clinical efficacy.

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

Cancer is a common age-related disorder. Its connection with ageing stands from the fact that we are continuously submitted to constant and multiples sources of damage that, when accumulated or persistent in our tissues/organs, cannot be repaired. This damage results in mutations that can eventually be oncogenic (affecting genes that have the potential to cause cancer when become active). However, increasing evidence suggests that although cancer-promoting mutations are required for tumour development they also need of a damaged, aged, and/or inflammatory “microenvironment” to initiate cancer. This environment is composed of multiple cellular types (e.g. immune system cells, vascular cells, structural cells, etc.) and connective tissue, which is like the concrete that glue the bricks (the cells) to make a wall (our tissues or organs). Importantly, this tumour-promoting microenvironment is currently incompletely and, in most of the cases, poorly understood. This knowledge is essential to develop more efficient and refined therapeutic strategies against cancer by targeting simultaneously (i) cancer cells and, also, (ii) the adjacent tissue to the tumour (tumour microenvironment or TME), but such an effective pharmacological combination therapies for precision medicine still remains a challenge.

Therefore, we expect to deliver our outputs at two levels:

1. **Fundamental Cancer Biology Research:**

a. Identification of crucial cellular types of the TME (adjacent tissue to the tumour) contributing to cancer initiation and progression. Analysis of the underlying molecular mechanisms.

b. Identification of key cellular states and processes contributing to cancer initiation and progression. Analysis of the underlying mechanistic insights.

c. Analysis of cancer-predisposed tissues upon injury/damage or inflammation. Analysis of the underlying processes and molecular mechanisms.

d. Dissection of the contribution and impact of ageing to cancer initiation and progression.

2. Preclinical Validation of Novel Detection and Therapeutic Strategies:

a. Development and validation of tracers and detection probes.

b. Development and validation of inhibitors and small molecules with therapeutic activity.

c. Development and validation of activatable drugs (prodrugs).

d. Development and validation of nantotechnologies and modular systems.

Our main outputs will be in form of scientific publications. We expect to gain insights into how damaged and/or aged tissues contribute to cancer initiation and progression, which is crucial for the development and validation of novel detection and therapeutic tools to tackle cancer. The principal route for dissemination will be through scientific literature and published on Open Access. Communications at international conferences [e.g. AACR and EACR International Conference, or International Cell Senescence Association (ICSA) Conference], as well as external and internal seminars series, will enable us to communicate our findings in real time with other scientists. Publication of outcomes will be accompanied by press releases, which additionally has a wide network of interactions with national and international journals. We will work proactively with the media to ensure that significant research findings reach the lay public. Although some discoveries may be subject to intellectual property protection and commercialisation we will prioritise public data sharing in free repositories to facilitate advances by other researchers.

In parallel to the publications and spread of our research, we also envisage the delivery of a translational toolkit consisting in novel detection probes and therapeutic tools for targeting the tumour microenvironment and cancer precision medicine. For the validation and delivery of this collection of novel therapeutics and detection tools for the management of cancer it is essential their validation in in vivo models that recapitulate, as much as possible, the human pathological conditions. To ensure relevance (**1.** Fundamental cancer biology research) and translatability (**2.** Preclinical validation of novel detection and therapeutic strategies) of our findings we will use a variety of mouse models of lung cancer allowing us to manipulate the tumour microenvironment. These include models of transplantation of lung cancer cells (either under the skin or in the lungs) and inducible models of lung cancer (so-called genetically-engineered mouse models). We expect to complete the preclinical validation of our translational toolkit of therapeutics by the end of the 5-year project. This is therefore a high return translational project that, in the long-term, aims at prioritising our most efficient detection and therapeutic tools validated in mice to early phase clinical trials with patients.

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

The potential beneficiaries of this project are multiple reflecting the fact that it aims to deliver practical knowledge at three fundamental levels:

1. Molecular level: (i) molecular biologists and **(ii)** oncologist consultants, which will have at hand new tools and datasets to detect alterations during cancer initiation resulting from the characterisation of molecular profiles of cancer precursor cells and tumour microenvironment cells; **(iii)** drug discovery programmes, after the identification of new drugable targets at the origin and progression of lung cancer.

