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NON-TECHNICAL SUMMARY

Generation and validation of mouse and avian models for the discovery of innovative antibodies with therapeutic potential

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

therapy, "next-generation" antibodies, cancer, mouse and avian models, bispecific antibodies

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult
Domestic fowl (Gallus gallus domesticus)	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult
Japanese quail (Coturnix japonica)	Embryo and egg, Neonate, Juvenile, Adult, Pregnant adult

Retrospective assessment

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> 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 generate and validate transgenic mouse and avian models capable of producing innovative formats of human antibodies that can potentially be used as therapeutic drugs.

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?

Over the past 30 years antibodies proved to be an extremely valuable therapeutic tool for the treatment of a variety of diseases, including many forms of cancer. Antibodies work as therapeutic agents by acting like smart "seekers" that find and attach to harmful targets in the body, such as viruses, cancer cells, or bacteria. Once attached, they either block the harmful activity directly or signal the immune system to attack and remove the threat, helping the body heal and protect itself more effectively. Most of the antibodies that have been approved so far for use in the clinic, were isolated from mice that are capable of producing human-like antibodies (a humanised mouse model). Despite the clinical success of therapeutic antibodies, there are still many pathologies that don't have an effective treatment and require the development of innovative type of drugs. "Conventional" antibodies (like the ones produced by healthy individuals) have limitations that restrict their use in some of the more advanced experimental therapies. For example, most drugs based on conventional antibodies struggle to reach targets located in the human brain because of their large size. Ongoing research has identified new, smaller antibodies that target diseased cells more effectively and penetrate better into specific types of human tissues. This is just an example of a growing family of "next-generation" antibodies that have the potential of revolutionizing the treatment of multiple diseases.

In this project we aim at generating and validating new mouse models that can produce "next-generation" antibodies. As we have done in the past with mouse models capable of producing fully human antibodies, our goal is to make them available to researchers around the world and expedite the development of much needed drugs. This would have a tremendous impact on the life of many patients that are waiting for new treatments to ameliorate or cure their conditions.

We also plan to complement our mouse models with equivalent avian platforms capable of producing fully human therapeutic antibodies. Avian models, such as chickens and quails, offer a significant advantage due to their uniquely evolved immune system, which generates antibody diversity through a molecular mechanism entirely different from that of mammals (e.g., mice and humans).

Our approach aims to leverage the avian immune system to identify antibodies with binding properties that may not be generated in mice or humans. Additionally, birds serve as an excellent model for developing antibodies against targets with high homology between mice and humans. Many disease-related proteins are highly conserved across these species, making it difficult for mice to generate effective antibodies against them. However, because birds are more evolutionarily distant from mammals, they can efficiently recognize these conserved targets, producing high-quality, high-affinity antibodies suitable for therapeutic applications.

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

At the end of this project, the primary outcome will be the development and validation of multiple mouse models that have the capacity to produce human antibodies with special properties that makes them different from the "normal" or "canonical" antibodies produced by healthy individuals.

Such mouse models will be used to discover new drugs for a variety of conditions like cancer, autoimmune diseases (a disease where the immune system of an individual attacks and damages its own cells) and infectious diseases (a disease caused by different types of microorganisms like bacteria and viruses). These new mouse models will also allow to learn more about the biology of this new class of "next-generation" antibodies.

An example, is the development of the so called "common light chain" mice. This type of mouse will be engineered to produce human antibodies that are particularly suited to be assembled into a new class of therapeutic antibodies called "bi-specifics". As the name suggests, bi-specific antibodies can recognize two different targets at the same time compared to a "canonical" antibody that only recognises and binds a single target at the time. Antibodies engineered to recognize two targets at the same time have already showed very promising results for the treatment of multiple diseases, in particular cancer. Another goal of this project is the development of mice able to produce a novel class of antibodies called “VHH” antibodies or “nanobodies”. This novel class of antibodies is gaining a lot of attention among scientists and clinicians because their properties are particularly promising for targeting diseases that currently have limited therapeutic options.

Unfortunately, producing this type of innovative drugs is not straightforward due to the lack of efficient discovery platforms, but also due to the limited understanding of their biology. For this reason, fully validated and easily accessible discovery platforms for this type of innovative antibodies would be of crucial importance to support the rapid development of new drugs.

