Society for Laboratory Animals of Slovenia (SLAS)

5th Congress of the SLAS and 3rd joint SLAS - CroLASA meeting

Improving nonclinical research practices: the way forward

Proceedings

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Ljubljana, Slovenia
15 June 2023

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5. kongres SDLŽ in 3. skupno srečanje SDLŽ in CroLASA

Ljubljana, Slovenia
15 June, 2023

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Society for Laboratory Animals of Slovenia (SLAS)

Croatian Laboratory Animal Science Association (CroLASA)

Society for Laboratory Animals of Slovenia (SLAS)

Location: Grand Hotel Union Eurostars

Ljubljana, Slovenia
15 June 2023

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felasa
FEDERATION OF EUROPEAN LABORATORY ANIMAL SCIENCE ASSOCIATIONS
Severity Classification Workshop

Scientific and Organizing Committee

Chair: Martina Perše

Treasurer: Simona Kranjc Brezar

Members: Tatjana Pirman, Duško Lainšček, Malan Štrbenc, Maša Skelin, Daša Seveljević-Jaran, Sara Trnski

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Location: Grand Hotel Union Eurostars

Garden Hall: Receptions, lunch, coffee breaks:

White Hall: Lectures

Orchid Room: Workshops

June 15	Lectures: White Hall	WS: Orchid room
7.30-8.30	**REGISTRATION (Garden Hall)**	**WS1: 8.00-10.45**
8.30-8.50	**OPENING: SLAS & CroLASA WELCOME**	**David Anderson**
8.40-8.50	**FELASA President**: A brief introduction to FELASA	FELASA workshop on classification and reporting of severity of procedures Farm Animal Models
8.50-9.20	**L1. Klas Abelson**: Refinement of painful procedures – are we good enough?	
9.20-9.50	**L2. Delphine Bouard:** Improving aseptic conditions in laboratory animals: a major refinement tool	
9.50-10.20	**L3. Belen Pintado**: Genetically altered animals through the lens of Directive 2010/63/EU	
10.20-10.40	**Simon Koren**: Reducing animal usage and revealing new insights: the power of *in vivo* imaging technologies in preclinical research	
10.40 -11.15	**COFFEE BREAK**	**WS3: 11.00-13.30**
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11.45-12.10	**L5. Aleš Belič**: Animal models: RNA expression analyses as an important source of irreproducibility and low translatability	
12.10-12.25	**Anemari Horvat**: Stress promotes lipid droplet accumulation in astrocytes (SL1)	
12.25-12.40	**Sara Orehek**: Antitumor immunity boosted by gasdermin D induced necrosis (SL2)	
12.40-12.55	**Polona Kovačič**: Extent of necrosis as a measure of acute pancreatitis severity in studies employing live cell imaging (SL3)	
12.55-13.05	**Carlo Demalde**: Tecniplast – your partner for animal facility	
13.05 -14.15	**LUNCH**	**WS2: 13.45-16.45**
14.15-14.40	**L6. Chris Van Ginneken**: Pigs in research, what about the piglet?	**David Anderson** **Dolores Bonaparte** FELASA workshop on classification and reporting of severity of procedures Rodent & Rabbit Models
14.40-15.05	**L7. Alenka Seliškar**: Anaesthesia and analgesia in calves, goats, sheep and pigs used for biomedical research	
15.05-15.30	**L8. Amir Rosner**: Equine species in research and education	
15.30-15.45	**Malan Štrbenc:** Successful rehoming as ultimate refinement for golden hamsters in SARS-CoV-2 vaccine research (SL4)	
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17.15-17.20	180s: MR1. **Manca Pečjak Pal**	
17.20-17.25	180s: MR2. **Tajda Gredar**	
17.25-17.30	180s: MR3. **Sara Trnski**	
17.30-17.35	180s: MR4. **Dominika Peskar**	
17.35-17.40	180s: MR5. **Katja Uršič Valentinuzzi**	
17.40-17.45	180s: MR6. **Sanita Maleškić Kapo**	
17.45-17.50	180s: MR7. **Maša Čater**	
17.50-18.00	**Kristijan Jurkovac**: Animalab - your trusted partner in life-science research	
18.00-18.30	**CHEESE AND WINE RECEPTION**	
18.30h	**SLAS AWARDS (the best 180s presentations)**	

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WELCOME

Dear Colleagues,

On behalf of the Scientific and Organizing Committee it is a great pleasure to welcome you to the 5th Congress of Society for Laboratory Animals of Slovenia (SLAS) and 3rd joint meeting of SLAS and CroLASA entitled *Improving nonclinical research practices: the way forward*.

In 2013, the first Congress of SLAS was organized aiming to gather people from all institutions in Slovenia working with experimental animals to network and present their research, methods, equipment, and experience. Recognizing the need for state-of-the-art knowledge SLAS has organized many scientific events and activities on burning topics such as legislation, environmental, dietary, microbiological, and genetic factors, communication with the public, welfare assessment, severity classification of procedures, use of analgesia and anesthesia in rodents, including factors that significantly influence the credibility and reproducibility of animal experimentation and reporting transparency of scientific publications.
Today, 10 years later, many positive changes in the field of laboratory animals in Slovenia have been achieved (in terms of legislation, equipment, technology, and knowledge) showing that SLAS has importantly contributed to the development of Laboratory Animal Science (LAS) awareness in Slovenia.

In this short period, the scientific community in Slovenia has recognized/accepted that it is our moral and professional obligation to give a high priority to ethical considerations and animal welfare and to follow constantly developing progress in the LAS field. I would like to express my sincere gratitude for all the achievements each of you – everyone has contributed hers/his own part in our community so that we can improve ourselves, our knowledge, and our scientific work.

For the first time scientific program of the Congress covers also farm animals and fish, besides rodents. At the event, the latest knowledge on the field will be presented by renowned and experienced experts from leading research organizations. I would like to take this opportunity to sincerely thank the speakers and workshop organizers for their time, efforts, goodwill, cooperation and valuable contributions to better science.

Finally, the event is supported by companies whose employees grow and learn with us and strive to be able to supply and provide us with quality services and equipment according to the latest standards. Sincere thank you to the sponsors for their attendance and support.

However, the animals are the stars of our work. Therefore, I would like to express my deepest respect and gratitude to all the animals involved in research and testing.

We look forward to welcoming you in Ljubljana,

Martina Perše
President of the Scientific and Organizing Committee

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LABORATORY ANIMAL SCIENCE ASSOCIATIONS/ORGANIZATIONS

LAS1. SLAS CELEBRATES 20 YEARS

Martina Perše
SLAS President 2012-2025

The idea of a Society for Laboratory Animals was conceived in 2001 at the scientific seminar in Ljubljana and two years latter, in 2003, the Society for Laboratory Animals of Slovenia (SLAS) was founded. However, the first activities started not earlier than in 2010, when SLAS (in collaboration with the Ethical Committee for laboratory animals of the Republic of Slovenia and the Committee of researchers working with laboratory animals) has taken an important part in the process of collaboration in the implementation of the Directive 2010/63/EU.

SLAS is an organization of researchers, scientists, and experts working with experimental animals in Slovenia. The objectives of the society are to encourage ethical work and good laboratory practice; to cooperate with national and international organizations and individuals from the field of Laboratoy Animal Science; to promote and disseminate the advances in laboratory animal science through electronic communications, webinars, and various events; to promote implementation of the highest ethics and scientific practices in the care and use of all animals used for testing, research or teaching purposes; to give opinions in the development of law or regulations regarding care and use of experimental animals. Society can be authorized by the Government for performing certain formal and legal tasks.

The mission of SLAS is thus to care for the humane use of experimental animals, to promote the 3Rs principle, and quality and credible research on animals.

SLAS organizes national and international events (congresses, workshops, round tables, webinars), where all burning and state-of-the-art topics are exposed and discussed. The association enables networking, cooperation, and the exchange of good practices between scientists and research organizations in Slovenia and abroad, mostly in the European area.

Today, SLAS is a member of FELASA (2016 -), ICLAS (2022 -), and EARA (2022 -).

www.slas.si

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Slov Vet Res • Ljubljana • 2014 • Vol 51 • Supplement 15 • 1-53
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Slov Vet Res • Ljubljana • 2018 • Vol 55 • Supplement 19 • 1-56

LAS2. CROLASA

Daša Seveljević-Jaran
CroLASA President 2020-2023

Croatian Laboratory Animal Science Association (CroLASA) started with its activities within the Section of the Croatian Society of Physiologists (Hrvatsko Drustvo Fiziologa) more than 40 years ago, in 1980. In September 1984, the "First Symposium on Laboratory Animals" lasting two days was held at the Faculty of Medicine in Zagreb, gathering 120 participants from all over the former state of Yugoslavia and two years later, in 1986, the „Society for Laboratory Animals“ in Zagreb was constituted.

In 1990, the Society held its founding assembly at which the statute was adopted, with Dr. Sc. Lidija Suman elected as its first president. In 1996, together with the Rudjer Boskovic Institute, the Society organized its 1st symposium with international participation "Experimental animals in scientific research". In 2007, the Society became a member of FELASA (Federation of European Laboratory Animal Science Associations), thus establishing new contacts with other European LAS (Laboratory Animal Science) associations. The CroLASA organized its 2nd symposium with international participation entitled: "Experimental animals in scientific research" in 2014 at the Faculty of Veterinary Medicine in Zagreb. In 2014, CroLASA begins publishing its annual Newsletter (Bilten). In 2015, CroLASA organized the first workshop entitled "Application of 3R approach in working with laboratory animals". In the same year, CroLASA got its new website, providing the Members and followers with all the necessary information related to the work and activities of the Association. In 2016, another workshop was organized as a continuation of workshops related to the implementation of 3Rs. The two-day workshop entitled "Preclinical Biological Imaging - BIOIMAGING" was held in cooperation with the Croatian Institute for Brain Research, the School of Medicine in Zagreb and the Rudjer Boskovic Institute with the participation of LAS colleagues from Slovenia.

Collaboration with SLAS (Society for Laboratory Animals of Slovenia) resulted in first joint educational workshop entitled "Microbiological and nutritional standards for laboratory rodents" in January 2017 at the Faculty of Medicine, University Ljubljana, following the June 2016 3rd SLAS Congress, as well as the first joint SLAS - CroLASA meeting. In the fall of 2018, the 3rd CroLASA and the 2nd joint Meeting of CroLASA and SLAS "Experimental Animals in Scientific Research" were held. The succesfull collaboration with SLAS continued on-line, despite COVID pandemic and lockdown, in 2022, in form of series of webinars conducted on-line under the title „Improving nonclinical research practices: way forward”, and this year in form of the 5th Congress of the Society for Laboratory Animals of Slovenia and 3rd joint SLAS - CroLASA meeting.

www.crolasa.com

LAS3. A BRIEF INTRODUCTION TO FELASA

Klas Abelson
FELASA President 2023-2024

The Federation of European Laboratory Animal Science Associations (FELASA) was founded in 1978. Since then, the federation has had the mission to further all aspects of laboratory animal science (LAS), by advocating responsible scientific research with emphasis on animal welfare with the 3Rs principle as the guiding star. This mission is accomplished by collaboration and networking with bodies at both the European and the global level, by establishing working groups for the publication of guidelines and recommendations, by accrediting LAS courses, and by organizing scientific conferences.

www.felasa.eu

LAS4. ICLAS

Martina Perše
ICLAS European Regional Committee member

The International Council for Laboratory Animal Science (ICLAS) is a scientific organization dedicated to advancing human and animal health and well-being by promoting the ethical care and use of animals in research worldwide. ICLAS works at all continents as ICLAS Regional Committees to ensure the diffusion of scientific knowledge within all regions of the world (i.e.Europe, America, Asia, Oceania, Africa).

The European Regional Committee (ERC) engages in activities aimed at strengthening and developing Laboratory Animal Science (LAS) in European countries with the emphasis on the 3Rs and animal welfare.

ICLAS ERC supports educational and scientific programs in the region, particularly in institutions or countries that want to develop modern Laboratory Animal Science (LAS) programs. Shares information on international standards relating to ethical oversight, research animal care and use, experimental design and reporting research results. Promotes harmonization in the care and use of laboratory animals with the emphasis on animal welfare and scientific quality as part of ethical and reproducible science.
Supports the development of LAS associations within the region. Encourages the exchange of information between associations within the region and with other regions around the world. Establishes and maintains appropriate links within local, national, or regional associations as well as other organizations concerned with LAS. Encourages openness and fostering of public understanding of the research process and the role of laboratory animals.

The objectives of ICLAS ERC are to facilitate meetings and training sessions (i.e. assisting in the organization and providing speakers); to sponsor education and training programs, supported by outside funding (i.e. ICLAS grant for Training the Trainer in LAS Education in Europe, ICLAS visiting grant for professional development in LAS in Europe); to seek financial or in-kind (e.g. hosting organizations) resources to support these activities; to share news on how ICLAS support was used, through follow-up reports of the grants.

ICLAS ERC cooperates with FELASA (Federation for Laboratory Animal Science Associations) through the FELASA-ICLAS Liaison Body. This Body explores and develops activities of mutual interest that may benefit the global progress in any area of laboratory animal science. This is achieved through participation in working groups, scientific meetings, and any other joint activity that is considered appropriate by both organizations.

www.iclas.org

INVITED LECTURES

L1. REFINEMENT OF PAINFUL PROCEDURES – ARE WE GOOD ENOUGH?

Klas Abelson

Department of Experimental Medicine, University of Copenhagen, Copenhagen, Denmark

Experimental procedures such as injections, blood sampling, surgery and induction of various models are considerable sources of anything from mild acute pain to severe chronic pain in experimental animals. Pain involves both physiological and emotional alterations to the animals, which has a detrimental effect on both the experimental results and on the welfare of the animals. There is thus a great need for refinement of painful procedures in all aspects of animal experimentation.

For refinement of the procedures, proper identification of the extent of pain and stress inflicted on the animals as well as relevant assessment is necessary, in order to determine what action needs to be taken for eliminating or minimizing the pain and stress in question.

This presentation will give a brief overview of the pain and stress inflicted by different procedures, how this pain can be identified and assessed and thereby alleviated by analgesic treatment, improvement of practical procedures as well as other means to minimize discomfort in the animals.

L2. IMPROVING ASEPTIC CONDITIONS IN LABORATORY ANIMALS : A MAJOR REFINEMENT TOOL

Delphine Bouard

Vetsalius, Surgery, Saint Didier, France

The principle of aseptic surgery was established at the end of the 19th century, more than 200 years ago. Eventhough progresses were made regarding strategies and available material, the fundamental principle remains the same: preventing any contamination of patients cavity with microorganisms during a surgery.

In the context of laboratory animals' operations, respecting strict aseptic conditions do not only allow to prevent healing issues of operated individuals but also to prevent other post-operative complications (catheter blockage for example) and to avoid any bias in collected scientific data.

Strict aseptic conditions are usually respected for large laboratory animals' surgeries. However, it remains significantly improvable when it comes to rodents.

During this presentation, the most recent "tips and tricks" regarding prevention of contamination from major potential sources (environment, animals, instruments, surgeons) will be presented. Usual problems related to lack of asepsis and some successful improvement strategies will also be described.