2. Cellular level: Our progress on better understanding the role and impact of cellular types and states operating in the tumour microenvironment will be useful for cell biologists (including immunologists) interested in cancer initiation and development, inflammation, cell damage (senescence) and plasticity, cancer precursor cells and cellular reprogramming. Satisfactory outputs may result in the identification of key (targetable) cellular populations, including cells of the TME (adjacent tissue to the tumour), precancerous cells, and cancer cells.

3. Pathological level: On the pathological front, a better understanding of (lung) cancer development and how it relates to damaged lungs or pathological conditions (e.g. fibrosis) is crucial to develop novel tools for early detection and the design of more efficient therapeutic interventions. Satisfactory results attained within this proposal may therefore provide us with the grounds for the diversification of our own research towards the development of pharmacological interventions and new strategies to target lung premalignant and cancer lesions, which in turn could eventually be of interest for translational researchers, engineers and trialists working in imaging, biomarkers and therapeutic approaches. In addition to cancer, damaged cells (senescent cells) are also associated with multiple chronic or aged-related pathologies making our proposal thus of interest to a broader research community operating in basic and translational projects both in the UK and overseas. Ultimately, if successful, patients and the NHS would be most notable beneficiaries of our research.

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

We will maximize the outputs by a variety of action points:

1. During the last five years I have managed and been the holder of a **Home Office PPL licence**. The accumulated experience is giving me the opportunity to: **(i)** develop a preclinical programme including animal experimentation, **(ii)** acquire and consolidate the required personal and laboratory skills, and **(iii)** deliver significant research outputs in terms of scientific publications and presentations in national and international conferences. Altogether, I will manage this new project supported by additional skills and with a more consolidated laboratory team with the appropriate expertise and training in mouse research, thereby helping us to increase the chances of achieving the aims and goals hereby stated.

2. After the transfer and rederivation of our mouse colonies to our new animal facility, we count now with additional state-of-the-art facilities for procedures and imaging, as well as the continuous support of a strong team of experienced staff. These resources and specialised staff will ensure the practical, technical, and ethical feasibility of our experimental tasks.

3. Our research is truly multidisciplinary, as it encompasses from cancer fundamental biology to translational applications. I have therefore forged a potent network of collaborators, including biologists, chemists, physicists, engineers and clinicians. They all contribute in a very positive manner to provide our projects with a translational orientation and ensure their progression to the highest standards. Remarkably, our network of collaborators allowed us to identify other groups interested in our animal tissues to address their own experimental question and the support of their projects, thereby making them available for other scientists (e.g. groups focused on the study of cellular damage in multiple organs or other cancer stages, such as the invasion of other tissues in advanced cancer).

4. Our results will be submitted (and eventually published) to international open access scientific journals, as requested by the University of Cambridge. These will include public or free online repositories. Our research articles largely contain positive results to support our hypotheses and central aims but also experimental results that fail to support our hypotheses or negative data, whenever possible. This also prevents duplication by ensuring that other laboratories do not spend resources, efforts and funding in ideas that have already been tested.

5. Our experimental activities will imply the generation and analysis of large-scale datasets. To maximize the outputs obtained from these complex datasets (e.g. genetic profiles), we collaborate and are supported by Core Units with experts in high-throughput analyses (genomic profiles, protein profiles, etc.), mathematical modelling, statisticians, 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 (e.g. gene expresion profiles) will be publicly available as part of the relevant research articles enabling new collaborative projects.

6. We will spread our observations from early stages, for example via national and international conferences, symposia, and in internal and external events. It will allow us to create new collaborations with experts in research fields beyond our own scope, and also to increase the training and communication skills and network of our postdocs and students.

7. We will share our science with lay members via public engagement activities. This will allow people to learn about novel aspects of cancer, ageing and new therapies, and will provide the opportunity for a productive exchange of ideas between the public, patients and scientists.