The mouse models generated in this project will hopefully become a very valuable platform for the early discovery and early development of new therapeutic antibodies however they won't be used to produce the final product. The final therapeutic antibodies initially discovered through these mouse platforms will be produced using exclusively non-animal based technologies like for example bioreactors.

A second outcome of this project is the generation and validation of avian models (chickens and quails) capable of producing different types of human antibodies. Avian models are very valuable tools for the discovery of therapeutic antibodies for two main reasons:

1) Birds have a unique immune system that generate antibodies through a mechanism that is completely different from mice (and humans). This allows to generate a broader range of antibodies and target specific portions of the targeted proteins that are not normally recognized by antibodies isolate from mice.

2) Humans are phylogenetically more distant from birds than mice meaning that on the evolutionary tree, they diverged from a common ancestor a long time ago and have many genetic and biological differences. This can be a great advantage because their immune system sees human proteins as more foreign (immunogenic) compared to mice allowing to identify antibodies against targets that are very similar (high homology) between mouse and human. There are several disease-related proteins that are almost identical between mouse and human and it's almost impossible to produce antibodies against this type of targets in mice because they are seen as "self" from the mouse's immune system. In such cases, using an avian platform can be a highly valuable alternative.

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

In the short term, mice and avian models generated in this study will allow us to learn more about the general biology of innovative classes of antibodies. This will be accomplished by our team during the validation process, but also by multiple research teams around the world with which we plan to share our mice. We also collaborate with academic groups that are involved in immunology and drug-discovery research and will benefit from the use of these new models for their studies. Whenever possible, the new findings will be published on scientific journals and the data shared with the wider scientific community. This will help disseminate data and additional information on these new types of antibodies and reduce the need of generating additional mouse models in the future (reduction).

On the medium and longer term, the goal is to create tools to allow the discovery of new, more efficient drugs, that can help improve patient's life. Mouse platforms for the discovery of innovative antibodies can significantly benefit a wide range of patients. For example, bi-specific antibodies, thanks to their ability to recognize two different targets simultaneously, can enhance the precision and effectiveness of cancer immunotherapy (a type of therapy that uses our own immune system to attack cancer cells). This unique dual targeting capacity can lead to improved recruitment and activation of immune cells at the tumour site, offering a powerful strategy against various cancers.

Patients with neurological disorders like Alzheimer's and Parkinson's disease would also benefit from innovative antibodies. Single domain antibodies, for example, can reach the brain more easily than conventional antibodies due to their smaller size. This makes them particularly suited for targeting this type of diseases. Preclinical studies with single domain antibodies in patients affected by Alzheimer's disease have shown a potential in preventing the onset of the disease an in improving cognitive functions.

Single domain antibodies have also shown an enhanced capacity in neutralising certain types of pathogens that can cause different types of infectious diseases. For example, specific types of single domain antibodies have shown promise in neutralising SARS-CoV-2, the virus responsible for COVID-19, by binding to multiple sites on the virus, thereby preventing it from entering human cells. These initial findings can lead to the development of next-generation drugs against a range of widely common viruses and bacteria.

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In this project, our minimum goal is to produce the following mouse models:

- Common Light Chain (CLC) mouse for the discovery of bispecific antibodies. We are aiming to produce 3-4 different versions characterized by alternative genetic designs. The goal is to generate a selection of transgenic mouse models that can be used for discovery projects against a wide range of human therapeutic targets.

- Single Domain (SD) mouse for the discovery of "VHH antibodies" / "nanobodies". Also in this case, we are aiming to produce different versions (2-3 strains) characterized by different genetic designs. The biology of VHH antibodies is still an area of active research and testing alternative designs will allow us to identify the best model(s) to support the discovery of this promising new type of therapeutic antibodies.

On the other hand, avian models represent a perfect tool to complement mice platforms and allow the identification of therapeutic antibodies against targets that prove to be particularly challenging or have a high homology (i.e. are very similar) between mouse and human.

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

Our company believes that our industry should compete on getting the best drugs to patients as quickly as possible by providing easier access to the best discovery platforms. Our mission is to make medicines together, by working collaboratively with the global scientific community. For this reason, our goal is to establish and validate platforms that can be widely used by academic groups, small biotech and big pharma that are involved in therapeutic antibody discovery.

Humanised mouse models that our team developed in previous projects are now deployed by hundreds of discovery teams around the world and, as of 2024, more than 12 antibodies discovered from such platforms already entered clinical studies and are tested in patients. The goal for this project is to replicate the success that we achieved in the past by developing and distributing new platforms that can support the discovery on "next-generation" antibody-based drugs.