L3. GENETICALLY ALTERED ANIMALS THROUGH THE LENS OF DIRECTIVE 2010/63/EU

Belén Pintado

National Center of Biotechnology (CNB-CSIC), Transgenesis Service, Madrid, Spain

Directive 2010/63/EU has represented a major turning point in the regulation of research carried out with animals. By establishing a robust legal framework that promotes their ethical and humane use, it also seeks to endorse high-quality science. The overall purpose of this legislation is to ensure that the use of these animals in scientific research is carried out in a way that is ethical, humane, and scientifically justified. The Directive established special provisions that apply to the use of genetically altered animals in scientific research. Genetically Altered Animals represented a 28% of the total number of animals used in the EU in 2019 and this percentage will increase in the future, since genetically altered models represent a refinement of animal models for the study of human diseases among other applications.

During the presentation the main provisions included in the Directive will be discussed with the support of the working document released by the Commission in November 2021 on Genetically Altered Animals to fulfil the requirements under the Directive. The presentation will cover general concepts like creation, using the traditional and new tools, establishment with the required preliminary welfare assessment and also practical aspects like general concepts on the statistical report of these lines in animal facilities.

All these concepts are of major importance in the process of a project design, evaluation and development. Even though 10 years have passed since the entry into force of the regulation, there are still some misinterpretations that the working document tries to explain and clarify with examples. This guidance deserves special attention from the competent authorities, researchers carrying out studies with these animals and animal welfare bodies responsible to ensure that all the studies are carried out in compliance with the legislation.

At last some general concepts to safeguard the quality of Genetically Altered Animals mice colonies will be discussed, paying special attention to the 3Rs in this specific scenario.

L4. PREPARE FOR BETTER SCIENCE: GUIDELINES FOR ANIMAL RESEARCH AND TESTING

Adrian Smith

Norecopa, Norwegian Veterinary Institute, Norway

It is widely accepted that there is still great room for improvement when planning and conducting experiments which appear to involve the use of animals. There are good scientific and ethical reasons for working to advance the three Rs: Replacement, Reduction and Refinement. In addition, we must ensure that those experiments which still have to use animals are correctly designed, producing data that is a true treatment effect, so that the results are transferable to other animals and to humans. At the same time we need to ensure the health and safety of other animals and people in the facility, working for a good culture of care and good communication of our work to stakeholders.

The path to better science is long and involves good preparation from day 1, starting with an evaluation of the possibility of using alternatives to animal experiments. These efforts must involve specialists in literature searches, experts within non-animal methodologies and, if animal studies are unavoidable, close collaboration with animal care staff who know the strengths and limitations of the facility.

To give scientists an overview of all the factors which can influence these decisions, and the validity and outcomes of animal research, we have developed a set of guidelines called PREPARE, based upon experiences in managing accredited animal facilities. PREPARE consists of a two-page checklist, available in 35 languages, and a comprehensive website with links to more information and the latest resources about each topic on the checklist (https://norecopa.no/PREPARE).

To encourage advances in animal research, we have also created a Refinement Wiki, which can be used to publish large or small improvements to housing, care and use (https://wiki.norecopa.no).

Together we can PREPARE for, and achieve, better Science.

Reference:
Smith, A. J. et al. (2018) 'PREPARE: guidelines for planning animal research and testing', Laboratory Animals, 52(2), pp. 135–141. doi: 10.1177/0023677217724823.

L5. ANIMAL MODELS: RNA EXPRESSION ANALYSES AS AN IMPORTANT SOURCE OF IRREPRODUCIBILITY AND LOW TRANSLATABILITY

Aleš Belič[1], Ana Unkovič[2], Emanuela Boštjančič[3], Martina Perše[2]

[1] *Statistics and Modelling, Technical Development Biologics, Novartis Technical Research & Development, Lek Pharmaceuticals d.d., Ljubljana, Slovenia*
[2] *Medical Experimental Centre, Institute of Pathology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia*
[3] *Institute of Pathology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia*

Animal research is facing numerous challenges. Besides methodological errors in conducting in vivo experiments, there are also fatal errors in conducting RNA expression analyses. Nowadays research on animals is used mainly to investigate underlying molecular mechanisms, most frequently with the use of RNA expression analyses such as microarrays and quantitative real-time polymerase chain reaction (qPCR). RNA technology is used to identify molecular targets in the pathogenesis of diseases as well as to evaluate the treatment effect in testing new agents and treatment strategies. qPCR is widely used and accepted for human and animal research and diagnostic purposes due to its relative speed and accessibility in comparison to conventional quantification methods. However, qPCR accuracy and reliability are significantly affected by the stability of the selected reference genes. The expression stability of reference genes is influenced by numerous/countless inter- and intra- experimental factors, such as genetic background, sex, the disease model, the disease state (phases of the disease), organ analyzed, circadian rhythms, etc. A recent systematic review showed that the majority of animal studies (particularly rodents) use "traditional" reference genes without any prior analysis of reference gene expression stability. In rodent studies the most frequently single reference gene is used such as GAPDH or ACTB although both have been shown as inappropriate in numerous experimental settings. Therefore, many qPCR results from past rodent studies can be misleading or false.

The presentation is aimed to demonstrate how different choices of reference genes for RNA expression analysis can significantly affect study results and conclusions.

The main purpose of normalizing the expression of target gene against reference genes (endogenous control) is to remove those differences in the expression of target gene that do not originate from the treatment effect or studied/experimental processes/conditions. For instance, the same type of tissue may have significantly different expressions of target gene just because the tissue originates from different animal or because the tissue is in different state of metabolism; such »non-treatment« contributions to target gene expression should be removed prior to any analysis of the target genes.

Let us define the variability of target gene that does not originate from studied processes (for instance metabolism) for the study. Currently, effects of metabolism are treated as multiplicative effects on target genes expressions originating from the studied processes.

$$Tg = Tg(studied\ processes) * Tg(metabolism)$$

Where Tg stands for measured expression of target gene, Tg(studied processes) stands for a part of expression variability of Tg that comes from studied processes and Tg(metabolism) stands for a part of

expression variability that comes from metabolism. Estimate of Tg(metabolism) is derived from the reference gene expression (Rg)

$$\frac{Tg}{Rg} = \frac{Tg(studied\ processes) * Tg(metabolism)}{Rg}$$

If the expression of reference gene or combination of reference genes describes the contribution of metabolism to target genes the resulting number closely resembles the effect of studied processes on target gene.

$$Rg \doteq Tg(metabolism)$$
$$\frac{Tg}{Rg} \doteq Tg(studied\ processes)$$

However, when we use inappropriate reference genes, then reference genes do not compensate the effects of metabolism on target gene expression. Moreover, inappropriate reference genes may even introduce additional metabolism variability previously not included in the target gene variability.

To better understand the purpose of reference genes or normalization, the presentation will focus on graphical demonstration of various types of possibilities using the example of TNFR1 and TNFR2 expression monitored in archived FFPE colon samples that were diagnosed with various levels of DSS induced inflammation in the colon. Samples were obtained from several experiments. The goal was to establish correlation between various known and controlled experimental factors such as histological picture (mild-severe inflammation, mucosal/transmural, erosion), DSS induction protocol and expression of TNFR1 and TNFR2, while taking into account effects of experiment and sex.
Several different normalizations of target genes will be shown and the effects on correlation will be presented.

We compared changes in multivariate correlation patterns for different normalization strategies.

We used Partial Least Squares method (PLS) to establish multivariate correlation between experimental factors and target genes expressions. Four different types of normalization of target gene were considered: raw (no normalization), normalization to reference gene with lowest variability, normalization to the combination of genes with lowest variability of geometric mean of expression data and normalization to the first principal component of the expressions of all five reference genes candidates. We will present and discuss results of all four normalizations. We will also present a univariate example to show, how forcefully reducing the experimental factors to only one while neglecting the effect of the remaining factors can influence conclusions.

Applying different normalization strategies results in quite different correlation patterns. Most significantly, contribution of experimental factors can shift from significant to non-significant and vice versa, the amount of contribution can change and in some cases (sex) the correlation can even change from positive to negative or vice versa, as we show in our example.

Reference:

Unkovič A, Boštjančič E, Belič A, Perše M. Selection and Evaluation of mRNA and miRNA Reference Genes for Expression Studies (qPCR) in Archived Formalin-Fixed and Paraffin-Embedded (FFPE) Colon Samples of DSS-Induced Colitis Mouse Model. *Biology*. 2023; 12(2):190. https://doi.org/10.3390/biology12020190

L6. PIGS IN RESEARCH, WHAT ABOUT THE PIGLET?

Chris Van Ginneken, Lieselotte Van Bockstal, Steven Van Cruchten

Veterinary Sciences, Laboratory of Comparative Perinatal Development, University of Antwerp, Antwerpen, Belgium

According to the European Commission's statistics, the number of pigs used for research purposes in Europe has been relatively stable over the past years (2016-2020: ± 78,200). In 2020, 0.93% of the animals used for research and testing were pigs. However, it is important to note that there may be variations between the individual EU countries and that over these past years, a differing number of countries were included in these statistics[1]. Nevertheless, this number of pigs kept for research falls short of the 142 million pigs kept as livestock[2].

Pigs are used in biomedical research due to their anatomical, physiological, and genetic similarities to humans. This makes them good choices for exploring and refining techniques (e.g., surgical) and testing drugs for toxicity and safety. In this respect, they are frequently used as the second species/choice, next to a rodent species required by regulatory bodies. Recently, much knowledge has been gained regarding drug metabolism and pharmacokinetics in minipigs, which are alike in humans. Their smaller size, the genetic standardization, and the fact that breeding included a selection for a pig's temperament more suited for their research tasks make (mini)pigs a popular and increasingly used strain in pharmaceutical research replacing dogs and non-human primates (NHP). Secondly, pigs seem a very useful species in juvenile studies as they pose practical advantages over the dog: piglets are easy to handle, they have a relatively large litter size, they reach sexual maturity earlier than dogs and NHPs, and their size allows the conduction of basic procedures such as blood sampling from an early age onwards. It should be acknowledged, however, that less historical background data regarding drug-induced toxicities are available compared to the dog and NHP. Next to their use for toxicity and safety testing for new drugs, pigs are used in basic and applied research to study various human health conditions such as cardiovascular disease, diabetes, and cancer. Piglets are particularly useful in research because they are born with an immature immune system and thus can be used to study the development of immune function, responses to infections, drug development, and the effects of different nutritional interventions on growth and development. In the ICH S11 guidelines[3] and the literature[4], many similarities at the level of organ development in pediatric age groups have been reported between humans and (mini)pigs. In general, developmental patterns of the gastrointestinal tract, the cardiovascular, the central nervous systems, and the eye are similar between both species, while renal, immune, and reproductive development occurs slightly earlier and more rapidly in humans than in pigs.

Whether to use a pig or a minipig will depend on the aims and context of the research. The animal's growth rate will be an important parameter depending on the research question and design. In the case of surgical training, the domestic pig's size will be perceived as advantageous to identify anatomical structures. In contrast, in nonclinical safety studies, their size will be a limiting factor due to the restricted availability of the test compound, which is often administered based on kg body weight^{-1}. The latter is the exact reason for the development of miniature strains. Finally, the research question will also be an important determinant as some human pediatric conditions have an equivalent disorder in agricultural settings, i.c. intrauterine growth restriction[5]. Such studies on the domestic piglet could serve a dual purpose, fitting into the One Health approach.

Nevertheless, it should be borne in mind that the pig is a precocial species, whereas the human is an altricial species. In this respect, it is important to note that certain developmental processes occur at a faster rate and thus within a shorter time frame, making it more interesting to study a precocial species from an economic perspective.

In conclusion, basic knowledge of the different developmental processes (biological, molecular, functional) in the perinatal piglet is of utmost importance to judge whether it can be used as an animal model or at least to know the pitfalls when using the piglet as a model.

In the presentation, this will be reviewed in studies focusing on the gastrointestinal tract covering the preterm piglet, the IUGR piglet, and the piglet in pharmacokinetic studies.

References:
1. https://webgate.ec.europa.eu/envdataportal/content/alures/section1_number-of-animals.html#
2. https://ec.europa.eu/eurostat/web/agriculture/data/database?node_code=apro_mt_ls
3. https://www.ema.europa.eu/en/ich-q11-development-manufacture-drug-substances-chemical-entities-biotechnological-biological
4. Evaert et al. 2017, http://dx.doi.org/10.1016/j.anifeedsci.2017.06.011
5. Van Ginneken et al. 2022, https://doi.org/10.1002/MRD.23614

L7. ANAESTHESIA AND ANALGESIA IN CALVES, GOATS, SHEEP AND PIGS USED FOR BIOMEDICAL RESEARCH

Alenka Seliškar (SLAS), Daniela Casoni (SGV), Eddie Clutton (LASA), Gabrielle Musk (LASA), Stéphanie De Vleeschauwer (BCLAS), Sabine Bischoff (GV-SOLAS)

FELASA Working Group and Veterinary Faculty, University of Ljubljana, Slovenia
https://felasa.eu/working-groups/id/13

This FELASA working group (WG) was established to provide guidelines for the selection of anaesthetic and analgesic techniques in calves, goats, sheep, and pigs intended for biomedical research. The goal of the WG was to review existing recommendations in veterinary (and medical) anaesthesia and analgesia and to develop well-defined evidence-based guidelines that combine the highest welfare standards with the limitations imposed by the need to meet scientific outcomes.

The guidelines describe:

1) procedures that should be performed under physical or chemical restraint or under regional or general anaesthesia;
2) peri-procedural monitoring adapted to the species and procedure;
3) methods for pain assessment; and
4) peri-anaesthetic complications and their management.

The recommendations from the WG are based on a comprehensive review of the literature and personal experience, resulting in guidance for the delivery of safe anaesthesia in experimental farm animals.

The WG concluded that it is vitally important to involve veterinary anaesthesiologists in planning projects and conducting research. The recommendations emphasise that anaesthesia should not be viewed as a series of instructions in which specific drugs are administered in a specific order and in predetermined doses by prescribed routes. The acquisition of anaesthetic competence should be achieved in consultation with, or involving more experienced collaborators. The anaesthetic and analgesic technique for an animal in a given experiment must be agreed upon between those responsible for animal welfare and those conducting the experiment.

The guidelines are expected to be finalised in 2023 and published in a peer-reviewed journal.

L8. HEALTH & WELFARE MANAGEMENT OF EQUINE SPECIES IN RESEARCH AND EDUCATION

Amir Rosner (ILAF), Eduard Jose-Cunilleras (SECAL), Grigorios Maleas (HBLAS), Imke Van Dijk (DALAS), Guillemette Petti (AFSTAL), Isabelle Desjardins (AFSTAL)

FELASA Working Group and IIBR, Gabera, Israel
https://felasa.eu/working-groups/present/id/52

Equine are used for experimental research in a variety of fields. Horses and ponies are the most commonly used equine species in research, followed by donkeys, mules and hinnies.

There is little published scientific literature that describes current or evidence-based recommendations for keeping and managing these species for research purposes.

There are advantages and disadvantages of using horses in research. Disease research conducted in horses might be translated to similar health conditions in humans but compared to rodents, horses might not inherently seem like great models for studying human physiology and disease because they need large living space, they are expensive to maintain and there are safety and emotional aspects we need to take into account.

Equine species require particular environmental conditions that allow them to perform their natural behavior and physiology needs. In this lecture we will present briefly the practices for ambient facility conditions; provision of bedding, food and water, environmental enrichment and allocation of facility spaces. The staff of the facility (Animal caretakers and veterinarians) must maintain ambient conditions; provide bedding, feed and water; and allow opportunities for exercise and environmental enrichment, when needed, to properly accommodate captive horses. Careful consideration of these factors can help to improve the welfare of equine subjects in research and to ensure high-quality experimental data.