Species and numbers of animals expected to be used

- Mice: 12195

Predicted harms

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

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

Mice are well used in cancer research and there are many genetic models that allow us to understand cancer, which is not feasible in other models. As mammalians, they are much closer evolutionarily to

human disease compared to non-mammalian model organisms, making the protocols able to better recapitulate human disease, and the results gained more translatable into early-phase clinical trials that could improve patient outcome. Mouse tissue structure are much more comparable to humans than in other model systems. Zebrafish for example do not have lungs and so studying lung tumours and their microenvironment are more accurate and reliable in mouse models. Cancer, particularly the types that we focus on, is a disease of adulthood and old age, which is why we need to use some adult and aged mice in our research to understand these important interactions that affect human cancer incidence, progression and responsiveness to treatment.

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

We use various methods of inducing cancer in our mice for our research. Most of our experiments will use either genetically modified animals to induce models of cancer in a highly optimised and refined manner.

To address our research questions, animals will typically undergo one (or a combination) of three main types of experiments:

1. Tumour initiation: Animals can have tumour-promoting genes activated in the tissues of interest at the time of interest, through exposure to modified viruses that activate inducible genetic alterations in the mice (e.g. activation of oncogenes, which are genes that cause cancer). Reagents altering cancer genes can be provided via the diet and supplemented with sweeteners to improve feeding behaviour. Alternatively, tumours can be formed through injection of carcinogens (cancer-causing compounds), or DNA containing tumour-promoting genes. In addition, tumour cells may be manipulated in a plate and then be engrafted into the mice, either subcutaneously (under the skin) or transplanted in the tissue where they usually grow (e.g. lungs). These mice are then monitored over time for tumour induction and growth through labelling and non-invasive imaging techniques (e.g. X-ray scans). Mice can be exposed to therapeutic compounds with translational relevance to change the rate of tumour growth and the components of the microenvironment (adjacent tissue to the tumour). Samples are collected at different times to capture how tissue transforms into a pro-tumour state and then how tumours form and grow, further modifying the tissue environment. Blood samples may be collected to identify potential markers that allow earlier and more reliable detection of tumours, potentially improving cancer detection in humans.

2. Tissue damage: Animals are exposed to tissue injuries and damage from a variety of environmental insults (e.g. chemical exposure, irradiation, infections, etc.). This is because we wish to understand how damage alters the tissue microenvironment, which can in turn promote inflammatory processes and tumour initiation or growth. Where possible, this will be carried out specifically on the target tissue (e.g. by providing a chemical or damaging agent directly into the lungs). Cells can be labelled to track changes in the tissue microenvironment, such as the immune response and healing. Subsequently, tissue samples will be collected following the humane killing of the mice at different stages of the damage protocol. A portion of mice may also undergo tumour initiation after tissue damage (methods stated above), thereby allowing us to better understand how damage can contribute to cancer.

3. Ageing: Animals will be occasionally aged before the tumour initiation stages mentioned above. This is because cancer is a prevalent disease in the adulthood and old age in humans (e.g. lung

cancer, which is a marginal disease in children or young individuals). Therefore, we find important to understand what are the crucial changes in aged tissues that can create a good niche or microenvironment to promote cancer initiation and progression. E.g. changes in the cellular types present such as immune system cells or the accumulation of damaged cells (senescent cells). After a certain age (e.g. 12 months, considering than mice can live for more than 2 years), mice will be monitored closely and scored to ensure that adverse effects are limited and well controlled. At different stages, mice may undergo tissue damage protocols or tumour initiation protocols and then be monitored over time (methods stated above). Since we are predominantly interested in early stages of cancer, the majority of animals will be humanely killed before showing any signs of suffering or distress. Samples will be collected to investigate how the tumour microenvironment changes with age and can lead to tumour initiation.

For any of these typical experiments, we may regularly treat some animals with drugs that help us understand the processes that govern tumour initiation and progression. This will also allow us to validate novel detection and therapeutic tools and modalities.

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

Most of the mice in our protocols will experience tumour growth or moderate tissue damage, over the course of days to weeks. Typically, we are interested in the early stages of cancer, and the tissue microenvironment that leads to and promotes cancer growth, and so the majority of mice are humanely killed before they develop adverse effects from tumour growth. Further, due to our ability to non-invasively image tumours, allowing us to following the tumour, most cases of clinical signs are avoided. Adverse effects of longer-term tumour growth include weight loss, shortness of breath, changes in the colour of the skin, accumulation of fluids in the abdominal cavity, or diarrhoea. Mice are checked twice a day, and when a mouse presents with any of these symptoms, they will be culled asap (15% weight loss is typically the limit). As the number of mice that develop a tumour burden that leads to clinical signs are low, the majority of adverse effects for the animals in the project will come from the cancer initiation techniques themselves (e.g. delivery of cancer-initiating compounds, or surgery).