In the longer-term, when possible, key findings will be made available to other scientists through publications in scientific journals for the benefit of the wider scientific community. This will include positive as well as negative outcomes. During this project, we will be testing different alternative designs to evaluate which specific configuration is more effective in supporting the development of non-canonical antibodies. Inevitably, some designs will prove to be better than others and sharing such information will inform other groups involved in similar projects and help avoid duplications and additional animal work (reduction).

Species and numbers of animals expected to be used

- Mice: The maximum number of mice that we expect to use over a period of 5 years is 10000.
- Domestic fowl (Gallus gallus domesticus): The maximum number of chickens that we expect to use is 6000.
- Other birds:
  - Japanese quail (Coturnix japonica): The maximum number of quails that we expect to use is 5000.

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.

Our project is divided in three major goals: 1) generation of new transgenic mouse models that can produce "non-canonical" antibodies with therapeutic potential and 2) validation and characterization of the newly generated mouse models 3) generation and validation of avian models (chicken and quail) for human therapeutic antibody discovery.

The generation of new transgenic mouse models will be performed using a well characterised procedure that uses mouse Embryonic Stem Cells (mESC). Mouse ES cells are cell lines that are already available in our lab and can be propagated and manipulated *in vitro* to introduce the desired genetic modifications that are investigated in this study. Once the mES cells are successfully modified, they get injected into an early-stage embryo called blastocyst (this is also performed *in vitro*). The blastocysts containing the injected mES cells can then be transferred into a recipient female mouse where the embryos can complete their normal development and produce transgenic pups. The genetic modifications need to be introduced into early-stage embryonic cells because they are easy to manipulate and have the unique property of being able to generate all the different tissues and organs of a fully developed mouse. This unique property is called "totipotency".

All the remaining steps of the work performed in this project, including the validation will be performed in adult mice. Breeding of mice will involve all stages of development as part of the normal life cycle of a mouse.

We chose mice as our animal model because their genetics are well understood, and current technologies enable the specific genetic modifications we need for this study. This wouldn't be possible (or it would be much more difficult) in other animal models. Mice have already proven to be an ideal model for discovery of clinical antibodies in previous studies, and we believe they will be particularly suitable for achieving the objectives described in this project.

The generation of transgenic birds will be performed using Primordial Germ Cells (PGCs) that are the avian equivalent of mouse Embryonic Stem Cells. Wildtype PGCs are available from international repositories or can be collected from embryos at a very early stage (2 - 2.5 days of incubation). At that early stage of development, the embryo is still not fully formed and there is no risk of causing pain or distress. PGCs are then manipulated in vitro and only once they pass all quality checks are injected back into a host embryo. The procedure is performed opening a little window in the eggshell without damaging the embryo. Once the microinjection of PGCs is completed, eggs are re-sealed and incubated at 37.5C until chicks are hatch (~21 days of incubation). This approach allows to perform precise editing of the avian DNA and at the same time is minimally invasive and performed exclusively within fertilized eggs.

All the remaining steps of the work performed on poultry will be performed on adult animals. Breeding of chicken and quails will involve all stages of development as part of the normal life cycle of a bird.

We chose chickens and quails as our animal models because the current technologies, despite not being as advanced as the ones available for mice, enable the generation of transgenic models with the properties we need for this study. Moreover, as mentioned earlier, avian models have unique properties that complement very well mouse models, and we believe will enable the discovery of therapeutic antibodies against challenging disease-associated targets.

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

The major procedures involved in the generation of new genetically altered mice are 2 minimally invasive surgical procedures. A small number of male mice will be vasectomized to render them sterile. This procedure only causes transient discomfort and has no long-term effects on the welfare of the mice. Non-surgical embryo transfer is the preferred method to implant the manipulated embryos into recipient females. Also in this case, the procedure is minimally invasive, and it only causes a transient mild discomfort.

The procedure to generate genetically altered birds is performed in fertilized eggs, microinjecting a particular type of cells (PGCs) into early-stage embryos. Modified embryos will be allowed to develop normally and hatch after an incubation period of approximately 21 days. The procedure being performed on early stage embryos and in vitro (PGCs manipulation), doesn't cause any type of pain or distress.