L9. FELASA RECOMMENDATIONS FOR HEALTH MONITORING AND EUTHANASIA OF LABORATORY FISH

Jean-Philippe Mocho

DanioVet, POTTERS BAR, United Kingdom

Fish recently became a major group of animal species used for research. FELASA, the Federation of European Laboratory Animal Science Associations, sponsored several Working Groups to propose recommendations for the care of laboratory fish. Here, we will focus on two sets of publications.

The FELASA-AALAS Working Group on health monitoring for fish in research allowed European and American fish veterinarians to write common recommendations for the monitoring and reporting of laboratory fish diseases and health status, with an emphasis on zebrafish (Danio Rerio). The publication was completed by a set of supplementary materials to provide templates for laboratories to describe their facility and husbandry practices. This supports the safe transfer of fish colonies between facilities, as described in the second publication of the working group on biosecurity in an aquatic facility, including prevention of zoonosis, introduction of new fish colonies, and quarantine. The results of a survey were also published and revealed the need for laboratory fish facilities to improve their quarantine practices. This survey was the opportunity to ask fish users about their methods of euthanasia and to identify gaps of knowledge and refinement opportunities. These are the pillars of the FELASA Working Group on methods of humane killing of laboratory fish. The draft recommendations of this working group are under review. It details advantages and disadvantages of a range of techniques (e.g., overdose of euthanasia, hypothermic shock, electrical stunning, concussion) according to species and developmental stages. Simple tips and protocols for good practice are proposed, and secondary methods for completion and confirmation of death as required by Directive 2010/63/EU are described.

References:

FELASA-AALAS Recommendations for Monitoring and Reporting of Laboratory Fish Diseases and Health Status, with an Emphasis on Zebrafish (Danio Rerio). Mocho JP, Collymore C, Farmer SC, Leguay E, Murray KN, Pereira N. Comp Med. 2022. 72(3):127-148. doi: 10.30802/AALAS-CM-22-000034

FELASA-AALAS Recommendations for Biosecurity in an Aquatic Facility, Including Prevention of Zoonosis, Introduction of New Fish Colonies, and Quarantine. Mocho JP, Collymore C, Farmer SC, Leguay E, Murray KN, Pereira N. Comp Med. 2022. 72(3):149-168. doi: 10.30802/AALAS-CM-22-000042

A FELASA Working Group Survey on Fish Species Used for Research, Methods of Euthanasia, Health Monitoring, and Biosecurity in Europe, North America, and Oceania. Mocho, J.-P. and von Krogh, K. Biology 2022, 11, 1259. https://doi.org/10.3390/biology11091259

WORKSHOPS

WS1 & WS2. FELASA WORKSHOP ON CLASSIFICATION AND REPORTING OF SEVERITY OF PROCEDURES

David Anderson[1,2], Dolores Bonaparte[1,3]

[1] *Core Trainer, FELASA Severity Working Group*
[2] *Pentlands Management Systems, DUNDEE, United Kingdom*
[3] *Fundação Champalimaud, Lisbon, Portugal*

Workshop 1: Farm Animal Models (8.00 – 10.45 h)
David Anderson

Workshop 2: Rodent & Rabbit Models (13.45 – 16.45 h)
David Anderson, Dolores Bonaparte

The Directive 2010/63/EU on the protection of animals used for scientific purposes entered into force in Member States on January 1st, 2013. As with the previous Directive 86/609/EEC, this Directive requires that experiments are designed to cause the least pain, suffering, distress or lasting harm to the animals used.
However, there are new and additional requirements which are that all procedures are assigned a severity classification in advance of the procedure being performed (prospective severity classification), and that the severity experienced by each individual animal must be reported in the Statistical returns (actual severity classification). This actual severity of any previous procedure is also a key consideration in determining whether or not an animal can be re-used in further procedures. The implementation of severity classification of procedures, both prospectively and at the end of procedures, is a big challenge in animal studies as it entails legal and ethical implications.

Prospective severity classification is an important and useful tool for properly evaluating research projects so as to implement the least constraining procedures, and thus keep the level of severity of procedures the lowest possible. Coherence in assigning actual severity classifications across Member States is very important to ensure harmonization in statistical reporting.

Correct reporting of the actual severity experienced by the animals will also help to improve communication with the public.

Formal retrospective assessment is required for projects which include severe procedures.

These three processes offer opportunities to implement the principles of 3Rs, aimed at improving animal welfare. In 2010, the FELASA/ESLAV/ECLAM Working Group elaborated on these new requirements with real examples which have been the object of many successful Workshops since the FELASA 2016 Congress. During these Workshops it became apparent that important differences in perceptions exist between participants when evaluating Severity.

The European Commission has since encouraged FELASA to create these ‘Road shows on Severity Classification of Procedures’ throughout Member states and beyond, so as to promote a coherent approach.

The two Workshops (one on rodents and one on farm animals) at the Congress will consist of presentations on the severity framework, group discussions on real examples and an interactive session on determining actual severity.

The workshop is suitable for Project Leaders, scientists applying for projects, veterinarians, animal care staff, advisors to and members of Animal Welfare Body, and those involved in Project Evaluation.

For these workshops we will use real-life research models:

- in **rodents** we will use a model evaluating the efficacy of an antitumour medication in mice and a model investigating brain pathways in decision making in the rat.
- in **farm animals** we will use a model to evaluate a novel treatment for cryptosporidiosis in calves and a model to evaluate the digestibility of novel Genetically Modified diets in sheep.

Learning resources:

Classification and reporting of severity experienced by animals used in scientific procedures FELASA/ECLAM/ESLAV Working Group report. Smith D, Anderson D, Degryse AD, Bol C, Criado A, Ferrara A, Franco NH, Gyertyan I, Orellana JM, Ostergaard G, Varga O, Voipio HM. Lab Anim. 2018 Feb;52(1_suppl):5-57. doi: 10.1177/0023677217744587

European Commission, Directorate-General for Environment, *Caring for animals aiming for better science – Directive 2010/63/EU on protection of animals used for scientific purposes : severity assessment framework*, Publications Office, 2019, https://data.europa.eu/doi/10.2779/068620

ETPLAS - https://learn.etplas.eu/all-courses/ - Free E-learning Module on EU 12 – The Severity Assessment Framework

David Anderson, BVMS MVM MRCVS

He is a veterinary surgeon with over thirty years experience in practice, academia and government service. He worked initially in a University teaching environment where he developed an interest and expertise in veterinary reproduction, and was Named Veterinary Surgeon under the Animals (Scientific Procedures) Act.

Until 2010, he worked in the UK Home Office in the Animals (Scientific Procedures) Inspectorate.

Since 2011, he has been working as a veterinary advisor for Pentlands Management Systems, where his main role is to provide technical support to the European Commission during the transposition of the new European Directive 2010/63/EU on the protection of animals used for scientific purposes.

David Anderson is a Vice-President of Institute of Animal Technologists and Past President of Laboratory Animal Science Association.

Dolores Bonaparte

She is a veterinarian who has been working in different areas of Laboratory Animal Science since 2001.

Currently the Designated Vivarium of the Champalimaud Foundation, in Lisbon, she is also responsible for assisting researchers in the refinement of experiments and for the Education & Training of staff and researchers.

Dolores is the Convenor of the FELASA Core Trainers Group responsible for further developing and ensuring the standards of the FELASA Severity Workshops. She is also involved in several Refinement and Education & Training initiatives at national and international level.

WS3. WORKSHOP ON SPECIFIC PROVISIONS OF DIRECTIVE 2010/63/EU ON THE USE OF GENETICALLY ALTERED ANIMALS IN SCIENTIFIC RESEARCH

Belen Pintado

GAA Taskforce
National Center of Biotechnology (CNB-CSIC), Transgenesis Service, Madrid, Spain

The main goal of the workshop is to help understand the explicit provisions of Directive 2010/63/EU regarding Genetically Altered Animals (GAA). It will focus on the Framework for the genetically altered animals under Directive 2010/63/EU on the protection of animals used for scientific purposes released by the Commission in November 2021.

The document can be downloaded in all languages at the following link
https://op.europa.eu/en/publication-detail/-/publication/7ff424e1-eb8f-11ec-a534-01aa75ed71a1/language-en/format-PDF/source-282223967

Participants are encouraged to read the mentioned document in advance.

The workshop will focus on GA rodents and it is dedicated to participants of 3 different functions:

- researchers using GAA in their scientific work,
- animal welfare bodies from facilities housing GAA, and
- Competent Authorities who authorize these projects.

It is desirable that participants are familiar with regulations and GA generation procedures: additive transgenesis, ES cell cased mutagenesis and CRISPR Cas/9 technology.

The estimated length of the workshop is 3 hours.

Desired minimum number of participants is 10 and a maximum of 15. Participants will be asked to fill out a short professional questionnaire in advance to help the facilitator to distribute them into the groups.

The first hour will include:

1) Presentation of attendants,
2) Presentation of workshop goals,
3) Refreshment of basic GAA concepts that are crucial for a good project design and reporting:
    a. Concept of line creation/establishment with specific mention of different GA generation methods.
    b. Breeding, genotyping and other management techniques

Attendants will be divided in 2-3 groups (estimated 4-5 people per group) to discuss and solve each one a practical case during 45 minutes:

A. Case 1 will focus on the creation of a new conditional GA line following different technologies, ES based or Crispr Cas9. The group will be asked to determine the number of animals that would be needed to be requested in the project authorization.

B. Case 2 will require to make the statistical report of a project of creation of a line already approved based on the data provided by the facilitator including severity. They will have to compare these data with the number and prospective severity in the original project.
C. Case 3 will involve estimating number of animals required to develop a project with harmful phenotype that needs to be imported from another facility, and that is going to be used to determine the potential tumour depression effect of a new molecule. With specific information provided in the case regarding the procedure, they will also have to make the statistical report

The 3 groups will join in a common session to present their results and discuss the outcome. The expected time devoted will be between 45 minutes-1 hour. During this session, the facilitator could present some specific cases and situations to be discussed in common if time allows.

Attendants will be asked to fill out an anonymous questionnaire in order to assess the impact of the workshop and identify areas for improvement. They will receive a certification of attendance describing the content of the workshop, learning outcomes and functions according to Directive as CPD activity.

SELECTED LECTURES

SL1. STRESS PROMOTES LIPID DROPLET ACCUMULATION IN ASTROCYTES

Anemari Horvat[1,2], Tina Smolič[1], Petra Tavčar[1], Urška Černe[1], Larisa Tratnjek[3], Mateja Erdani Kreft[3], Maja Matis[4,5], Toni Petan[6], Robert Zorec[1,2], Nina Vardjan[1,2]

[1] *Laboratory of Neuroendocrinology-Molecular Cell Physiology, Institute of Pathophysiology, Faculty of Medicine, University of Ljubljana, Slovenia*
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[3] *Institute of Cell Biology, Faculty of Medicine, University of Ljubljana, Slovenia*
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[5] *Cells in Motion Interfaculty Centre, University of Münster, Münster, Germany*
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The content of brain lipid droplets (LDs), lipid storage organelles, increases when the brain is in a pathological state, mainly in neuroglial cells and, to a lesser extent, in neurons. LDs have an important role in cellular lipid turnover and stress response. They provide substrates for energy metabolism, building blocks for membranes and signaling molecules, and protection of cells against lipotoxicity and free radicals. The characteristics of LDs and the molecular mechanisms driving LD accumulation in astrocytes, an abundant and heterogeneous subtype of neuroglial cells with key homeostatic functions, under stress conditions are poorly understood. Here, we studied the (sub)cellular localization and the content of LDs in isolated and brain tissue rat astrocytes, and in *Drosophila melanogaster* brain, after exposure to various stress stimuli associated with brain pathologies. LDs were examined by transmission electron microscopy and fluorescence microscopy in combination with the neutral lipid stains, Nile Red or BODIPY493/503. We found that in resting astrocytes, LDs have a diameter of ~450 nm and are located close to mitochondria and the endoplasmic reticulum in the soma and processes. Following attenuation of *de novo* LD biogenesis by inhibitors of DGAT1 and DGAT2 enzymes, the number of astrocytes decreased by ~40%, indicating the importance of LD turnover for cell survival and/or proliferation. When astrocytes were exposed to metabolic stress (nutrient deprivation, excess of extracellular free fatty acids and L-lactate) or hypoxic stress (1% pO_2), the content of LDs increased >2-fold, indicating accumulation of LDs. Similarly, increased LD accumulation was also observed with chronic exposure of astrocytes to noradrenaline, a neuromodulator of the brain stress response system. Noradrenaline triggers LD accumulation via activation of β- and α_2-adrenergic receptors and cAMP signaling. In addition, the accumulation of LDs was also increased in the brains of *Drosophila* exposed to hypoxic and nutrient stress conditions *in vivo*. Together, our findings show that noradrenergic activation and metabolic and hypoxic stress, which are associated with many brain pathologies, trigger LD accumulation in astrocytes. The observed response may serve to support energy metabolism and/or provide neuroprotection against lipotoxicity, which may increase the viability of stressed astrocytes and neighboring cells[1,2].

References:
1. Smolič T. *et al.* Astrocytes in stress accumulate lipid droplets. *Glia*. 2021. doi: 10.1002/glia.23978.
2. Smolič T. *et al.* Pathophysiology of Lipid Droplets in Neuroglia. *Antioxidants*. 2022. doi: 10.3390/antiox11010022.

SL2. ANTITUMOR IMMUNITY BOOSTED BY GASDERMIN D INDUCED NECROSIS

Sara Orehek[1], Duško Lainšček[1,2], Mojca Benčina[1,2], Roman Jerala[1,2] and Iva Hafner Bratkovič[1,2]

[1] *Department of Synthetic Biology and Immunology, National Institute of Chemistry, Ljubljana, Slovenia*
[2]*EN-FIST Centre of Excellence, Ljubljana, Slovenia*

Pyroptosis is a programmed form of immunogenic cell death mediated by gasdermin D (GSDMD) pore formation downstream of inflammasome activation. Induction of immunogenic cell death has proved promising in the context of cancer therapy. In the present work, we investigate the effect of GSDMD-induced pyroptosis of cancer cells on boosting anti-tumor immunity of solid tumors.

We have designed various GSDMD variants with different pore-forming activities and divergent modes of activation, where GSDMD variants are either transcriptionally regulated or GSDMD cleavage is chemically regulated. GSDMD variant pore-forming activity was tested *in vitro* and *in vivo* in a B16 mouse melanoma model, where tumors were injected with GSDMD variant-encoding plasmids and subsequently electroporated.

Pyroptosis-induced inflammation via tightly regulated GSDMD pore formation resulted in cytotoxic $CD8^+$ T-cell tumor infiltration, tumor regression, increased survival rate and remission. GSDMD treatment in combination with IL-1β, IL-18 or IL-12, which have known function in T-cell mediated antitumor immunity, further increased survival rate of treated mice.

In conclusion, stringently controlled formation of GSDMD pores leads to cancer cell death through release of immunostimulatory components, promotes infiltration of immune cells into the tumor microenvironment and stimulates a potent antitumor response.

SL3. EXTENT OF NECROSIS AS A MEASURE OF ACUTE PANCREATITIS SEVERITY IN STUDIES EMPLOYING LIVE CELL IMAGING

Polona Kovačič[1], Maša Skelin Klemen[1], Andraž Stožer[1], and Jurij Dolenšek[1,2]

[1] *Faculty of Medicine, University of Maribor, Slovenia*
[2] *Faculty of Natural Sciences and Mathematics, University of Maribor, Slovenia*

Acute pancreatitis (AP) is a severe clinical syndrome that involves autodigestion and destruction of pancreatic tissue and is brought about by an abrupt inflammatory injury to the pancreas. Long-established risk factors for acute pancreatitis include alcohol, endoscopic retrograde cholangiopancreatography, genetic factors, medications, and pancreatic ductal obstruction due to gallstone-mediated obstruction. AP can progress rapidly and cause severe symptoms, such as a systemic inflammatory response. Further, patients with AP can develop a severe and very painful inflammatory response, accompanied by multi-system organ failure and characterized by a high mortality rate.