Animals may be aged in our protocols, such that they reach a maximum of 30 months of age. Potential clinical signs of ageing and frailty that impair normal mouse behaviour, movement or feeding include the skin (e.g. dermatitis or alopecia), physical/musculoskeletal (e.g. tumour growth or lameness), digestive/urogenital (rectal/vaginal prolapse or malocclusions, meaning teeth too large for the mouth), respiratory (e.g. altered breathing rate) and discomfort (e.g. pain). We have developed End of Life criteria and a Score System for ageing colonies based on previous studies (Ullman-Cullere, Lab Anim Sci. 1999 Jun;49(3):319-23). Green scoring usually requires no actions, whereas Amber signs will be assessed by specialised animal facility staff (e.g veterinary surgeon). If treatment can be provided to alleviate these signs, or if the signs are providing minimal suffering or distress, then they may be allowed to be monitored and culled only if signs worsen. Mice presenting with red signs will be killed via a schedule 1 method.

The adverse effects presented here are based on our previous experience using these methods and genetically-altered animals. Our technicians are extensively trained on the techniques before carrying them out on live animals and understand monitoring procedures to ensure the welfare of the animals. Generally, the genetic modifications are not expected to show adverse effects themselves.

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:

Sub-threshold 5%.

Mild 64%.

Moderate 30%.

Severe 1%.

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

- Killed
- 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?

Cancer initiation and progression is a complex multi-step phenomenon that is modulated by different causal stimuli (damage, activation of cancer-initiating genes, inflammation, etc.), the intrinsic properties of malignant precursor cells, and the surrounding tissue. Tissue microenvironment may involve numerous cellular types and states (e.g. immune cells, senescent cells, pluripotent cells, etc.). Such triggers, mechanisms, processes, and interactions, that lie at the origin of cancer cannot be modelled accurately in vitro (cell cultures) at present. While we are studying in vitro the interplay between cellular damage, ageing and cancer we need to extrapolate and to confirm our results in an in vivo model, thereby reproducing more realistically the microenvironment and complexity of events that contribute to cancer in humans.

Rodents have already been widely used in cancer research and provide unique opportunities to dissect out the precise role of cellular damage and ageing in cancer and to implement the use of novel diagnostic and therapeutic tools. Importantly, mouse cancer models (such as oncogene-driven genetic models and chemically- induced models) have been shown to recapitulate accurately the human disease so they constitute an ideal platform to develop our aims and to reach the proposed goals.

A key component of this project is to improve the diagnosis and treatment of human cancers; therefore, it is crucial to test and validate novel tools in preclinical models that involve animals. This is an essential step before moving to future early-phase clinical trials.

While we cannot fully recapitulate human cancer outside of animal models, we are continually attempting to, at least partially, replace animal models as technologies develop. Experiments will be only performed in vivo with solid functional or correlative data from in vitro systems (cell cultures with cancer cells) or clinical samples.

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

We have considered the following alternatives for replacing animals in cancer research:

1. Cells grown in vitro (cell cultures in plates, also known as petri dishes), including co-cultures (mixes) of cell types.

2. Cells grown as organoids (three-dimensional cultures of cells), these include tissue slices and in vitro growth of cells using commercially available matrices (scaffolds for the cells, thereby mimicking the tissue architecture), and bioprinting.

3. Using clinical samples (including biopsies) from cancer patients.

Why were they not suitable?