During the validation process, both mice and poultry will receive injections of different types of compounds. This is done to induce an immune response and evaluate the capacity of producing certain types of antibodies. This approach is very similar to vaccination in human patients and it might include multiple boosts to achieve an optimal immune response. The substances injected are not expected to cause any harm or cause the development of any type of disease. When needed, small samples of blood will be collected from peripheral veins to test the production of antibodies or to test other clinically relevant parameters.

Injections will be performed by highly trained technicians with the appropriate equipment and are expected to only cause transient discomfort with no long-lasting effects.

The following routes for injections will be used during the different procedure performed in this project:

- Subcutaneous (mouse and poultry)

- Intravenous (mouse and poultry)

- Intradermal (mouse)

- Intraperitoneal (mouse)

- Intramuscular (mouse and poultry)

Before the start of an immunization study, mice might be microchipped for identification purposes. Microchips are about the size of a grain of rice and are implanted under the skin of the mice using a special type of syringe. A combination of local anaesthetic cream and general anaesthetic is normally used to minimize discomfort.

In the UK there is a limited number of suppliers that can distribute purpose-bred quails and access to these birds is often restricted to commercial entities. To prevent unnecessary production of wildtype quails in house, we might source these birds from other facilities that already breed quails for other purposes (e.g. food industry).

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

Mice and avian models generated in this project, are not expected to develop any type of disease or pathology. They are expected to be healthy and behave like unmodified animals. Some of the genetically modified mice might have mild forms of immunodeficiency (conditions where the body's defence system is not fully functional) but that won't be associated with any harmful effect. All mice will be kept in pathogen-free facilities to avoid any potential risk of infection. All the substances injected into the experimental animals are non-toxic and not expected to cause pain, suffering, distress or lasting harm.

The only time when the animals might experience mild discomfort is during procedures such as injections or small surgeries. Animals subjected to these small surgery procedures will be treated with the most appropriate anaesthetic regimen and be provided with pain-killer medications if needed. The nature of the discomfort is expected to be of transient nature (less than 24 hours) and never exceed a moderate severity.

The administration of substances will be performed through different routes of injection that should cause no more than momentary pain.

Some immunisations will be performed mixing the antigen of interest with an adjuvant. An adjuvant is a substance that is added to help enhance the body's immune response to the antigen of interest. In simple terms, it acts like a booster, making the immunisation procedure more effective. A particular type of adjuvant called FCA (Freund's Complete Adjuvant) may be associated with small, non-painful nodules at the site of injection or in worse cases, in local tissue necrosis and ulcerations. FCA will be used no more than once and when used, mice will be monitored closely to ensure that any potential adverse reactions are identified promptly.

Insertion of microchips under the skin of mice, can cause localized inflammation and swelling that normally disappear within 24 hours. In rare cases, the body may form a granuloma, which is a small area of inflammation, around the microchip. This might persist for a few days post insertion. In very rare cases, if proper aseptic techniques are not followed, infection can be observed at the site of the microchip insertion. This can lead to localized swelling, redness, and in severe cases, abscess formation.

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: 34.56%

Mild: 51.84%

	Moderate: 13.60%
	Severe: 0%
Chickens	Sub-threshold: 31.5%
	Mild: 23.5%
	Moderate: 5%
	Severe: 0%
Quails	Sub-threshold: 40.5%
	Mild: 4.5%
	Moderate: 5%
	Severe: 0%

What will happen to animals used in 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?

Therapeutic antibodies can be identified using two different approaches: *in vitro* and *in vivo*. "*In vitro*" refers to experiments or procedures that are performed outside of a living organism, usually in a controlled laboratory environment. The most common *in vitro* methods used for antibody discovery are display technologies or computer-generated algorithms like artificial intelligence or machine learning.

On the contrary, "*in vivo*" refers to experiments or procedures that are performed within a living organism, such as a transgenic mouse.

Unfortunately, there is no *in vitro* system currently available that, on its own, can be used to generate appropriately modified antibodies with the diversity, specificity and properties that the *in vivo* models offer.

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

Display technologies, like phage display, are powerful tools for discovering new antibodies, but they have limitations that mean they are not always sufficient on their own, to guarantee finding the best therapeutic antibodies. This is particularly true in the case of more sophisticated "next-generation" antibodies. We carefully consider all possible *in vitro* discovery platforms that can replace or reduce the use of *in vivo* models. We have a full team of scientists specialised in display technologies (phage, yeast and mammalian) and we often achieve the best results by combining these with *in vivo* platforms. Our team keeps improving display technologies implementing all the technical advancements that become available and also by applying what we learn from the use of animal models. In the long term, the goal is to be able to reduce as much as possible, and even replace animal work whenever is not strictly needed.