Appropriate preclinical experimental models are crucial resources for understanding the pathophysiology of AP due to the ethical restrictions on inducing a disease state in humans for research purposes. As many mouse models reflect pathophysiological characteristics of AP comparable to the ones in humans, they are a valuable resource for the study of the origin, development, and course of the disease, as well as the investigation of the molecular mechanisms causing it.

The common approach to induce AP in animal models is supraphysiological pharmacological overstimulation of acinar cells. During such stimulation, high levels of intracellular calcium concentration are achieved and follow non-oscillatory dynamics, which leads to pathological basolateral exocytosis, fusion of exocytotic granules with other organelles, such as lysosomes, premature activation of digestive enzymes, and gland autodigestion, followed by an inflammatory response, interstitial edema, and necrosis of the acinus. A common and widely accepted mouse model for the induction of AP involves repeated applications of cerulein, a cholecystokinin (CCK) analogue that stimulates pancreatic acinar cells to secrete digestive enzymes. Supramaximal cerulein doses lead to vacuolization within acinar cells, followed by regeneration of the pancreas; higher doses cause pancreatic interstitial edema and inflammatory cell infiltration, together with a significant increase of the amylase levels in the blood. The model produces reproducible results and has strong clinical relevance, as it is the most appropriate model for the study of AP induced by organophosphate poisoning in humans.

Despite growing knowledge on the AP pathophysiology gained from the animal models, little is known about the AP-induced changes on the endocrine pancreas and its pathophysiology. There is mounting evidence indicating that the exocrine and endocrine functions of the pancreas are strongly interconnected, with exocrine failure having an impact on exocrine function, and *vice versa*. Calcium imaging of endocrine cells in live acute mouse pancreas tissue slices was demonstrated to be an effective approach to study the exo-endocrine interactions in the pancreas. However, there is inherent incompatibility between live cell calcium imaging in slices and the classic histological assessment of the severity of AP, where either whole body or tissue samples need to be fixed. Here, we set out to apply a novel approach to enable assessment of AP severity in acute tissue slices that would be compatible with calcium imaging on the same slices or neighboring slices from the same preparation.

To this end, we used the commercially available fluorescence LiveDead assay to assess AP severity, and endocrine function was examined with confocal calcium imaging, circumventing the need to fix the tissue to evaluate pancreatic damage. We validated our approach against the gold standard, i.e., classical histological grading of AP severity. The main advantage of our approach is the ability to practically simultaneously perform live cell imaging and assessing tissue damage in a quick and simple way on same specimens, decreasing biological variability and further decreasing the number of animals involved in a study.

The cerulein-induced AP in 8-16-weeks-old NMRI mice resulted in extensive damage to the acinar tissue and increased plasma levels of amylase while preserving the morphology of the islets of Langerhans. The signs of AP included pancreatic edema, necrosis, vacuolization, and inflammatory infiltration. The cerulein-induced changes did not differ between the duodenal, gastric, and splenic parts of the pancreas. Importantly, we found a good correlation between the classical histological grading of the AP and the extent of necrosis assessed on the living pancreatic slices. As the tissue was not subjected to histological fixation, we were able to functionally measure endocrine activity in parallel to measuring the extent of tissue damage, which would not be feasible with the classical histological AP characterization. In the stable plateau phase, when beta cells exhibit dependable, rapid calcium oscillations, we examined the active time, frequencies, and lengths of the oscillations. We noticed an increase in activity time and frequency with rising glucose concentrations in the AP group of animals. At any of the stimulating glucose concentrations, there were no statistically significant differences between the two groups when examining the duration of oscillations during the plateau phase.

In sum, assessing the extent of necrosis using the commercially available LiveDead assay may be used as a measure to assess AP severity in studies employing live cell imaging, and is expected to become a standard tool in future studies of AP pathophysiology in animal models.

SL4. SUCCESSFUL REHOMING AS ULTIMATE REFINEMENT FOR GOLDEN HAMSTERS IN SARS-COV-2 VACCINE RESEARCH

Malan Štrbenc[1], Urška Kuhar[2], Duško Lainšček[3], Brigita Slavec[4], Uroš Krapež[4], Nina Kočar[4], Joško Račnik[4], Jelka Zabavnik Piano[1], Tadej Malovrh[2], Gregor Majdič[1]

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[4] *Institute of Poultry, Birds, Small Mammals and Reptiles, Veterinary Faculty, University of Ljubljana, Slovenia*

In 2020, Slovenia joined the global effort to develop effective vaccines and drugs to treat SARS-CoV-2. The golden (Syrian) hamster is the recommended species for preclinical research on the disease because it is the only known non transgenic rodent species that is susceptible to the virus due to its high levels of sequence homology with human ACE2 receptors. They develop strong viral titers within 48 hours of inoculation and develop transient pneumonia with mild clinical signs.

Forty-eight hamsters were vaccinated with promising vaccine candidates testing different vaccination regimens. Twelve animals were used as controls with viral challenge only. Animal work with viral challenge was conducted under appropriate biosafety conditions in a BSL-3 facility equipped for animal husbandry and appropriately licensed. Refinements to reduce animal pain and distress were observed all the time, all applications (intranasal, intramuscular, subdermal) were performed under general isoflurane anesthesia, and blood samples were collected simultaneously from the junction of the cranial vena cava, external jugular vein, and subclavian vein. ELISA tests were performed to determine end-point serum titers for specific antibodies to select the best vaccination regimen.

Further refinement was then introduced to check the efficacy of the specific antibodies. It was first tested in vitro with a virus neutralization test on VeroE6 cells and the SARS-CoV-2 virus strain Slovenia/ SI-4265/20. Since all tests with serum from vaccinated hamsters were negative even after booster vaccinations and vaccine adjuvants, we concluded that these specific antibodies do not have sufficient neutralizing properties in hamsters. It is likely that they differed in conformation from antibodies produced in prior tests in mice, but this was not further investigated with additional procedures in animals. Given the negative results of the in vitro method, we put into practice the next R of the three Rs - we replaced 42 animals in a moderate procedure, they were not transferred to the BSL3 facility and were therefore subjected to only a first set of procedures with mild severity. We can also claim that the total number of animals in the project was reduced because no further preclinical experiments were conducted.

The institutional animal welfare officer was consulted to approve the reuse or rehoming of the now surplus animals. The institutional designated veterinarian certified that most of the animals were in good health, so they could be reused or rehomed, even though they were about halfway through their life expectancy at that point. In the meantime, simple socialization measures were carried out. A few animals did not get used to handling well, so we deemed them unsuitable for rehoming as pets.

In the end, 19 animals were rehomed. We aimed for procedure to be transparent; interview between the person responsible for the animals and potential adopters was done initially, a simple adoption

contract was signed, and voluntary follow-up meetings were held in the following months. Initial adopters were internal – staff of the Veterinary Faculty, and there was significant external interest and very positive feedback. The FELASA Recommendations for rehoming of animals used for scientific and educational purposes deal mostly with farm animals and larger companion animals. However, based on our positive experience we have concluded that rehoming is also possible for rodents, and calls for careful planning of further project proposals.

SL5. IMPROVING BIOMEDICAL RESEARCH BY AUTOMATED BEHAVIOUR MONITORING IN THE ANIMAL HOME CAGE (COST TEATIME)

Maša Čater

Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Slovenia

Laboratory animals are used throughout the biomedical field of research to study physiological and pathological conditions in living organisms with the ultimate aim to understand the mechanisms and to develop treatments for human and animal disorders. More than 70% of the total number of animals used for this purpose in Europe are mice and rats. This work is guided by the principles of the 3Rs (Replacement, Reduction, Refinement), where developing refined experimental conditions could substantially improve animal welfare and, importantly, enhance the translational value by improving the quality and reproducibility of the data. There are also implications regarding Reduction, as more accurate and reliable data reduces the waste of animals in unreproducible research. Additionally, the ability to monitor animals without disturbance can reduce variability caused by handling stress, thus allowing for a better 'signal-to-noise' ratio, with fewer animals thus being required to detect a given effect size.

COST TEATIME Action, which started at the end of 2021 and will last until 2025, combines experts in mouse behaviour, laboratory animal science and data science, animal welfare specialists and industry representatives from 34 European countries to critically and transparently assess the potential of home cage monitoring (HCM) technologies. The main aims of the Action are (i) to develop a framework for enhancing the sensitivity, reliability, and reproducibility of behavioural and physiological measures from laboratory rodents in a home cage; (ii) to strengthen pan-European preclinical research by establishing standards and guidelines in promoting emerging tools for home-cage monitoring; and (iii) to improve data relevance and reproducibility in compliance with animal welfare and ethical considerations.

The COST_TEATIME action pursues several goals. First, the Action aims to identify currently unmet community needs for further technological development in HCM. COST_TEATIME surveyed researchers in mouse behaviour, laboratory animal science and data science from both academia and industry. The purpose of this survey was to assess the unique opportunities for HCM by gathering the views to inform future developments and challenges in the field. A broader aim of the Action is to expand our network by connecting researchers across various disciplines who are using and developing HCM systems, as well as those in industry, and manufacturers, and to bring together a critical mass of European experts in this emerging technology. This endeavour will also result in the establishment of communication channels to expand the possibilities for knowledge transfer, which will also be valuable for other activities within the Action.

Complementary to the survey, a systematic review of the literature on existing HCM systems was prepared, allowing a comparison of their features, potential and limitations. In addition, Action participants are exchanging knowledge about the various HCM systems available to them, compare experimental designs and parameters measured in the members' laboratories and share baseline data collected. This is also helping to determine how datasets from different HCM systems can be integrated. Both the systematic review and direct comparisons will contribute to identifying future requirements for these systems. Eventually, our activities aim to develop new lasting forums to bridge

behavioural and data science to achieve breakthroughs in the integration and analysis of complex datasets, which will be useful for other projects in biomedical research beyond HCM in the future.

The scientific community can benefit from the activities of the Action in multiple ways. Apart from the systematic review, a catalogue of available HCM systems with standard operating procedures established by Action members has been developed and is available on the Action's website. The goal of this activity is to reduce the fragmentation of HCM development and to share best practices on HCM system use with the wider research community. However, the most important activity is the COST_TEATIME training programme – workshops, webinars (recordings available at COST TEATIME YouTube channel) and short-term scientific missions (funding of short research visits to another lab/country), fundable through the Action. These measures will build capacity, not only about the emerging HCM technologies but also by establishing a sustainable, interconnected and well-trained European network of mouse behaviour analysts spanning all ages and career levels. The Action encourages representation and active participation of Early Career Investigators and researchers from inclusiveness target countries with fewer resources in the field of HCM and other related research areas.

MY RESEARCH in 180s

MR1. NUTRITIONAL STUDIES OF DIETARY- AND HEAT-INDUCED OXIDATIVE STRESS IN BROILERS: DEVELOPING EFFECTIVE STRATEGIES FOR AMELIORATION THROUGH ANTIOXIDANT SUPPLEMENTATION

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Poultry production worldwide is often challenged with various external factors, including nutritional and environmental stressors that negatively impact the performance, health, and welfare of broilers. These stressors cause excessive production of reactive oxygen species (ROS), resulting in decreased antioxidant defense and increased oxidative stress, which in turn leads to oxidative damage to biological macromolecules.

Supplementing broiler diets with n-3 polyunsaturated fatty acids (PUFA)-enriched plant oils can enhance the nutritional value of chicken meat by increasing the n-3 PUFA content and reducing the n-6 to n-3 PUFA ratio, which is beneficial for human health. However, increased intake of n-3 PUFA, which are more susceptible to oxidation, may increase oxidative stress in birds, leading to lipid oxidation and rapid depletion of both, endogenous and dietary antioxidants. Moreover, broilers are highly susceptible to high ambient temperatures, leading to heat stress due to their anatomical characteristics and genetic selection, as modern broiler genotypes generate more body heat owing to their high metabolic activity. Heat stress can negatively affect broiler performance and lead to behavioral, immune, and physiological disorders, resulting in increased mortality and economic losses. Various management and nutritional approaches have been proposed to mitigate the negative effects of oxidative stress, and previous studies have shown that dietary supplementation with vitamins and minerals provides promising results.

At the Chair of Nutrition, Department of Animal Science, Biotechnical Faculty, we conduct nutritional studies in broiler chickens in our animal research facility. The research facility consists of two separate compartments, with 12 deep litter pens of identical dimensions (0.95 m × 1.26 m) per room. Each pen is equipped with a plastic feeder and an automatic nipple watering system. In nutritional studies, we use feed mixtures where the required nutrient ratios and amounts are calculated considering the composition of the feed ingredients before the experimental feed mixtures are mixed in the registered feed mill at the Department of Animal Science. Our recent nutritional studies in broilers have focused on investigating the effects of high n-3 PUFA intake and cyclic heat stress, either alone or in combination, on broiler growth performance, oxidative status, and gut health. To ameliorate oxidative stress caused by both external stressors, we supplemented broiler diets with vitamins E and C, as well as selenium, as these are important antioxidants that can act synergistically. Performance parameters such as body weight, body temperature, feed intake, and feed conversion are monitored weekly. To prevent any health-related problems, clinical observations of broilers are performed daily in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes.

Towards the end of the experiment, which usually lasts until 42 days of age, birds are randomly selected from each experimental group for blood collection to perform the Comet assay. This assay is used to determine the DNA fragmentation of lymphocytes in the blood. At the end of the experiment, we perform a blood gas analysis to determine blood chemistry, hematology, and acid-base balance,

which helps us determine if the broilers were under heat stress. Once the selected birds are slaughtered at our experimental slaughterhouse, we collect blood, organ, intestinal and meat samples for further analysis. In our analytical laboratory we analyze the composition of feed, bones, tissues, and litter, as well as macro- and microminerals using AAS. We also perform analyses of α- and γ-tocopherol, vitamin C, and the product of fat oxidation, malondialdehyde, using HPLC. In addition, we perform analyses of the oxidation products of lipids and DNA (F2-isoprostanes and 8-OHdG), Hsp70, and corticosterone concentrations using ELISA kits, and fatty acid analyses using GC. Finally, we evaluate the antioxidant capacity of water-soluble and lipid-soluble compounds using the PhotoChem method.

Conducting nutritional studies in broilers is crucial for developing effective strategies to mitigate the negative effects of external stressors that are common in broiler production and can cause oxidative stress. Maintaining broiler health is an essential factor in improving performance parameters, especially when these strategies are implemented under practical farming conditions. In addition to improving broiler health and performance, the development of efficient nutritional strategies could also be of great interest to the poultry industry to minimize economic losses. Therefore, future research on this topic will focus on further investigating the efficacy of different nutritional strategies to mitigate oxidative stress in broilers and maintain their welfare.

MR2. THE USE OF BLOOD SMEARS TO ASSESS HEALTH AND STRESS IN CAPTIVE NON-MODEL AMPHIBIAN, THE OLM *PROTEUS ANGUINUS*

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The olm (*Proteus anguinus*) is a neotenic amphibian endemic to the underground waters of the Dinaric Karst. It is classified as vulnerable in the Red List of Threatened Species of the International Union for Conservation of Nature (IUCN) and is protected by national laws in all countries within its range. Due to its specific biology with a low reproductive rate, its longevity (its life expectancy is over 70 years) and its subterranean habitat in porous limestone, the olm is considered highly susceptible to pollution and pathogens responsible for the global decline in amphibian populations. Considering all of this, it is important to develop non-destructive methods to assess the effects of environmental stressors on this species and to maintain healthy captive populations of olm that can be used in research for conservation purposes and for the purpose of repopulation in the event of local extinction.