(1) Cells can only grow well in petri dishes if they become immortalised, this causes genetic changes that cause them to not act as they would in the environment of the body. Important components of the tumour microenvironment such as damaged cells (senescent cells), immune cells, vasculature, pluripotent cells do not grow as readily in a dish due to space limits in one dimension. As well as these growth limitations, cell types can often no longer act the way they would in an organism because of the absence of the normal tissue microenvironment (adjacent cells or cellular “ecosystem”). Fibroblasts (structural cells) are a reliable cell type for growing in a culture dish, but this is a minuscule snapshot of the myriad of cell types we need to study. Focusing on these cell types, though useful, is limiting and does not provide the full information required to develop understanding of how cancers grow in an organism and manipulate their environment.

Co-culturing is useful as this can allow us to study the interactions between two cell types grown together, but this still does not consider the cross talk that occurs between multiple cell types, both proximal (close) and distal (far) from the cells of interest, and so will not recapitulate these vital components with co-culture alone.

(2) Organoids are useful as they are a step closer to modelling a living organism, they can more accurately show a more 3D picture - for example with vasculature and immune cells interweaving between tumour cells. But organoids are frequently challenging to be developed for some tissues or tumour stages (e.g. in particular for the lung) and they also cannot currently fully recapitulate the complexity of whole living organisms (e.g. lack of high-fidelity of cellular types, limited maturation, atypical physiology and structure, or lack of compartmentalisation). We are following this research field closely and will replace animal research where we can as new technologies develop and are tested and established.

(3) Using clinical samples from human cancer patient’s ex vivo (out of the body) is again of significant use and will prove supportive to this project. The reason they cannot fully replace animal

models is that both fixed and fresh samples provide only a snapshot of the disease. This is of particular significance for studying early cancers, as there are very few early-disease samples available (they are difficult to get by biopsies as they are usually small and when available they commonly have to be use for diagnostic purposes rather than research). Also, the cancers we are focused on are often diagnosed until much later when symptoms present (e.g. lung cancer), this is when the samples would usually be obtained.

While these approaches are not suitable to fully replace animal models yet, we are using these methods to reduce the numbers of animals used to the minimum required.

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?

This is the second project license being held by the lab. We estimate the number of animals based on our usage during the tenure of the previous license. Also, due to our new aims and objectives of this project, some protocols may be used more or less than in the previous license due to changes of focus, this is considered too. We have experienced statisticians with whom we consult to determine our experimental cohort sizes and ensure our results obtained have enough statistical power to draw meaningful biological conclusions. Most of our estimated usage (~60%) comes from the breeding of genetically altered mice. Our breeding strategies are deemed optimal, but we keep our approaches under review.

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

To the extent possible, we will use in vitro cellular models and human tissue samples to limit the number of animals required for in vivo studies.

To reduce the numbers of animals in our experimental **protocols (5-11)**, we take the following steps:

- Carry out pilot studies (4-6 mice per group) where to optimise the experimental design through identification of uncontrolled variables. This information can be discussed with local statisticians to help us appropriately power our experiments and use the correct number of animals to draw statistical conclusions.
- The experimental approaches described have been used extensively within our Department and Animal Facility, so we have the necessary expertise to develop the proposed research effectively.

- If necessary, we will consult statisticians based in our Institution. Additionally, we use online tools such as the NC3R's Experimental Design Assistant (EDA).
- Where there is published experimental data, we will use that to estimate the numbers of animals required for our procedures.
- Power calculations are used to determine sample sizes. For most of the quantitative experiments, power analyses will be generally set by using a significance level of 5%, a power of 80%, and a minimum practicable difference between groups of 20-25%.
- We will pay special attention to controlling sources of variability related to the environment, animals, animal handlers and the experimental procedures. Examples include using randomised experimental designs, assigning animals at random to a treatment.
- Blinded operators will perform procedures and subsequent data analyses where possible to avoid biases.
- Where possible, we split tissue for different experimental outputs (e.g. using different lobes of the lung). This reduces the number of animals used to answer these hypotheses.
- Subcutaneous injections of cancer cells are carried out on each flank of the mouse, halving the number of mice required to carry tumours.
- We use a Computerised Tomography (CT) X-ray scanner for non-invasive imaging and monitoring of internal tumours in the same animals over time, reducing the number of animals used.
- In vivo imaging system (IVIS) allows an alternative non-invasive imaging of labelled-tumour growth over time in the same animal.