Artificial Intelligence (AI) and Machine Learning (ML) are also promising technologies that are already used by our scientists to facilitate the discovery of new therapeutic antibodies. Both technologies (AI and ML) are very helpful in complementing or facilitating the use of both *in vitro* and *in vivo* platforms but, at the current state of development, it's unrealistic that new therapeutic antibodies can be discovered with an *in silico*-only approach. As of 2024, no drug has been approved that was generated exclusively by AI or other *in silico* methods.

Why were they not suitable?

There are multiple reasons that limit the use of display technologies on their own. Below are some examples:

- Artificial biological context

Display technologies often occur in a test tube or on the surface of a virus, which doesn't fully replicate the complex environment inside an animal body. Antibodies identified through display technologies might not work as effectively in real-life biological conditions because they haven't been selected in a living system with all its complexities. Phage display bypasses the natural immune system's selection mechanisms, which consider how antibodies interact with other parts of the immune system, such as T cells and various signalling molecules. As a result, antibodies identified from *in vitro* approaches have less chances to become functional drugs.

- Lack of functionality and diversity.

While display technologies can identify antibodies that bind tightly to a target, this high affinity doesn't always translate into effective therapeutic action. For an antibody to be therapeutically useful, it needs to not only bind to its target but also induce the desired biological effect, such as blocking a receptor or inducing cell death.

The human immune system has a remarkable capacity of generating an astonishing number of antibodies. Considering all the different mechanisms deployed by the immune system to generate antibodies, it is estimated that the human body can produce on the order of 12^{12} to 10^{15} different antibody specificities! This incredible diversity can be replicated in mouse models (which our group achieved in the past) but it's much more challenging to achieve the same diversity in an *in vitro* system. Even with the most modern technologies, it is hard for a phage display library to go over 10^{11} antibodies. This is multiple orders of magnitude lower than an *in vivo* system.

- Specificity issues

Antibodies identified through display technologies might bind to unintended targets (off-target binding), potentially causing side effects. The immune system of a mouse or human has a quality control system that negatively select the antibodies with low affinity against the specific target or for any antibody that targets (and damage) the body itself (auto-reactive antibodies). This means that in general, antibodies isolated from *in vivo* platforms have a better safety profile while antibodies isolated from display technologies require a more comprehensive testing to ensure specificity and safety.

- Developability

A major bottleneck in translating experimental antibodies into clinical antibodies is their developability (propensity to become a commercial drug). Antibodies identified through phage display can encounter several developability problems that need to be addressed before they can become effective therapeutic agents. These challenges include issues related, for example, to their physical stability or aggregation. On the contrary, antibodies isolated from *in vivo* platforms have been already selected by the immune system not only for their binding and functional properties but also for their stability and lack of aggregation.

- Affinity vs. Functional Maturation

While additional rounds of biopanning can increase the affinity of phage library candidates, this process focuses primarily on binding affinity, often at the expense of other critical factors, such as antibody stability, specificity, or effector function. In vivo systems allow for a more holistic approach to antibody maturation, where the functional relevance of an antibody (e.g., its ability to trigger immune cell engagement, cross the blood-brain barrier, or neutralize a pathogen) is co-selected alongside affinity. This is particularly important for non-canonical antibodies, such as bispecific and single-domain antibodies, where function often depends on more complex interactions than simple high-affinity binding.

In conclusion, *in vitro* methods for antibody discovery are very powerful but on their own are limited by a multitude of issues that undermines their ability of isolating antibodies with clinical properties. The combination of *in vitro* and *in vivo* approaches gives the best chances of success and for this reason the development of animal platforms is of paramount importance.

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 estimation is based on previous experience on similar research projects and on the type of goals that we plan to achieve in this project. Our research team has successfully worked on similar projects where transgenic mice capable of producing human antibodies were developed and validated. This helped us estimate the number of mice that will be required to generate a minimum of 5 new transgenic models and perform the validation and characterization of the newly developed strains.

The estimated numbers of chicken and quails are based on similar considerations and take into account the number of animals required to generate a minimum of 3 GA chicken strains and 1 GA quail strain. In addition to introducing genetic alterations, both chickens and quails will need to go through several rounds of cross-breeding to reach the desired genetic composition. The final validation of the newly generated models will be done through immunizations and analysis of their immune response.