Health status and exposure to chronic stressors can be assessed using various hematologic parameters. The advantage of using hematologic data is that blood sampling is a nondestructive and minimally invasive method (only one drop of blood is needed), while the preparation of a blood smear is simple, easy, and inexpensive. Based on the morphology of the blood cells and the differential count of leukocytes, chronic diseases or acute infections can be detected even if the animals do not show obvious signs of disease. In addition, the neutrophil to lymphocyte ratio (N:L ratio) can be used to assess animal stress, which can have a major impact on animal fitness, reproduction, and experimental results. Each type of leukocyte has a specific function in the immune system, and the role is almost identical in all vertebrates, making it possible to detect abnormalities and sometimes to identify the cause of a deviation from the normal leukocyte profile. Such a deviation was recently described during an infection with an opportunistic pathogenic fungus, in which the number of monocytes was greatly increased to 46% (Bizjak Mali et al., 2018).

The aim of our work was to gain basic knowledge of the hematology of this endarged species and to establish reference values for leukocyte differential counts in captive animals. Using blood smears from both recently captured and long-term captive animals, we examined for the changes of leukocyte counts during captivity. In this study, however, we were limited by the number of animals that could be captured and held in captivity under the permits. Over the course of five years, we were able to analyze the blood samples from 11 animals from the largest known population of the olm from the Planina-Postojna cave system.

The olm leukocyte profile shows a typical urodelan pattern with lymphocytes being the most abundant ($80.4 \pm 9.7\%$), followed by neutrophils ($10.2 \pm 5.0\%$), while the count of eosinophils and monocytes is lower ($6.9 \pm 4.1\%$ and $2.5 \pm 4.5\%$, respectively) and basophils are almost absent. Among our examined olm specimens, we found no abnormalities in blood cell morphology, and we detected none of the blood parasites commonly found in wild populations of amphibians and other ectothermic vertebrates. The leukocyte profiles of the observed olms also indicate that no detectable infection or disease was present.
Differential counts also allow us to detect physiological stress, which can be assessed by a higher N:L ratio compared with baseline levels. Such changes in leukocyte proportions are particularly useful for analyzing long-term stress, e. g. long-term captivity, because the high N:L ratio persist longer than

elevated stress hormones (reviewed in Davis and Maney, 2018). In non-stressed amphibians, the reported N:L ratio ranges from 0.01 to 0.67, while a threefold increase in the ratio has been observed in stressed salamanders (Davis, 2009). The average N:L ratio in olms was low in both recently captured animals (considered stressed due to handling and transport to captivity) and captive animals, and was within the reported range for other amphibian species. Although the N:L ratio was slightly higher in recently captured olms, there was no statistical difference in the N:L ratio between the two groups. Given the literature that capture and handling cause stress in animals, we are not convinced that the N:L ratio in olms actually reflects stress as is it known in other vertebrates. In other words, we were unable to define captive animals as non-stressed based on hematological data, and further research is needed, including analysis of stress hormones. Nonetheless, the olms that have been kept in the captivity for years, are fed and handled regularly and are presumably habituated to the captive environment. In addition, the growing oocytes and maturing testes indicate that reproductive cycles are not inhibited, as is known to be the case with chronic stress.

Although much work has been done in this study and the reference values obtained can be used to evaluate the physiological status of the animals, several aspects still need to be investigated. Further studies should include age, sex, and reproductive status to allow a more comprehensive interpretation of the hematological data. In addition, the health status of various natural populations needs to be studied to better understand the impacts of environmental stressors on this vulnerable species. Importantly, blood analysis is a useful tool to improve the housing, handling, and procedures of captive animals.

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MR3. CHANGES OF EXTRACELLULAR MATRIX IN THE MIDCINGULATE CORTEX AFTER PERINATAL HYPOXIA IN RATS

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This research investigates the effect of moderate non-invasive short-term perinatal hypoxic injury in rats on the critical components of perineuronal nets (PNNs) in the midcingulate cortex (MCC) in adult rats. In total, 30 Wistar Han (*RccHan: WIST*) rats at P1 were sex-determined, weighed, and randomly divided into hypoxic or control groups, keeping both sexes represented equally per experimental group (three females and three males). Rats were placed in a heated hypoxic chamber together with nest and dams' cage bedding, a thermometer, and a hygrometer and subjected to hypoxic conditions: partial pressure of oxygen (pO_2) 9.7325 kPa (73 mm Hg); atmospheric pressure (p^{ATM}) 46.6628 kPa (350 mm Hg) or control conditions: pO_2 21.1983 kPa (159 mm Hg); p^{ATM} 101.3250 kPa (760 mm Hg) for 2 hours. Subsequently, all rat pups were marked by a permanent toe tattoo, returned to the cage with the dams, and used later in the study. Animals were sacrificed at the age of 3.5 months for immunofluorescence (IF) analysis of molecular components of PNNs – aggrecan, neurocan, versican, and their colocalization around parvalbumin (PV) neurons. IF analysis revealed differences in the PNNs' morphology, distribution, and composition in the MCC in the hypoxic group compared to the control group.

Improvement of this research was made in terms of animal welfare. The 3R principles (*Replacement, Reduction, and Refinement*) were applied in the research design and while conducting the experiment. Although an alternative model could not be used, the reduction of the number and reuse of animals as well as refining the procedure for conducting the experiments was imperative. The refinement of this research was made from the aspect of better utilization of animals and the application of current knowledge about the welfare of laboratory animals – health supervision, better housing conditions, personnel, and the researcher education, and application of modern methods aimed at reducing pain, suffering and/or anxiety of laboratory animals. In this aspect, we used the least invasive method of marking, the nest and dams' cage bedding in all experiments, the shortest possible separation of dams from pups. Also, pregnant females were kept in an enriched environment with nesting material while raising pups with edible cellulose tunnels and wrapped thick toilet paper sticks with shallow dust content. In addition to the 3R principles, the 4A's of responsible experiments on laboratory animals were also taken into account in the research design – 1) *Awareness* of the suffering experienced by the experimental animal during the experiment, 2) to *Assess* all procedures on animals to 3) *Avoid* or 4) *Alleviate* the suffering of the animals in the experiment.

This research is co-financed by the Croatian Science Foundation project: "Brain extracellular matrix in development and perinatal hypoxia" IP 2019 04 3182, and the Scientific Centre of Excellence for Basic, Clinical and Translational Neuroscience project: "Experimental and clinical research of hypoxic-ischemic damage in perinatal and adult brain" GA KK.01.1.1.01.0007 funded by the European Union through the European Regional Development Fund.

MR4. CBD AS A POTENTIAL THERAPEUTIC AGENT OF INTERSTITIAL CYSTITIS /BLADDER PAIN SYNDROME

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Interstitial cystitis/bladder pain syndrome (IC/BPS) is a chronic aseptic bladder inflammation that predominantly affects women. The disease is clinically presented as increased urinary frequency, persistent pain in the pelvic area, painful urination, nocturia, vulvodynia, and even depression and anxiety. The pathophysiology of IC/BPS remains elusive, which has resulted in unsuccessful treatment. Cannabidiol (CBD), a phytocannabinoid extracted from *Cannabis sativa,* is known to possess various anti-inflammatory and pain-relieving properties and is readily available as dietary supplement. However, its mechanisms of action are still poorly understood. The aim of our study was to evaluate the effects of CBD treatment on the improvement of IC/BPS-related symptoms in a cyclophosphamide (CYP)-induced mouse model of the disease.

IC/BPS was induced in 20 adult female C57BL/6J mice using CYP at a dosage of 80 mg/kg applied *i.p.* 4-times over 10 days (*Figure 1, blue syringes*). The CBD group (n=10) received 1.5 mg/kg CBD *i.p.* daily for 9 days (*Figure 1, yellow syringes*), while the control animals (n=10) received corresponding volumes of the vehicle. During the experiment, animals were monitored for burrowing behavior to assess the presence of pain (*Figure 1, bBUR: basal measurement; eBUR: experimental measurement*). Euthanasia was conducted on the 10th day of the experiment. Urinary bladders were excised and processed for tissue sectioning, immunolabeling of Ki67 and active caspase 3, *ex vivo* transepithelial electrical resistance (TEER) measurements, scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

The monitoring of burrowing behavior showed increased activity in CBD-treated animals compared to controls, demonstrating an analgesic effect of CBD. After the disruption of urothelium with 15 min exposure of the urothelium to 0.01% poly-L lysine in an *ex vivo* system for TEER measurements, the drop of TEER was less severe in the CBD-treated group (58 % decrease in TEER value) compared to vehicle-treated controls (77 % decrease in TEER value). At the end of the regeneration phase urothelial TEER recovered to 75 % and 57 % of the baseline value in the CBD group and the vehicle-treated controls, respectively. SEM showed a well-preserved luminal surface of the urothelium in the CBD-treated group with maintained tight junctions and only individual exfoliated superficial cells. In contrast, superficial cells were extensively exfoliated in the vehicle group with areas of deep urothelial injury reaching the basal lamina. Disrupted urothelial cell junctions in the vehicle group were also observed with TEM, revealing increased intracellular spaces in all urothelial cell layers. Immunolabeling revealed a higher urothelial proliferation and apoptotic activity in the CBD-treated group versus the control group, indicating an increased urothelial turnover and accelerated regeneration after CBD treatment. Moreover, edema of the lamina propria was reduced in the CBD-treated group compared to the vehicle-treated group.

CBD treatment aided in pain relief, restoration of the urothelial barrier via fast re-establishment of cell junctions and urothelial turnover, protected the urothelium against severe CYP-induced injury, and reduced inflammation in the bladder lamina propria. Therefore, the analgesic and anti-inflammatory effects of CBD may be exploited in the treatment of IC/BPS in the future.

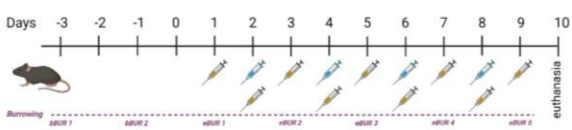

Figure 1*: Timeline of the experiment.*

MR5. *IN SITU* VACCINATION WITH ELECTROCHEMOTHERAPY

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Apart from its direct cytotoxic effects, electrochemotherapy (ECT) can also elicit indirect antitumor effects by stimulating the immune system response. This occurs through increased expression of immunologically significant molecules on tumor cells and the induction of immunologically recognizable cell death, accompanied with the release of tumor-associated antigens and the accumulation of damage-associated molecular patterns (DAMPs). This process can lead to an *in situ* vaccination effect. Since recently, ECT is commonly combined with immunotherapies. To enhance responses and outcomes, it is crucial to carefully time the combination therapies. Thus, the primary objective of this study was to compare and describe the timeline of immunologically relevant biomarkers after intratumoral ECT with bleomycin (BLM), cisplatin (CDDP), and oxaliplatin (OXA). We examined the cytotoxic and immunomodulatory effects of ECT and determined whether it is sufficient in accomplishing effective *in situ* vaccination.

In this study, we investigated the immunological effects of ECT using equivalent doses of BLM, CDDP, or OXA on three distinct murine tumor models (B16F10, 4T1, and CT26), both *in vitro* and *in vivo*. *In vitro*, we evaluated the type of cell death and expression of MHC-I, MHC-II, PD-L1, and CD-40 in tumor cells. *In vivo*, the antitumor effect of ECT was compared between wild-type mice and immunocompromised (NUDE) mice, and immune-related mechanisms such as cell death and infiltration of tumors by different immune cells were compared. The latter was evaluated using cytometric and histological analyses. To assess the systemic effect, the study was also performed on a metastatic model established through induced lung metastases.

We showed that the three tumor models are differently sensitive to ECT; ECT was more effective in more immunogenictumors, with CT26 demonstrating the greatest sensitivity followed by 4T1 and B16F10 tumors. The involvement of T cells in the antitumor effectiveness of ECT with CDDP was confirmed through the use of immunocompromised mice. Additionally, histological analyses revealed that ECT attracted other immune cells intratumorally. ECT also altered the tumor's expression of MHC I, MHC II, PD-L1, and CD40, as well as induced DAMPs associated with immunogenic cell death. However, the kinetics of these changes were specific to the cell line and drug used. Finally, it is noteworthy that ECT with CDDP and BLM resulted in an abscopal effect.

The objective of this study was to conduct a systematic evaluation of the effects of ECT with CDDP, BLM, or OXA in three tumor models. Our findings revealed that the response of the three models to ECT differed, with immunogenic tumors showing a greater response to all three drugs. Additionally, we confirmed the involvement of the immune system in the response, with the level of immune system activation being dependent on the drug and tumor model type. Hence, the selection of the drug for ECT should primarily be based on the tumor's immune status. The results obtained from this study will enable us to design a more effective combinatorial treatment protocol.

MR6. COLLAGEN-INDUCED ARTHRITIS IN RATS - CHALLENGES AND LIMITATIONS

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Collagen-induced arthritis (CIA) is the most used model of this disease in rats, characterized by joint inflammation and cartilage destruction. This model is recommended for the evaluation of the potency of topically applied nonsteroidal anti-inflammatory drugs (NSAIDs).

The aim of the study was to evaluate the effect of topical application of NSAID on the concentration of tissue immune parameters and pathohistological tissue changes in CIA in male Wistar rats and to define limitations of this model.

Arthritis was induced by subcutaneous injection of Bovine collagen type II (CII) and Incomplete Freund's Adjuvant (IFA) emulsion in 24 rats. Control group (n=6) received 0.9% NaCl. Two weeks later, rats with induced CIA were randomized into 3 groups with 6 rats and patches containing diclofenac, ketoprofen or piroxicam were applied for 5 consecutive days. The positive and negative control group were treated with placebo patch. On day 19, the tissue for measurement of interleukin 17 (IL-17), prostaglandin E2 (PGE2) and the transporter signal transducer 3 (STAT3) (ELISA method), as well as the tissue for pathohistological analyses were taken after the animals were sacrificed on day same day. Statistical data processing was performed using the SPSS Statistics v 20.0 software.

There was a statistically significant difference in PGE2 values in the diclofenac-treated group compared to ketoprofen group as well as negative control. There were no statistically significant differences between the groups in levels of IL-17 and STAT3. Although the study was performed in accordance with the 3R principles, including the use of a small sample size to minimize harm to the animals, this animal model showed certain challenges and limitations. Statistically significant differences were recorded in the values of pathohistological findings for oedema and inflammation of the synovium between the groups. There was no statistically significant difference between groups for bone erosion, cartilage erosion and neutrophil accumulation.

Diclofenac was suggested as the preferred first-line treatment based on its effect on the levels of PGE2. However, further research is needed to enhance our understanding of how immunological parameters contribute to the pathophysiology of the disease Due to the absence of more severe microscopic changes in the joints we recommend designing studies with the period of induction longer than two weeks.

MR7. CELEBRATING OPENNESS ABOUT ANIMAL RESEARCH WITH EUROPEAN ANIMAL RESEARCH ASSOCIATION AND #BOARD23

Maša Čater

European Animal Research Association, London, United Kingdom

The European Association for Animal Research (EARA) is a non-profit communication and support organization whose mission is to defend the interests of biomedical research and health development in Europe. EARA provides accurate and evidence-based information that demonstrates the achievements made through the use of experimental animals in biomedical research. In this way, EARA informs, educates, and unifies the different audiences that support animal research and promotes a transparent debate about the use of experimental animals.