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

- Cryopreservation is used to preserve important mouse strains when they are not required, reducing the holding of live animals for extended periods.
- Genetically altered animals are maintained with homozygous genetic alterations (two identical modifications of a particular gene) to reduce the number of offspring mice having unwanted genotypes. We only have one genetic allele (KRas mutated, a well-known cancer-initiating oncogene) where this is not possible due to embryonic lethality.
- We are utilising methods for producing genetic alterations without the need for breeding genetically altered (GA) animals at all. This includes viral administration of DNA & injections of chemicals.
- Offspring with unwanted genotypes can be used for in vitro experiments (using tissue outside of the animal), training, validation and pilot studies where possible.

- We share our animal lines with other researchers and will place in international repositories, where appropriate, in order to reduce the number of animals used globally to derive these lines. We are part of local email lists where we can share mice locally that are unused. Further, we share tissues with other researchers to reduce wastage.

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 for this project, including genetically modified mice. Our fundamental research is focused on the mechanisms and processes that lie at the origin of cancer and, consequently, tumours will not usually reach advanced malignant stages, so most of the protocols are categorised as ‘moderate’. Most genetic alterations in our mice are inducible, meaning they do not lead to adverse effects until they are activated in our experimental protocols. As most of our expected animal usage comes from breeding genetically altered animals (~60%) developing tumours in an inducible manner rather than constitutive (chronic) manner, then this reduces suffering for many mice as they live healthy most of their lives. We have refined our approaches based on our previous experience, the experience of our colleagues and collaborators, as well as published data on these models. We can effectively monitor tumour growth and have well established humane end points that result in the least suffering both in terms of intensity and time spent with adverse effects.

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

Mice are evolutionary the most useful form of vertebrates that can be used to study cancer biology and they are the mammals in which the gene manipulation technology (transgene technology) is more developed and works and works in a more reliable manner. Mice are widely considered as the model organism of choice for studying human diseases (such as cancer and other chronic disorders), with whom they share 99% of their genes (Rosenthal and Brown, 2007). Less sentient animals can develop cancer, but there are questions as to how well these organisms recapitulate human disease. The proposed genetically.altered and chemically-induced mouse models recapitulate accurately human (lung) cancer and the effects of the manipulation (or mutation) of tumour promoting proteins (e.g. Ras) and tumour suppression proteins (e.g. p53, p16/p19ARF or p21) (for review see Hynds et al., 2022; Wang et al., 2022). Processes such as cellular damage (senescence), the immune system and the tumour microenvironment (adjacent tissue to the tumour) have been extensively studied in mice (for review see Gonzalez et al., 2018; Fane et al., 2020; Di Micco et al. Nat Rev Mol Cell Biol 2021), and can differ substantially in organisms other than mammals and, especially, in invertebrates. The types of cancer that we are focusing on are mainly diseases of adulthood and old age, therefore it is essential that we use adult stage and aged animals. The important interactions of the microenvironment with cancer development, such as ageing, is important in human disease but poorly understood.

Consequently, conventional wild type, genetically-altered and immunodeficient mouse models will be used.

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

From our experience we have made multiple refinements, amending our previous license to minimise welfare costs to the animals. Some examples are listed below.

General refinements

- 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.
- Humane endpoints are well described and are continually updated according to our experience and guidelines to promote animal welfare.
- Home cages are enriched with tubes, bedding, chew sticks and is monitored according to the animal's wellbeing. For example, to mitigate for possible aggressive encounters between mice, we include extra enrichment, such as tubes, in the home cage.
- Non-aversive handling techniques are utilised wherever possible, such as cupping and tunnelling.
- When mice are ordered from an external supplier, we allow at least 7-14 days for the animals to habituate into their new environment.
- Our animals are mostly genotyped by TransnetYX providing quicker and more reliable results to avoid unwanted phenotypes and genetic backgrounds. The TransnetYX miniMUGA genetic report facilitates fewer generational mouse crosses to be required.
- We always try to house animals in groups, unless strictly necessary due to experimental reasons, in order to improve their quality of life.
- Claws are clipped where necessary to avoid excessive scratching.
- Welfare scoring has been set up for mice with subcutaneous tumours (see **Protocol 7**), and we have develop scoring systems for animals over 15 months of age to minimise potential suffering and distress, which we have been established using End of Life criteria for ageing colonies: Based on Ullman-Cullere, Lab Anim Sci. 1999 Jun;49(3):319-23. Amber signs in such scoring systems may be assessed on a per mouse basis together with the Named Animal Care & Welfare Officer (NACWO) and/or Named Veterinary Surgeon (NVS). If treatment can be provided to alleviate these signs, or if the signs are providing minimal suffering or distress, then they may

be allowed to be monitored and culled only if signs worsen. When using the above or a similar score system mice presenting with red signs will be killed via a Schedule 1 method.