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

During the experimental design of our validation experiments for both mouse and avian models, we make sure to select the correct number of animals. The goal is always to use the smallest possible number of animals that allows to address our scientific objectives. We often use online resources to help us selecting the most appropriate cohorts size like for example, the Experimental Design Assistant (EDA) from the NC3R webpage (https://www.nc3rs.org.uk/our-portfolio/experimental-design-assistant-eda). When planning experimental animal work we make extensive use of the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines (https://norecopa.no/prepare).

We also use data collected from previous experiments to help guide us in the design of similar studies. We have access to a team of experienced bioinformaticians and statisticians that we consult when we plan *in vivo* studies to make sure the typical variation observed in our own previous experiments is taken into account to calculate minimum number of animals to be used whilst ensuring that the results are statistically significant.

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

Different measures will be adopted in this project to optimise the number of animals used:

1) For the generation of new genetically altered mice we will use mouse ES cells that have been previously characterized for their ability to produce high percentage chimeras (mice where both modified and unmodified cells and tissues are present at the same time) that have high potential to transmit their genetic modifications to the offspring (germline transmission). This will reduce the number of breeding steps required and prevents the generation of surplus animals.

2) Breeding of transgenic strains will be carefully planned and monitored to avoid overproducing mice or birds that are not required for the purpose of this project.

3) At the end of each validation study all relevant tissues will be collected and cryopreserved (stored at very low temperatures) even if they are not needed at the time of the experiment. Additional data might be generated from these tissues at a later stage preventing the use of additional mice or birds.

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4) Wildtype (non-transgenic) animals that have been overproduced or that are the result of breeding experiments will be made available to other research groups in the facility whenever possible and following consultation with the facility veterinarian.

5) When possible, mice and birds will be challenged with multiple antigens simultaneously to reduce the overall number of animals used. This will only be considered when the presence of multiple antigens doesn't interfere with the scientific outcome of the experiment and when potential toxicity effects have been completely ruled out.

6) For the generation of transgenic birds, we will use the PGC methodology that has several advantages: a) higher efficiency in producing transgenic offspring compared to older methods such as DNA microinjection in the embryo. This results in fewer eggs and animals being used to achieve the same goal. b) Ability to screen and select cells before use, reducing the number of birds that need to be hatched and screened later, decreasing unnecessary animal use. This approach will be applied to both chickens and quails.

7) If suitable, quails will be sourced from local facilities that already breed quails for other purposes (e.g. food industry). This way, we will avoid maintaining a flock of wildtype birds in house preventing the risk of overproducing wildtype animals.

All measures described here are aimed at reducing the overall number of animals without compromising the scientific integrity of the output generated from these studies. All measures will be implemented under ASPA-compliant conditions to preserve animal welfare without compromising data quality.

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.

The generation of new genetically altered mice will be performed by introducing the desired modifications into ES cell lines with well-defined *in vitro* procedures. The engineered ES cells will then be used to produce early-stage embryos containing the desired modifications. All these steps don't cause any type of pain since they are performed on cell lines or on very premature embryos. To achieve full development of the embryos, they will then be implanted into recipient females. This procedure, whenever possible, will be achieved through a non-surgical procedure that is minimally invasive and causes very mild distress. In some circumstances, a surgical approach might be chosen and in this case the mice will be treated with an appropriate anaesthetic regimen in combination with pre- and post-operative analgesic treatment if required. All procedures will be performed by experienced and trained operators who will ensure best practises are followed. This way, the distress and the risk of complications will be reduced to the very minimum.

Adult mice will be tested for their capacity of mounting an immune response and produce the types of antibodies that are investigated in this project. This will be done by injecting the mice through standard routes (e.g. subcutaneous injections) in a way that replicates immunizations in human patients. The type of substances injected, the route of injection and the frequency of injection is optimized to achieve a good scientific output and, at the same time, limit as much as possible the distress of the animals. Immunizations are not expected to cause any long-lasting pain nor cause diseases to the mice. The handling of the mice and the injection itself is expected to produce a transient mild discomfort that will resolve within a few hours. During procedures, mice will be closely monitored to identify any early sign of distress or pain. In very rare cases where unexpected severe side effects are reported, the mice will be humanely killed.