The association has close to 130 member organizations from public research institutions, representative bodies, and private biomedical research, as well as breeders and other suppliers to the sector, in 23 European countries and five other countries worldwide.

Annually, EARA organizes an event 'Be Open About Animal Research Day' (#BOARD). This global, 24-hour social media campaign celebrates openness by biomedical institutions in communicating animal research. This year, #BOARD23 is happening on June 15, 2023, when biomedical institutions from around the world will share their stories and experiences of being open with the public. EARA will coordinate the 24-hour global campaign supported by the wider biomedical community, from both public and private research, across the continents of the world.

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POSTERS

P1. ADDITION OF A TLR COSTIMULATORY DOMAIN ENHANCES THE ACTIVATION OF CD19 CAR T CELLS

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Chimeric antigen receptor (CAR) T cell therapy is a type of immunotherapy that has recently emerged as an effective treatment against several hematological malignancies. Intracellular signaling domains of genetically modified CARs derive mainly from T cell receptor and their costimulatory receptors. In addition to the conventional signaling molecules, Toll-like receptors (TLRs) can also function as costimulatory signals to enhance T cell activation, proliferation and survival. Therefore, TLR-derived signaling elements could be potent costimulatory domains in the structure of CAR T cells. In our study, we developed anti-CD19 CARs that besides the 4-1BB costimulation domain also contain TLR activation domains.

We designed and generated various CAR constructs with specific elements of adaptor protein MyD88, TRIF, and additionally some constructs with truncated TLR4. First, we made the selection of our CARs by measuring hIL-2 production from Jurkat cells that had been previously electroporated with plasmid constructs and co-cultured with target CD19 positive cells. Constructs that generated higher activation compared to second-generation CAR were selected for further experiments. Human T cells transduced with selected CAR construct were found to be highly potent at inducing hIL-2, hIFN-γ, hTNF-α and cytotoxicity when co-cultured with several CD19 positive tumor cell lines. The analysis with the CD19 negative cell line did not show any cytokine secretion. No significant difference in immunophenotype was observed between our CAR T cells and second-generation CAR T cells. Finally, the efficacy of our CARs was evaluated in vivo using an NSG mouse model.

Our findings suggest that TLR signaling enhances the activation of the CAR T cells and that the additional TLR costimulatory domain did not induce constitutive activation.

P2. MULTI-APPROACH STUDY OF *PLA2G4E* INVOLVEMENT IN OBESITY DEVELOPMENT IN UNIQUE FAT AND LEAN MOUSE LINES

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Obesity, controlled by a combined effect of genetic and environmental factors, has been studied intensively in recent decades. Yet, the mechanisms of action that trigger metabolism disorders have yet to be fully discovered. In our study, we used fat (F) and lean (L) mouse lines representing unique polygenic models of obesity and leanness. Previous transcriptome studies on these lines fed standard maintenance chow diet identified gene *Pla2g4e* as differentially expressed. Our experiment aimed to study *Pla2g4e* on RNA and protein levels in an independent investigation comparing these lines on a high-fat (HFD) or a low-fat diet (LFD) that were isocaloric. *Pla2g4e* is involved in nutrient metabolism and energy expenditure and is mainly expressed in the heart, thyroid, brain, skin, and testis. Additionally, it is highly expressed in the skeletal muscles and involved in their metabolism and function. Thus we used *musculus gastrocnemius* for *Pla2g4e* expression analyses and *m. gastrocnemius*, *m. soleus* and *m. tibialis* for immunofluorescence staining of its protein product, cPLAε.

Obesity in F mice has developed in both diet treatments despite the same feed intake between the mouse lines. The effect of genetic background and diet on body weight was more significant in females than in males. F mice were 40 % (males) and 44 % (females) heavier than L mice fed with LFD, and 57 % (males) and 69 % (females) heavier than L mice when fed with HFD.
The real-time PCR was used to quantitate *Pla2g4e* mRNA in *m. gastrocnemius* and the results have been normalized to the expression of housekeeping genes *Gapdh* and *Actb*. *Pla2g4e* expression in *m. gastrocnemius* in F mice was several-fold higher than in L mice, regardless of the diet. *Pla2g4e* fold change in *m. gastrocnemius* of F mice was 3.3 (females) and 3.8 times (males) greater than in L mice fed with LFD, and 4.4 (females) and 4.5 times (males) greater than in L mice fed with HFD. A differential *Pla2g4e* expression pattern may be due to several polymorphisms in the *Pla2g4e* gene between F and L lines. Whole-genome sequencing and bioinformatic analyses revealed genetic variants within predicted miRNA target sites in 3'UTR and CTCF binding motifs in F but not in L mice. Fewer miRNA and protein silencing mechanisms may affect *Pla2g4e* expression in the muscles of F mice than in L mice, resulting in higher *Pla2g4e* expression and fat phenotype.
cPLAε expression in different skeletal muscles, determined by fluorescent immunostaining, was revealed to be muscle fiber type-specific. Only the oxidative fibers of type I (slowly contracting) and type IIa (fast contracting) expressed cPLAε. This finding is the first of its kind and points to the critical involvement of *Pla2g4e* in skeletal muscle aerobic respiration, which malfunctioning can contribute to obesity development.
These results point to the importance of genetic background in obesity development and suggest that *Pla2g4e* is one of the potential novel genetic factors highly linked to obesity that deserves further functional and mechanistic research.

P3. SARS-COV-2 CELL ENTRY AND ITS INHIBITION USING SHORT ANTIOXIDANT PEPTIDES

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After three years of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, the virus appears here to stay and has yet to settle into a predictable pattern. The virus can evolve and adapt quickly, which results in many new fast-spreading variants and can cause long-term post-COVID with persisting symptoms. Since the interaction between the SARS-CoV-2 spike protein and angiotensin-converting enzyme-2 (ACE2) receptor is essential to viral entry, interference in such binding represents an important therapeutic strategy. Spike protomers are rich in cysteine residues, many forming disulfide bonds that have an essential structural role in allowing a high-affinity interaction with the receptor. Previously studied redox-sensitive viral fusion proteins had been shown to be impacted by antioxidants, making antioxidants beneficial not only in preventing oxidative stress-related disorders but also in influencing viral cell entry. Described herein, we demonstrate the importance of disulfide-thiol balance within the spike protein for viral infection. By utilizing cell-cell fusion and in vitro (pseudo)viral systems, we showed reduced (pseudo)viral cell entry and syncytia formation in the presence of several reducing compounds in a dose-dependent manner. Results were complemented with in vivo experiments in which we showed significant inhibition of pseudovirus infection in mice that received tested compounds for two consecutive days before infection. Furthermore, lowered neutrophils and eosinophils levels and decreased cytokine levels in treated LPS-challenged mice indicate the anti-inflammatory activity of tested peptides. Our results confirm the efficiency and potential therapeutic value of tested antioxidant compounds. Understanding the cellular and inhibiting molecular mechanism of viral cell entry is essential in controlling the ongoing pandemic and possibly averting them in the future.

P4. THE EFFECT OF SURGICAL TREATMENT OF BRACHYCEPHALIC OBSTRUCTIVE AIRWAY SYNDROME ON THERMOREGULATORY RESPONSE TO EXERCISE IN FRENCH BULLDOGS

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Due to altered anatomy of the upper respiratory tract, brachycephalic dogs exhibit increased airway resistance and reduced surface area for evaporative heat loss, predisposing to respiratory and thermoregulatory problems, a syndrome referred to as Brachycephalic Obstructive Airway Syndrome (BOAS). It has been established that brachycephalic breeds are 2.1 times more susceptible to heat-related injuries even at low ambient temperatures and relatively low levels of physical activity. Surgical treatment alleviates clinical signs, potentially also improving the thermoregulatory ability of the dogs with BOAS. The aim of our study was to investigate the thermoregulatory response in French bulldogs, representatives of extreme brachycephalic breeds with BOAS.

The study was approved by The Animals in Experiments Welfare Commission of the Veterinary Faculty, University of Ljubljana (approval number 18-3/2022-1). We included 13 privately owned French bulldog whose owners gave an informed consent for participation in the study. Dogs were exposed to dynamic exercise on a treadmill and the dynamics of their rectal temperature (RT) and heart rate (HR) was assessed reflecting their thermoregulatory response. Exercise testing was performed at the Laboratory of Physiology of the Faculty of Sports, University of Ljubljana in two independent sessions, before and (six to nine months) after the surgical treatment. Exercise test consisted of two consecutive 5-minute walks at a speed of 2.5 km/h, in the first part at an inclination of 0% and in the second part at an inclination of 5%, and a 30-min recovery period. RT and HR were measured before (t0) the start of the test, at the end of the first (t1) and the second part (t2), and 15 minutes (t3) and 30 minutes (t4) in the recovery to exercise. During the surgical procedure which was performed at the Small Animal Clinic of the Veterinary Faculty, University of Ljubljana, stenotic nares and an elongated and thickened soft palate were corrected under general anesthesia.

During exercise and in the recovery, we observed a significant increase of both RT, and HR , compared to the baseline resting values, implying that the intensity of exercise was sufficient to challenge the thermoregulatory response. The increase of RT was significantly lower during both parts (t1, $p = 0.004$; t2 $p > 0.001$) of the exercise test after the surgical treatment than before the treatment. RT was also significantly lower at the end of the exercise test after the surgery ($p = 0.033$) compared to before the surgery. A similar trend was observed for the HR, i.e., the HR was significantly lower during exercise after the first ($p = 0.020$) and the second part ($p = 0.011$) of exercise after the surgery than before the surgery. Based on the results of our study, we might conclude that surgical treatment of BOAS can improve thermoregulation during exercise in French bulldogs with BOAS.

P5. MOLECULAR MARKER WUR10000125 ASSOCIATED WITH GENETIC RESISTANCE TO PRRSV INFECTION

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Porcine reproductive and respiratory syndrome (PRRS) is a major threat to pig production worldwide and is characterised by reproductive disorders in sows and respiratory disease in pigs of all ages, resulting in enormous economic losses and compromising animal welfare. The porcine reproductive syndrome virus (PRRSV) mutates very rapidly, and many different strains are circulating worldwide. The PRRS virus was introduced after Slovenia joined the EU (2004) by importing pigs from other EU countries, and now there are a large number of genetically different PRRSV strains in Slovenia. Today, health is the most important factor for animal welfare, productivity, and profitability in pig production. In recent years, selection goals have been lean growth, feed efficiency, meat quality and reproduction, and disease resistance. Animal genotyping methods are becoming increasingly valuable, both in terms of animal welfare and progress in research itself. We processed genotyping results for the GBP1 gene associated with resistance to PRRSV as one of the genotyping options. We analysed the genotyping results for the SNP with the commercial name WUR1000125 (the accession number in SNP databases is rs80800372). According to literature, allele G is associated with PRRS tolerance. Pigs carrying allele G had lower viral loads and better daily gains during PRRS outbreaks, and allele G reduced the proportion of abortions and loss of piglets during suckling period. Allele A is associated with daily gains in healthy pigs but is unfavourable during PRRS outbreaks because it is associated with higher PRRS virus titers and daily gains. Our objective was to determine the frequency of allele A and allele G, as well as the genotypes AA, AG and GG in our pig populations and to determine if selection for the favourable allele G would help us to limit and control the disease. The results show that the frequency of the AA genotype in the purebred pigs ranged from 73.4% to 95.8% and the AG genotype ranged from 0.8% to 25.6%. The frequency of the GG genotype was 0.5% in the Pietrain breed and 2.2% in the Krškopolje pig breed, while this genotype was absent in the other breeds. The frequency of allele A varied from 86.2% to 96.3% and the frequency of allele G ranged from 0.4% to 13.2%. The frequency of the genotype AA in the hybrid pigs ranged from 64.7% to 100%. The frequency of the genotype AG ranged from 0.0% to 20.7%. The frequency of the genotype GG was 2.0% in the Hybrid 54, while this genotype was not present in the other hybrid pigs. The frequency of allele A ranged from 89.7% to 100% and the frequency of allele G ranged from 0.0% to 18.6%. The population is in Hardy-Weinberg equilibrium. The allele frequencies of WUR10000125 SNP in our study were similar to the results of other studies, which showed that the unfavourable allele A (in case of PRRSV infection) was most common in commercial lines selected for increased growth rate. The frequency of allele G was the highes in the autochthonous Krškopolje pig breed compared to improved pig breeds. According to the literature, in autochthonous breeds, the effect of natural selection, and thus adaptation to harsh environmental conditions may have increased the frequency of disease resistance alleles. Pig breeders may benefit from selection of the WUR10000125 polymorphism to develop more effective means of disease control, not only because of economic losses, but also to reduce antibiotic use, improve animal welfare, increase food safety, and improve breeding technology.

P6. VITAMIN A SUPPLEMENTATION SUPPORTS UROTHELIAL REGENERATION IN A MOUSE MODEL OF ACUTE NONBACTERIAL CYSTITIS

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Vitamin A or retinoids are a group of hydrophobic substances that mainly affect cell proliferation, differentiation and apoptosis. They also possess anti-inflammatory properties. Retinoids are used in the treatment of various cancers and non-cancerous diseases. However, their potential use in the treatment of cystitis has not been adequately explored. In acute nonbacterial cystitis, the urothelium is damaged and the bladder wall is inflamed. In this case, the disruption of the urothelium is caused by chemical or physical injury. The undamaged urothelium forms a tight blood-urine barrier, which must be rapidly restored after disruption. Our study aimed to investigate the effects of a vitamin A-enriched diet on the regeneration of the urinary bladder urothelium in a mouse model of acute nonbacterial cystitis.

Eighteen female BALB/c mice (8 weeks old) were used for the experiment. Cystitis was induced by a single i.p. injection of cyclophosphamide (CP; 150 mg/kg; 12 animals). The control group (C; 6 animals) received the same volume of sterile saline. One week before CP application, the diet of the animals was changed from normal food to food enriched with vitamin A in half of the CP and C mice. The mice had ad libitum access to drinking water and food (normal or vitamin A-enriched food). The animals were euthanized 24 and 72 hours after administering CP and 24 hours after administering saline. Urinary bladders were collected and prepared for paraffin embedding, hematoxylin-eosin staining, and scanning electron microscopy.

In the hematoxylin-eosin stained samples, we observed destruction of the urothelium and inflammation of the lamina propria in mice treated with CP, regardless of the diet. SEM showed a higher degree of differentiation of the urothelial surface in mice fed a vitamin A-enriched diet compared to control animals fed a normal diet.

Our results show that the vitamin A-enriched diet does not reduce the extent of urothelial surface damage. On the other hand, we can assume that the higher intake of vitamin A causes slower desquamation and faster differentiation of the superficial urothelial cells and therefore might help in the therapy of acute nonbacterial cystitis. However, additional methods such as qPCR and immunohistochemistry should be used to further validate these results.

P7. THE DEVELOPMENT OF A FOUR-POINT TRANSVERSUS ABDOMINIS PLANE BLOCK TECHNIQUE IN PIGS FROM AN ANATOMICAL POINT OF VIEW

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The four muscles of the abdominal wall are supplied by the ventral branches of the caudal intercostal and lumbar nerves. Transversus abdominis plane (TAP) block is an interfascial block used to desensitize the nerves of the abdominal wall. The local anaesthetic is administered in the plane between the internal oblique muscle (IOM) and the transversus abdominis muscle (TAM). Two- and three-point TAP block techniques have been described previously in pigs. The aim of this study was to assess the anatomy of the abdominal wall in pigs to enable the development of a four-point TAP block technique.