Experimental refinements

- Most of our protocols use well established and extensively described methods, minimising uncertainty of how regulating agents and compounds affect animal wellbeing. Users are extensively trained in these protocols before carrying out techniques on live animals.
- The choice of the application route of the regulating agent will generally be the one that causes the minimum stress and discomfort on the mice. For example, using the intratracheal route to activate cancer genes means that these genetic alterations are only in the target tissue (in this case in the lungs).
- In some experiments diet composition may be altered. We will use commercially available high-fat and low-fat diets, avoiding food restriction when possible.
- The use of appropriate anaesthetics and analgesia, in the context of surgical procedures, are used according to best practice guidelines.
- The majority of our genetically modified animal models are activatable and so are not affecting animal welfare outside of essential experimental contexts. For instance, to regulate the expression of a lung cancer initiating oncogene (KRasG12V) we will provide the inducer (viral recombinase or AdenoCRE) by intratracheal administration, which will permit cancer onset in the tissue of interest (the lung).
- Providing Nesquick (sweet substances) with Tamoxifen (a gene modulator) pellets has improved the feeding behaviour and limited weight loss from maladaptive eating.
- Due to previous experience, cancer cell transplantation in the target tissue (orthotopic cell grafting) is now only carried out on 7 weeks old mice or older, weighing at least 18 g, which has reduced adverse events from rapid tumour development such as weight loss.
- To preserve the structure of the lung, mice are sometimes culled by perfusion fixation under terminal anaesthesia. We have found that, in some cases, animals can instead be culled by Schedule 1 before re-inflating the lung post-mortem to preserve the structure for histological analysis.
- Transplantation of lung cancer cells via the intratracheal route, rather than through tail vein injection, facilitates engraftment (take root) in the target tissue (lungs) and limits their spread in off-target areas.
- Non-invasive imaging techniques such as Computerised Tomography (CT) based on 3D X-rays have been developed to better assess internal tumour growth over time, such that we know when to increase monitoring of animals and minimise adverse events.
- We have refined our approach to bleomycin exposure (protocols 6, 7, 8, 9) by providing palatable food (e.g. peanut butter) and hydrogel before and throughout the whole expected weight loss and dehydration period.

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

The scientific literature includes journals dedicated to the publication of protocols, which often represent best practice and include tips for refinements to techniques. We also are in close communication with other labs that perform the same techniques as us, including discussing any refinements they have made and/or are currently making.

Unless otherwise specified, the work in this project will be undertaken in accordance with the principles set out with in the following guidelines and published research:

- Guidelines for the Welfare and Use of Animals in Cancer Research: British Journal of Cancer (2010) 102:1555-1577

- 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.

- 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

- ARRIVE Guidelines: https://arriveguidelines.org/

- LASA Guidelines. Principles for Preparing for and Undertaking Aseptic Surgery (2010): https://www.lasa.co.uk/current_publications/

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

The UK has a national centre for the 3Rs (NC3Rs). They produce newsletters that we are subscribed to and organise seminars that we attend. Our technicians routinely meet with managers of the animal housing facility, including Named Animal Care & Welfare Officer (NACWO) and the Name Veterinary Surgeon (NVS), where they update on the NC3Rs guidelines and constantly review breeding strategies such that we are ensuring our approach is the most refined it can be. We receive regular updates via email and have our own 3Rs search tool with a multitude of resources for implementing and being updated on the 3Rs (https://www.ubs.admin.cam.ac.uk/3rs/3rs-search-tool). We also discuss with other groups doing similar research and have an open policy whereby new advances are disseminated with one another.