The generation of new genetically altered birds will be performed by introducing the desired modifications into primordial germ cells (PGCs) using well-established in vitro procedures. The genetically modified PGCs will then be introduced into developing embryos to produce chimeric birds that carry the desired genetic modification in their germline. These procedures are conducted entirely in vitro or at early embryonic stages, ensuring that no pain or distress is caused to the animals.

Once modified PGCs are microinjected into a recipient embryo, the eggs will then be incubated under optimal conditions until hatching. Hatchlings will be carefully monitored for health and normal development. All procedures will be performed by experienced and trained operators following best practices to minimize any potential distress and ensure the highest welfare standards.

Once mature, transgenic birds (together with wildtype controls) will be tested for their ability to mount an immune response and produce the types of antibodies investigated in this project. This will be done through standard immunization procedures, such as intramuscular or subcutaneous injections, mimicking immunization strategies used in human patients. The type, route, and frequency of injections will be carefully optimized to achieve the desired scientific outcomes while minimizing any distress. Immunizations are not expected to cause long-lasting pain or disease in the tested animals. Any transient discomfort experienced during handling and injection is expected to resolve within a short period. Birds will be closely monitored throughout the study to detect any early signs of distress or discomfort. In the rare event of unexpected severe side effects, humane euthanasia will be performed in accordance with ethical guidelines.

Despite the biological process responsible for antibody generation being quite similar in both chickens and quails, there are key differences between the two species which makes the use of both models necessary and complementary for the goals of this project:

- Genetic modification efficiency and stability differs between the two species with chicken being a more well-established model than quails. However, certain antibody modifications or immune responses might be better suited to quails rather than chickens. Testing both species will allow to identify the best suited model and learn more about the biology underlying these processes.

- The two species have a very different maturation and breeding efficiency. Chickens reach sexual maturity in 5-6 months while quails reach sexual maturity in 6-8 weeks, allowing faster generation times and quicker evaluation of transgenic lines. If quails prove to be biologically equivalent to chickens, they will offer significant advantages in terms of antibody discovery timelines.

- While both species secrete IgY antibodies, variations in glycosylation patterns and immune system characteristics might make one species preferable for certain therapeutic targets. Exploring both models will allow to select the best possible platform for each therapeutic target.

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

To achieve the overall goal of this project it is not possible to use animals that are less sentient than mice or birds for several reasons:

- Mice and birds have a complex immune system that is more similar to humans compared to less sentient animals like invertebrates. This similarity includes the structure and function of immune cells and antibodies, making them a more suitable model for studying immune responses and developing therapeutic antibodies.

- Mice can generate a diverse array of antibodies through different complex processes called "V(D)J recombination", "somatic hypermutation", and "class switching". Humans and mice share the same mechanisms for generating antibody diversity, enabling mice models to accurately replicate the human immune response. Birds create antibody diversity by "V(D)J recombination", "somatic hypermutation" and also a peculiar mechanism called "gene conversion". Less sentient animals often lack these complex immune mechanisms.

- Mice and birds can be genetically engineered to express human immune components (humanization), which allows for the discovery of fully human antibodies for therapeutic use. This process is not feasible with less sentient animals like invertebrates.

- Mice and birds cannot replace less sentient models like fish for several reasons: 1)Antibody structure: Fish antibodies (mainly IgM and IgD) are simpler and often form large complexes (e.g., pentamers) rather than the refined, high-affinity IgG antibodies found in mammals and birds that are crucial for human therapies. 2) Fish have a much more limited or even absent process of affinity maturation (the refinement of antibodies to bind better to their targets). Mice and birds both undergo strong affinity maturation, which is critical for generating high-quality therapeutic antibodies. 3) Immune repertoire: The diversity and complexity of the antibody repertoire in fish is far lower than in mammals and birds. This limits the ability to discover rare, highly specific, and high-affinity antibodies. 4) Genetic engineering: It's much harder to create genetically engineered fish models (like humanized antibody platforms) compared to mice or chickens, where sophisticated transgenic technologies are well-established. 7) Immunization protocols: Immunizing fish for antibody discovery is less practical and less efficient than with mammals or birds, due to physiological differences and the aquatic environment.

We can’t use embryos or very young animals as their immune system is immature and doesn’t respond to antigenic stimulation in the way mature animals do.

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

Mice will be kept in specific pathogen-free facilities and will be handled by highly trained animal care staff to ensure that any type of distress is minimised. Animals will be given environmental enrichments

including plastic houses and cardboard tubes and, when possible, will be group housed to reduce isolation stress. The mice will be handled in such a way as to reduce stress (for example using cupped hands or cardboard tubes). If mice are imported from another facility, they will be allowed to acclimate prior to any procedures.