Ten pigs *(Sus scrofa domesticus)* were included in this study. TAP block was performed on 2-day-old carcasses (n = 3), freshly euthanized carcasses (n = 3, 45.67 ± 3.21 kg), and anaesthetized pigs (n = 4, 40.00 ± 9.18 kg) used primarily to study the effects of electroporation on the hepatic vasculature. The study was approved by the National Veterinary Authority (approval number U34401-2/2021/5, approval date 3.3.2021) and conducted in accordance with Directive 2010/63/EU and ARRIVE 2.0 guidelines. After TAP block application with methylene blue (1 mg/kg diluted in 5% glucose solution), dissection was performed according to previous reports with few modifications. The thoracic nerves were identified according to their position to the last rib. Anatomic dissection of both hemiabdomens was performed 60-90 minutes after euthanasia. Tissue staining was assessed 5 minutes after removal of the IOM from TAM. The number of ribs (phase II and III) was determined at the end of the dye spread evaluation.

The external oblique muscle (EOM) and TAM are mostly muscular. The muscular portion of the IOM is small and restricted to the paralumbar region, with an extensive aponeurosis running cranioventrally and ventrally. Because of the exchange of aponeurotic fibers, the thin aponeurosis is inherently connected to the aponeuroses of the EOM ventrally, the TAM cranially, or to the peritoneum ventrocaudally. In phase II, two pigs had 16 ribs and one had 15 ribs; in phase III, all pigs had 15 ribs.
In pigs, the muscular part of the IOM is smaller and its aponeurosis more extensive than in other domestic animals, such as dogs. Methylene blue dilution successfully stained the tissue in freshly euthanized and anaesthetized animals and enabled evaluation of the TAP technique.

P8. MICROBIOLOGICAL AIR QUALITY ASSESSMENT AS A REFINEMENT MEASURE FOR THE WELFARE OF LABORATORY ANIMALS

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Refinement is the part of the 3Rs principle that could contribute most to improve the welfare of research animals housed in appropriate animal-assisted research facilities, since there is always an opportunity to improve the immediate environment of the research animals.
Animal research facilities house laboratory animals for breeding, experimentation, and resale. Air quality in such facilities is one of the most important parameters for a healthy environment. Good air quality in breeding facilities is necessary for the health and welfare of animals and for the health protection of personnel. Stable and clean ambient air is also important for a stable and fluctuation-free environment for animal-assisted research. The main components of air quality in such facilities are particulate matter, volatile organic compounds, carbon dioxide, and ammonia. However, microbiological air quality can also play an important role in both animal welfare and a stable research environment.

In the present study, microbiological air quality was investigated in two animal research facilities. The main purpose was to evaluate the microbiological air quality and to exclude the possibility of spreading pathogens among laboratory mice via potentially contaminated air. Air in various sections of the laboratory animal facilities, including sanitary barriers/corridors, breeding and experimental compartments, was filtered onto an agar plate, and viable microbial colonies were counted and checked for numbers and species.

Indoor bioaerosol measurements were made in the two experimental animal housing facilities. Airborne concentrations of bacteria and fungi were measured as CFU per 100 L of air. Samples of the bio-aerosol were taken using a Merck MAS-100 NT bio-aerosol sampler. After collecting the air samples, the Petri dishes were incubated in the laboratory and the viable colonies were counted after the incubation period.

The total number of bacteria and fungi in the air of the studied facilities did not exceed 300 CFU per 1 m^3. The results indicate good microbiological air quality in both facilities studied, which could be due to excellent husbandry as well as adequate housing systems, although neither facility was housed in buildings primarily intended for laboratory animal husbandry. Air quality monitoring results generally indicate good husbandry conditions at the experimental facilities. In addition, air quality monitoring could be a simple and, from the animals' point of view, non-invasive method to evaluate housing quality and one of the parameters indicating animal welfare.

P9. OPTIMIZING FLOW CYTOMETRY FOR GENOME SIZE ESTIMATION IN SALAMANDERS: A CALL FOR NONDESTRUCTIVE METHODS IN AMPHIBIAN GENOMICS

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Regenerating limbs, large cells, and lifespans measured in decades - the extraordinary peculiarities of salamander biology are underpinned by some of the largest genomes among vertebrates, which can range from 9.3–120 gigabases (Gb). Although the gargantuan salamander genomes have at times hampered genomic sequencing efforts, the genome size of an organism can serve as a rich trove of information in its own right. Genome size, expressed as a C-value (the total amount of DNA in the unreplicated haploid nucleus) has been shown to strongly influence embryonic developmental time as well as the physical body and cell size and may act as an evolutionary constraint on the ecological habitats of salamanders. Additionally, genome size is highly variable even among closely related taxons of salamanders and may serve as an important tool in research pertaining to the evolution, phylogenetics, and speciation of salamanders. Intraspecific genome size variance can also help to elucidate taxonomic relationships among different subpopulations of species such as the endangered Proteus anguinus.

Despite its great potential, research in the field of salamander genome size estimation has often been hampered by inconsistent, laborious, and inappropriate methods. Sorely needed is a reliable protocol that provides consistent, comparable, and accurate results. In our work, we present an efficient and simple method for estimating genome size in salamanders using flow cytometry.

Flow cytometric estimation of genome size is a simple and relatively inexpensive method with a very high throughput that allows for the analysis of a very large number of nuclei. Cell nuclei are isolated from the sample and then stained with a fluorescent DNA-binding dye (eg. DAPI and propidium iodide) along with reference standard nuclei. Fluorescence intensity is measured using a flow cytometer. DNA content is calculated by comparing fluorescence intensity values between sample nuclei and the known internal standard. While flow cytometry is a well-established technique for estimating genome size in plants and has largely supplanted the much more laborious, lower-throughput Feulgen densitometry, its utility in estimating genome size in salamanders is limited by inconsistent and often inappropriate methods. A common problem is the selection of an unsuitable genome size standard, such as chicken or trout erythrocytes, as the genome size of these species is significantly smaller than that of most salamander species (the difference between standard and sample genome size should not exceed fourfold). DAPI, which is sometimes used as a fluorescent DNA-binding dye9, is also not an acceptable dye for salamander genomes as it preferentially binds to A-T-rich genomic regions – leading to inaccurate measurements of genome size.

Our proposed protocol for flow cytometric measurements of salamander genome size aims to address such flaws and inconsistencies. Our protocol is based on non-destructive methods, using whole blood as the source of sample nuclei - as opposed to destructive tissue or whole larval samples. Nondestructive methods are particularly suitable for obtaining blood samples from endangered species, such as P. anguinus.

The Iberian ribbed newt (Pleurodeles waltl) and the axolotl (Ambystoma mexicanum) were selected

for optimization of the flow cytometry protocol. Both species are commonly used as model organisms for studies of developmental biology and limb regeneration. Animals were anesthetized with 0,03-0,5% tricaine methanesulfonate (MS-222), buffered with sodium bicarbonate to a pH of 7.0-7.5. Safe blood volume was estimated from body mass. Blood was collected from the ventricle using a heparinized 26 G needle and a 1.0 mL syringe. All specimens survived the blood taking; full recovery varied between individuals in the time frame of 10-40 minutes.
Nuclei of blood cells were extracted in Galbraith's lysis buffer for 30 minutes on ice and then stained with 100 µg/ml propidium iodide for an additional 30 minutes on ice and in the dark. Fluorescence was measured using a BD FACSMelody Cell Sorter. Genome size measurements were cross-checked using Feulgen densitometry. The morphology of the stained nuclei was examined by fluorescence microscopy to verify the success of cell lysis.

We focused on the following parameters:
1. Comparison between DAPI and propidium iodide. Samples stained with 100 µg/mL of propidium iodide were compared with samples stained with a DAPI concentration of 4 µg/mL and 8 µg/mL. Compared to propidium iodide staining, DAPI staining resulted in poor separation of P. waltl and A. mexicanum peaks.
2. Comparison of different lysis treatments: Galbraith buffer, commonly used for extraction of plant cell nuclei, was compared with a modified lysis buffer of 0,1% sodium citrate in 0,1% Triton X-100, which has been suggested as the preferred buffer for extraction of vertebrate cell nuclei. The modified lysis buffer did not prove to be a suitable substitute as it resulted in large cell nuclei aggregates.
3. Comparison of four cell fixation methods. A reliable fixation method is of utmost importance for the analysis of field samples – such as blood taken from wild-caught amphibians. Blood samples were fixed using four different treatments (70% EtOH, 3:1 methanol acetic acid, frozen at -20 °C in PBS with 1mM EDTA, 10% formalin). Samples were analyzed 7 and 14 days after fixation. This experiment is still in progress.

We used our flow cytometry protocol to measure blood samples of P. anguinus, using P. waltl as an internal reference standard. Initially, fluorescence microscopy showed poor cell lysis in P. anguinus cells. At the suggestion of our esteemed biochemist colleague Dr. Kristina Sepčić, we then attempted to lyse P. anguinus cells in a 37 °C water bath (the P. waltl standard was lysed separately at 4 °C – the isolated nuclei of the two species were then combined prior to staining). We obtained satisfactory results using this modified protocol. It should be noted that lysis of P. waltl blood cells at 37 °C did not yield good flow cytometric measurements, suggesting that P. anguinus blood cells differ in some way from those of other salamanders, reflecting its particular ecology and biological nature. To confirm this, we plan to analyze the lipid composition of this enigmatic species.

P10. AN IN VITRO MODEL REPRODUCING THE INTERACTIONS BETWEEN MURINE PERITONEAL MACROPHAGES AND OVARIAN CANCER CELLS

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Tumor-associated macrophages (TAMs) are major components of the ascites microenvironment in ovarian cancer, affecting spheroid formation and tumor growth. However, established techniques for studying the impact of TAMs on the biological behavior of ovarian cancer cells in vitro are limited. The objective of this study was to create a 3D in vitro model that can reproduce the in vivo interactions of TAMs and ovarian cancer cells.

To mimic the ascites microenvironment, murine ID8 p53 *null* ovarian cancer cells and IL-4 induced peritoneal macrophages PMJ2-R were used. The expression of TAMs specific markers (CCL3, CD206, F4/80) was studied using RT qPCR. Ultra-low attachment surface coated- and 0.5% Matrigel coated 96-well plates were used to establish a spheroid formation of co-cultured PMJ2-R and ID8 p53 *null* at a ratio of 1:10. Separately cultured PMJ2-R and ID8 p53 *null* were used as a control.

IL-4 successfully induced PMJ2-R towards tumor-promoting TAMs phenotype by overexpressing CD206 and F4/80. Ultra-low attachment co-culturing method promoted formation of one single spheroid, while Matrigel co-culturing method promoted formation of multiple smaller spheroids, in both cases expanding the growth of the spheroid, which was not seen in the control groups.

We have described two different co-culturing methods for spheroid formation where the *in vivo* behavior of macrophages and ovarian cancer cells could be reproduced. This model may be beneficial for studying the interactions of TAMs and ovarian cancer cells *in vitro*.

P11. UNDERSTANDING THE INFLUENCE OF HETEROCHRONIC NON-MYELOABLATIVE BONE MARROW TRANSPLANTATION ON AGED MURINE RECIPIENTS: FOCUS ON IMMUNE SYSTEM, FRAILTY, HEALTH, AND LONGEVITY

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The stem cell theory of aging postulates that stem cells become inefficient at maintaining the original functions of the tissues. We, therefore, hypothesized that transplanting young bone marrow (BM) to old recipients would lead to rejuvenating effects on immunity, followed by improved general health, decreased frailty, and possibly life span extension.

We developed a murine model of non-myeloablative heterochronic BM transplantation in which old female BALB/c mice at 14, 16, and 18(19) months of age received altogether 125.1 ± 15.6 million nucleated BM cells from young male donors aged 7-13 weeks. At 21 months, donor chimerism was determined, and the immune system's innate and adaptive arms were analyzed. Mice were then observed for general health and frailty until spontaneous death, when their lifespan, post-mortem examinations, and histopathological changes were recorded.

The old mice developed on average 18.7 ± 9.6% donor chimerism in the BM and showed certain improvements in their innate and adaptive arms of the immune system, such as favorable counts of neutrophils in the spleen and BM, central memory Th cells, effector/effector memory Th and Tc cells in the spleen, and B1a and B1b cells in the peritoneal cavity. Borderline enhanced lymphocyte proliferation capacity was also seen. The frailty parameters, pathomorphological results, and life spans did not differ significantly in the transplanted vs. control group of mice.

Although several favorable effects are obtained in our heterochronic non-myeloablative transplantation model, additional optimization is needed for better rejuvenation effects.

P12. SEX RELATED IMPACT OF TFF3 DEFICIENCY ON MORPHOLOGY OF MOUSE INTESTINE UPON LONG TERM HIGH-FAT DIET

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Trefoil factor family protein 3 (Tff3) is a small peptide (59 amino acids; 7 kD) and is a member of the trefoil factor family (Tffs) proteins, which include Tff1, Tff2, and Tff3. The major site of Tff3 secretion is the intestinal epithelial surface, where Tff3 is involved in gastrointestinal mucosal protection through several mechanisms, including epithelial restitution, influencing apoptosis, and immunologically relevant signaling pathways. The mode of action of Tffs ranges from a simple increase in mucus viscosity, cytoprotection, and anti-apoptotic and chemotactic effects to more complex immunoregulatory functions. The wide distribution in the body, including liver, bile, pancreas, blood, exocrine, and endocrine modes of action, as well as the possibility of binding to different partners, contribute to the complex mode of action of the Tff3 protein in different tissues. Of particular interest is its effect on the gut-liver-brain axis (GLB), where it is found. Here, we describe the effects of Tff3 deficiency on intestinal tissue in the case of a prolonged high-fat diet (HFD), a widely accepted model of metabolic stress that deregulates the GLB axis. Several mouse models with specific Tff protein deficiency have been used to investigate the physiological role of Tffs.

We developed a new Tff3-deficient mouse strain from an existing mixed background strain with an sv129/C57BL/6J background. The widespread use of SNP analysis in mouse breeding has shown that various mutations have been developed and fixed in specific isolated laboratory mouse substrains. Mice with C57BL/6J background have an additional genetic variation in the nicotinamide nucleotide transhydrogenase (Nnt) gene, which encodes the mitochondrial redox-induced proton pump that links NADPH synthesis to the mitochondrial metabolic pathway. The Nnt mutation itself modulates metabolism and immune response and conceals the physiological function of the candidate protein under study. The mixed genetic background raises the problem of finding a suitable Wt control group due to the random combinations of genetic variations. Using the Tff3-/-// C57BL/6NCrl mouse model, we overcame the problems of the mixed genetic background and the additional mutations of the C57BL/6J strain.

We observed the systemic effects of Tff3 deficiency on the GLB axis and present here the effects of prolonged HFD exposure on the gut tissue of a new congenic strain without additional metabolically relevant mutations (Tff3-/- /C57Bl6Crl strain). Because Tff3 is regulated by estrogens and metabolic processes are sex-dependent, we examined the effects of prolonged HFD exposure (30 weeks) on male and female wild-type (Wt) and Tff3-deficient mice. Specifically, we examined the effects of Tff3 deficiency on gut morphology and ultrastructure and short-chain fatty acid content as indicators of the microbiome. Metabolic pathways related to Tff3 function (oxidative and endoplasmic reticulum stress (ER), apoptosis, and inflammation) were monitored by Sybergreen-based QPCR expression analysis.

Data were analyzed using REST© software (ΔΔCt method) and changes were presented as log2 (fold change) using the stable reference genes (ACTβ and B2M).

Prolonged HFD induced differential effects on gut structure in Tff3-deficient male and female mice. For the first time, we found a sex-specific difference in duodenal morphology. HFD exposure resulted in a reduction in the height of microvilli in Tff3-deficient females compared with Wt females, suggesting a possible effect on actin filament dynamics of microvilli. These changes could not be attributed to alterations in the monitored genes of ER and oxidative stress, apoptosis, and inflammation. Tff3-deficient males exhibited a reduced depth of the cecal crypt compared with Wt males, which was not the case in females. Microbiome-related short-chain fatty acid content was not affected by Tff3 deficiency in either males or females exposed to HFD. The sex differences due to Tff3 deficiency make it necessary to consider both sexes in future studies on the role of Tffs in gut function.

P13. EFFECT OF TREFOIL FACTOR 3 DEFICIENCY IN HIPPOCAMPUS OF MICE ON SHORT-TERM HIGH FAT DIET

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Trefoil factor 3 (Tff3) is a small secretory peptide found primarily in the gastrointestinal tract, where it plays a role in epithelial protection through its involvement in various physiological processes, including cell migration and immune response. However, Tff3 is also expressed in neurons in many regions of the adult and developing brain, as well as in astrocytes and microglia in primary cultures.

In recent years, it has become apparent that Tff3 may play an important role in metabolic and neurodegenerative diseases, including Alzheimer's disease (AD). Tff3 was significantly reduced in the cerebrospinal fluid of AD patients, and low levels correlate with brain atrophy, hippocampal atrophy, and ventricular dilation. Intraperitoneal administration of Tff3 improved learning and memory performance in mouse models. In addition, liver-derived Tff3 has been shown to cross the blood-brain barrier and to act neuroprotective after experimentally induced brain injury in mouse models. It is clear that Tff3 plays a role in the central nervous system (CNS), but the exact functions and mechanisms of action are still unknown, so systematic research is needed to elucidate the role of Tff3 in these complex diseases.

The high-fat diet (HFD) model is commonly used to induce metabolic syndrome in animal models and, in addition to metabolic syndrome in the periphery, may also induce various neurophysiological changes in the CNS that may directly or indirectly affect hippocampal integrity. The hippocampus is an area of the brain that is particularly vulnerable to damage in the early stages of AD. Moreover, it is a well-characterized adult neurogenic niche where new neurons develop throughout life and are integrated into existing neural circuits where they contribute to their functionality. Adult neurogenesis and neuroinflammation are important pathophysiological features of AD, and a short-term HFD has been shown to be sufficient to cause significant disruption of adult neurogenesis capacity and increased inflammation in the hippocampus. Finding therapeutic options before advanced neuronal loss could be critical for the development of more effective pharmaceutical solutions. Therefore, our research objective was to investigate possible phenotype differences due to Tff3 deficiency in the early phase of neurodegenerative process activation, focusing on hippocampal integrity (adult neurogenesis/neuroinflammation) after short-term HFD.

In this study, newly developed congenic male and female $Tff3^{-/-}$ mice on a C57BL/6N genetic background and corresponding WT controls were exposed to 9 weeks of HFD treatment. We analyzed relevant markers of neurogenesis and neuroinflammation in the hippocampus of the mouse models. After 9 weeks of HFD, we observed a significant decrease in Dcx protein levels (markers of immature neurons) in the hippocampus of $Tff3^{-/-}$ males compared with WT males but, surprisingly, a significant increase in Dcx and NeuN protein levels (markers of mature neurons) in the hippocampus of $Tff3^{-/-}$ females compared with WT females. Gene expression analysis of markers of neurogenesis again showed a more pronounced effect of Tff3 deficiency in females than in males, suggesting enhanced adult neurogenesis upon HFD. In addition to neurons, we examined the expression of other important cells of the nervous system using markers for astrocytes (Gfap) and microglia (Iba1). After 9 weeks of

HFD, Iba1 protein levels were significantly reduced in the hippocampus of Tff3$^{-/-}$ males compared with WT males.

Analysis of gene expressions of neuroinflammation markers showed a significant increase in only Cxcl1 gene expression in the hippocampus of Tff3$^{-/-}$ mice compared with WT. No changes were detected in the hippocampus of mice fed a standard diet, suggesting that the observed changes caused by Tff3 deficiency were due to HFD stress. We found for the first time that Tff3 can play a role in adult neurogenesis under relevant metabolic conditions. This opens a new field of research with the aim of elucidating the precise role of Tff3 in these processes and potentially discovering new therapeutic targets. Moreover, the observed phenotypes differ significantly by sex, highlighting the need to include both sexes in future research on the role of Tff3.

P14. A MOUSE MODEL OF HPV-POSITIVE HEAD AND NECK CANCER: DEVELOPMENT AND CHARACTERISATION

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Head and neck cancer is a heterogeneous group of tumors, including oral squamous cell carcinoma (OSCC). One of the risk factors for the development of OSCC is persistent infection with high-risk human papillomavirus (HPV), such as HPV-16. Clinical data show that some patients with HPV-positive OSCC have a better prognosis and respond better to various treatments, including radiotherapy and immunotherapy. Due to the species specificity of HPV (only infecting humans), immunocompetent models of HPV-positive OSCC are rare. Therefore, preclinical studies of these tumor types, and treatment evaluation, especially with therapies based on activation of the acquired immune system, are difficult. Thus, the aim of our study was to establish and characterize an HPV-positive mouse model of OSCC with stable expression of the E6 and E7 HPV-16 oncogenes. Using retroviral transduction, we established monoclonal HPV-positive cell lines (MOC1-HPV K1 and MOC1-HPV K3) from the parental murine OSCC cell line MOC1. In both cell lines, we confirmed stable expression of E6 and E7 at mRNA and protein levels. In vitro characterization of the established cell lines showed that the cell lines (parental and both HPV-positive) did not differ in growth rate, but did differ in morphology and in the rate of cell migration, which was determined by the wound healing assay. Radiosensitivity was determined both in vitro and in vivo. At the in vivo level, differences in the tumor microenvironment were determined by immunofluorescence staining of frozen tumor sections. The presence of hypoxia (EF5), proliferation (EdU), vascularity (CD31) and infiltration of CD4 and CD8 T cells and macrophages (F4/80) in the tumors, was determined. At the in vitro level, no statistically significant differences in the radiosensitivity of HPV-positive cell lines compared to the parental line by clonogenicity assay after irradiation with increasing doses was observed. In vivo, however, the radiosensitivity of the MOC1-HPV K1 tumor model was higher after irradiation with a single dose of 15 Gy, resulting in a longer growth delay compared with the parental MOC1 model and the HPV-positive MOC1-HPV K3 model. Immunofluorescence staining demonstrated a lower level of hypoxia and a higher proportion of proliferating cells in MOC1-HPV K1 tumors, suggesting a possible mechanism for the increased radiosensitivity of the MOC1-HPV K1 tumor model. We also characterize the new cell lines at the transcriptome level. Both in vitro and in vivo results are consistent with the results of the transcriptome analysis of the cell lines, where the 20 most enriched GO terms include processes related to cell migration and angiogenesis. In conclusion, we have developed and characterized a novel immunocompetent mouse model of HPV-positive OSCC, which shows enhanced radiosensitivity and allows studies of immune-based treatment approaches for HPV-positive OSCC.

P15. THE FEASIBILITY OF GENE ELECTROTRANSFER FOR VACCINATION AGAINST COVID-19

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DNA vaccination is one of the emerging approaches for vaccination against COVID-19 and other diseases. The aim of this study was to investigate the feasibility of Gene Electro Transfer (GET) in the context of vaccination against COVID-19.

Two plasmids encoding SARS-CoV-2 spike (S) or nucleocapsid (N) protein were used as the source of antigens. Additionally, an interleukin 12 (il-12)-encoding plasmid was used as an immunological adjuvant in the *in vivo* experiments. The expression of N and S proteins after *in vitro* GET in mouse myoblast cell line C2C12 and fibroblast cell line L929 was assessed by RT-qPCR and ELISA assays. Vaccination was performed via GET of 45 μg of plasmid DNA in the skin or muscle tissue of C57BL/6J mice on days 0 and 14 (boost). Two weeks after the boost, blood, spleens and transfected skin or muscle tissue were collected. Expression of *S, N, il-12* and *interferon γ* (*ifn γ)* in the transfected tissue was determined by RT-qPCR, and ifn *γ* in serum by ELISA. Induction of antigen-specific IgG antibodies was determined by ELISAs, and induction of antigen-specific cytotoxic T cells by tetramer assay on isolated splenocytes.

We confirmed that both *in vitro* and *in vivo* GET leads to expression from transfected plasmids. Production of N was further confirmed on protein level, while S was not detectable using available ELISA kits, indicating problems with its proper presentation on the cell membrane. Indeed, vaccination with S did not lead to the induction of S-specific antibodies. Vaccination with N, however, led to a significant induction of N-specific antibodies. Intramuscular vaccination proved superior to skin vaccination, therefore test for cell-mediated immunity were performed only after intramuscular vaccination using only the plasmid for N. Final results confirmed that intramuscular vaccination with GET of N-encoding plasmid led to formation of antigen specific antibodies against the presented antigen as well as induction of N-specific cytotoxic T cells, which was significantly higher when IL-12 was used as immunological adjuvant.

Overall, vaccination using GET was well tolerated and led to a significant induction of humoral and cell-mediated immunity, confirming the feasibility of GET platform for DNA vaccination against infectious disease like COVID-19.

P16. BIOTECHXCHANGE 3RS PLATFORM

Daša Seveljević-Jaran; promotional poster presentation on behalf of BiotechXchange_BX

1. https://ec.europa.eu/environment/chemicals/lab_animals/pdf/SWD_part_C.PDF
2. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52020SC0015

P17. ANIMAL MODELS OF HUMAN PATHOLOGY: REVISION, RELEVANCE AND REFINEMENTS

Martina Perše

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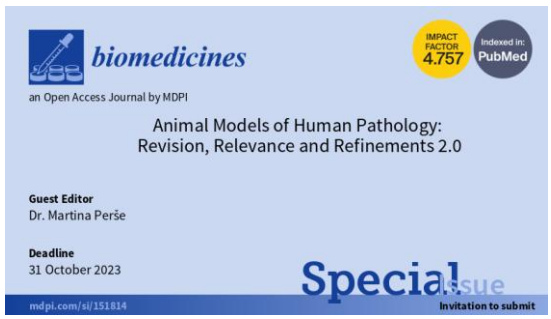
Use of animal models of human pathology is accepted on the assumption that it benefits humans. However, recent publications have shown that research on animals faces serious challenges. The increasing number of potential targets, molecular pathways or treatment strategies, which have been recognized as promising in animal models, have failed when translated into human trials. We have reached the point where the clinical relevance of animal models needs urgent clarification.

Multiple methodological problems in animal research have already been exposed, such as poor experimental design, inadequate use of fundamental statistical principles (i.e., randomization, blinding, inadequate power, inadequate sample size, pseudo-replication, etc.), and nontransparent reporting, which results in low scientific validity and irreproducibility of results. However, research on animal models also requires comprehensive knowledge about the model, as well as an understanding of the complex pathogenesis of diseases, which involves both local and systemic effects in the body. Every animal model has its own characteristics, advantages, and limitations. There are factors specific to the disease or animal model that can influence not only the severity of the disease but also underlying mechanisms, and, when these factors are not taken into account, research may result in the discovery of new targets and disease pathways that are of no scientific or clinical value. There are also animal-model-specific factors that can seriously affect the results and lead to false conclusions and failed translation. Although overall animal health and welfare issues—such as animal clinical state, morbidity, mortality, humane endpoints and humane interventions, whole body necropsy findings, sampling principles, pathohistological diagnosis—are of vital importance in the interpretation of the molecular mechanisms or treatment strategies in an organism, this information is usually lacking or rarely properly addressed in animal model studies.

The purpose of a Special issue is to promote submissions of high-quality papers of basic research using animal models to understand diseases and underlying mechanisms or to investigate new treatment strategies in various human diseases such as cancer, bowel diseases, kidney injury, Parkinson's disease, etc. New approaches towards the use of animal models or refinements of particular animal models of human pathology as well as methodological and welfare principles (such as experimental design and welfare or supportive measures in animal models) are also welcome.



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REDUCING ANIMAL USAGE AND REVEALING NEW INSIGHTS: THE POWER OF IN VIVO IMAGING TECHNOLOGIES IN BASIC AND PRECLINICAL RESEARCH

Simon Koren[1], Ron Koop[2]

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Conventional animal imaging requires sacrificing multiple animals at numerous time points, then slicing and staining tissue to identify the location and state of molecules of interest at a point in time. In recent decades, rapid advancements in high-resolution *in vivo* imaging techniques have enabled unparalleled new approaches to studying biological processes in living organisms, both at molecular and higher levels.

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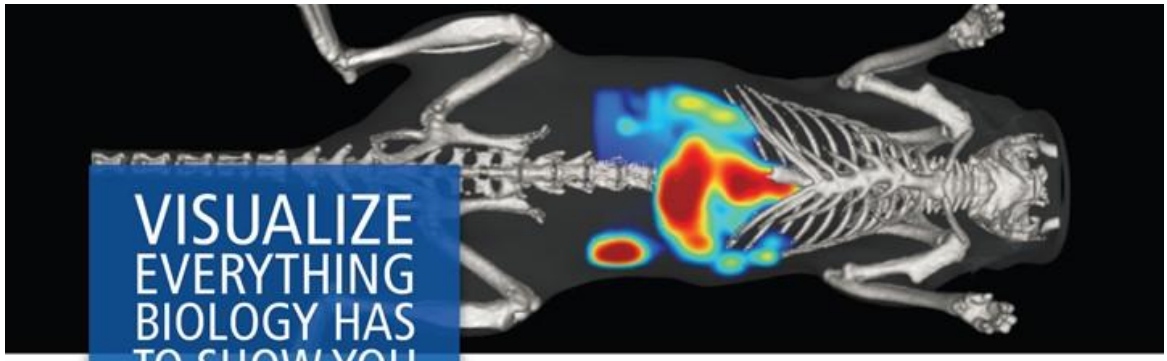

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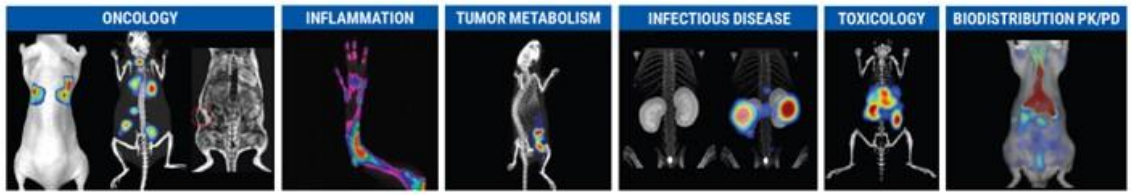

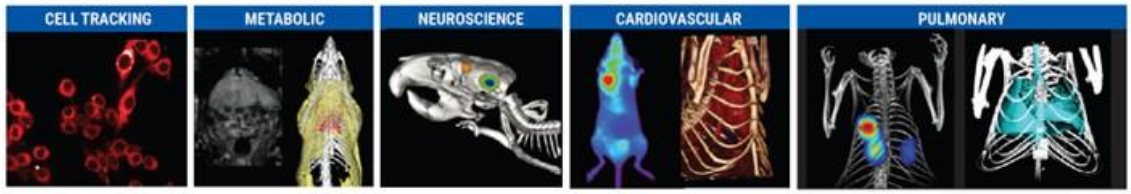

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