Chickens and quails will be kept in specific pathogen-free facilities and will be handled by highly trained animal care staff to ensure that any distress is minimized. Animals will be provided with environmental enrichments, including perches, dust baths, and nesting materials, to encourage natural behaviors and promote well-being. When possible, chickens will be group housed to support their social nature and reduce stress from isolation. Handling will be performed in a way that minimizes distress, using gentle restraint techniques and avoiding excessive confinement. If chickens are imported from another facility, they will be given an appropriate acclimation period before any procedures to ensure their well-being and adaptation to the new environment.

The route of injection used in this project, including intraperitoneal and subcutaneous injections, have all been shown to cause no adverse effects whilst inducing effective antibody responses in most cases. Animal suffering will be minimised by limiting the numbers of boosts and the duration of the experiment. The levels of antibodies present in the circulating blood (serum titres) will be used to determine the number of boosts and therefore the scientific endpoints. All substances used for mice immunizations will be tested to ensure they are sterile, pure and that endotoxins (an immunogenic contaminant derived from bacterial cells) are not present. Injections will be performed by trained technicians to minimise stress and discomfort.

Quails may be sourced from local breeders that already maintain flocks for other purposes. This approach avoids the need to establish and maintain a purpose-bred colony in-house, thereby reducing the risk of overproduction and unnecessary culling of surplus animals. The use of local breeders also represents an additional refinement, as it minimizes long-distance transport, which could negatively impact animal welfare due to transport-related stress. Whenever live animals are transported between facilities, all applicable animal welfare regulations will be strictly followed.

We will develop standardised species-specific check-list scoring sheets to monitor the health status of mice and birds after immunisations to ensure that no adverse reactions are overlooked. The score card will include several parameters, below is an example:

Mice check-list

General appearance

- weight loss, coat condition, posture, dehydration. The mouse grimace scale (https://nc3rs.org.uk/3rs-resource-library/grimace-scales/grimace-scale-mouse) will be used to assess post-procedural pain as part of the clinical assessment.

Behaviour

- activity level, grooming, social interaction, eating and drinking.

Additional parameters

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- breathing rate and effort, tremors or seizures, diarrhoea, abdominal distension, red or swollen eyes, nasal discharge, sensitivity to touch, vocalizations.

Chickens / quails check list

General Appearance

- Weight loss, feather condition (ruffled, missing, or unkempt feathers may indicate illness or distress), posture & stance (hunched posture, reluctance to move, or wing drooping), dehydration (assessed by checking skin elasticity and eye brightness), comb & wattle colour (pale, bluish, or excessively red comb and wattles may indicate circulatory or respiratory issues).

Behaviour

-activity level, preening and grooming, social interaction, reduced food and water intake or difficulty swallowing.

Additional parameters

- breathing rate and effort, tremors or seizures, diarrhoea or abnormal droppings, abdominal distension, red or swollen eyes, nasal discharge, sensitivity to touch, vocalizations.

Very few, if any, animals will undergo surgical procedures. However, if they do, then pre and post-operative pain relief will be given.

For immunizations, small pilot studies will be conducted to ensure that the methods used minimise harm while meeting the study objectives.

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

Best practice guidance from different sources will be followed. This will include:

-PREPARE guidelines (http://journals.sagepub.com/doi/full/10.1177/0023677217724823), very helpful to assist the planning of animal research.

- ARRIVE (Animal Research: Reporting of In Vivo Experiments) Guidelines (https://arriveguidelines.org/), provides a comprehensive framework for reporting scientific results and ensure studies are conducted rigorously and transparently.

- Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (UK Home Office)

- NC3Rs best practice guidelines (https://nc3rs.org.uk/)

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

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We will stay informed about advances in the 3Rs by checking regularly the NC3Rs webpage and by signing up to alerts and newsletters from relevant organizations like for example NC3Rs, AWI (Animal Welfare Institute), and FRAME (Fund for the Replacement of Animals in Medical Experiments). The University Biomedical Services (UBS) runs an in-house 3R's enquire list to which I am planning to become a member.

We will participate in online courses and webinars that provide updates and training on the latest 3Rs methodologies and technologies and have regular interactions with experts in our network like NACWOs and NVSs.

Whenever new developments in the 3Rs become available we will do our best to implement them straight away in our experimental project.

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