



Research Modernisation Deal

A Strategy for Ending Animal Experiments



People for the Ethical Treatment of Animals (PETA) Nederland

People for the Ethical Treatment of Animals (PETA) Nederland is a charity dedicated to establishing and protecting the rights of all animals. Please visit www.peta.nl for more information.

PETA encourages the sharing and downloading of the content within this document for personal and non-commercial use. If you wish to use any of the document materials (including text, images, photographs, etc.) for any other purpose, you must obtain our express written consent before doing so by contacting info@peta.nl.

© PETA Nederland

Version 2.0 • last updated 22 February 2024.

Samenvatting

Vooruitgang in onderzoekstechnologie heeft voor een revolutie op het gebied van biomedisch onderzoek en regelgevende testen gezorgd, en er wordt nog veel meer vooruitgang verwacht in de komende jaren.

De overgang van onderzoek dat gebaseerd is op het gebruik van dieren om menselijke ziekten te modelleren of als instrumenten om menselijke reacties op medicijnen of andere stoffen te voorspellen naar op de menselijke biologie gebaseerde methoden verbetert beleid en praktijk wereldwijd. Onderzoeksfondsen worden zich steeds meer bewust van het feit dat falende methoden met dieren, zowel voor het vaststellen van werkzaamheid als van toxicologische risico's, de ontwikkeling van potentiële geneesmiddelen en ons vermogen om de gezondheid van mens en milieu te beschermen belemmeren. Bijvoorbeeld, in het bestaande paradigma gebaseerd op dierproeven, duurt het 10 tot 15 jaar voordat nieuwe geneesmiddelen de markt bereiken, met een kostprijs van meer dan €1,9 miljard per geneesmiddel, waarvan meer dan 95% de klinische proeven niet doorstaat. Deze cijfers kunnen economisch noch ethisch worden verantwoord, en er zijn dringende inspanningen nodig om het onderzoekssysteem te transformeren.

Neem de volgende belangrijke punten in overweging:

- Systematische reviews die zijn gepubliceerd in wetenschappelijke tijdschriften hebben beperkingen blootgelegd in de vertaling van resultaten van dierproeven naar behandelingen voor de mens voor tal van ziektegebieden. Minder dan 10% van de veelbelovende basiswetenschappelijke ontdekkingen leidt binnen 20 jaar tot een klinische toepassing.
- Tussen 50% en 89% van het preklinische onderzoek, waarbij dierproeven een ernstig probleemgebied vormen, is niet reproduceerbaar.
- Grote wetenschappelijke doorbraken op ziektegebieden zoals diabetes en borstkanker zijn gebaseerd op onderzoek bij patiënten; op basis van dierproeven zouden deze doorbraken er niet gekomen zijn.

Naast het groeiende bewijs dat experimenten op dieren zich niet goed laten vertalen naar mensen of andere dieren – evenals de ontwikkeling en implementatie van technologie die het gebruik van dieren in laboratoria vervangt – heeft onze samenleving ook een groeiende morele bezorgdheid over het gebruik van dieren in experimenten.

Wij bevelen de ontwikkeling van een strategie aan die de volgende cruciale stappen omvat:

1. Beëindig onmiddellijk het gebruik van dieren op gebieden waar al is aangetoond dat dieren slechte en onbetrouwbare voorspellers voor mensen zijn en vooruitgang hebben belemmerd.
2. Voer kritische wetenschappelijke beoordelingen uit om gebieden te identificeren waar het gebruik van dieren de gezondheid van mens en milieu niet heeft bevorderd en daarom zou moeten worden beëindigd.
3. Voer transparante, robuuste prospectieve en retrospectieve evaluaties in voor alle projecten waarin dieren worden gebruikt, met een openbare commentaarperiode.
4. Werk wereldwijd samen met organisaties en instanties aan de harmonisatie en bevordering van de internationale acceptatie van diervrije testmethoden voor wettelijk verplichte testvereisten.
5. Verhoog de fondsen voor dierproefvrij onderzoek en verminder de fondsen voor dierstudies.
6. Zorg voor gedegen opleidingen en trainingen voor onderzoekers en regelgevers over de voordelen van en hoe gebruik te maken van diervrije onderzoeks- en testmethoden.



Table of Contents

I. Introduction	4
II. Limited Predictive Value of Research Using Animals	6
Lack of Validity	6
Lost in Translation	7
III. The Need for a Paradigm Shift	8
IV. Opportunities for Economic Advancement	9
The High Cost of Drug Development	9
Employment and Economic Growth in the Technology Sector	11
V. Opportunities for Regulatory Toxicology	12
VI. Public Opinion and Animal Sentience	13
VII. World Leadership	14
VIII. Plan of Action: Recommendations to Modernise Scientific Research and Assessment	15
1. Immediately eliminate animal use in areas for which animals have already shown to be poor and unreliable predictors for humans and their use has impeded scientific progress.	15
2. Conduct critical scientific reviews to identify the areas in which the use of animals has failed to advance human or environmental health and should therefore be ended.	15
3. Implement transparent, robust prospective and retrospective project evaluations, as required by Directive 2010/63/EU on the protection of animals used for scientific purposes, and allow a public commenting period so that external experts can contribute to them. This must apply to all projects.	15
4. Work with organisations and agencies globally to harmonise and promote international acceptance of non-animal testing methods for regulatory testing requirements.	16
5. Increase funds for non-animal studies and decrease funds for animal studies.	17
6. Educate and train researchers and regulators on the benefits of and how to use non-animal testing approaches.	17
References	19
Appendices	22



I. Introduction

“When you read about advances in medicine, it often seems like long-awaited breakthroughs are just around the corner for cancer, Alzheimer’s, stroke, osteoarthritis, and countless less common diseases. But it turns out we live in a world with an awful lot of corners.”¹



The observation expressed above by best-selling science journalist Richard Harris echoes in the hearts and minds of every person suffering or who knows someone suffering from an incurable disease. The US National Institutes of Health (NIH), the world’s largest funder of biomedical research, reports that “failure rates [for novel drugs] occur in about 95 percent of human studies”,² even though these drugs showed success in preclinical experiments using animals.

The transition away from using animals to model human disease or as tools to predict human responses to drugs or other substances and towards human biology-based methods is changing policy around the world.

In the EU, the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) — part of the Joint Research Centre, the European Commission’s science and knowledge service — is working to replace the use of animals in both biomedical research and toxicological testing. Indeed, EURL ECVAM launched a study to review the use of alternative methods in biomedical research, noting that encouraging the uptake of alternative methods is important and because “alternative methods offer the promise of recapitulating human physiology more effectively than many animal models, shifting to new animal-free methodologies and research strategies can in fact enhance the understanding of human-specific biology and disease”.³

Acceptance of non-animal approaches in one region or country is an open door to facilitate international harmonisation. Over the past two decades in particular, significant progress has been made in the development, validation, implementation, and regulatory acceptance of non-animal technology for the assessment of human health endpoints such as skin and eye irritation and corrosion, skin sensitivity,

skin absorption, and phototoxicity. We’ve also seen an end to notoriously cruel international test guidelines such as the Organisation for Economic Co-operation and Development (OECD) Test No 401, also known as the lethal dose (LD₅₀) test.

Opportunities exist to increase the use and acceptance of valid non-animal test methods for regulatory assessment, and by taking them, we can achieve better protection of human health and the environment.

In light of the European citizens’ initiative “Save Cruelty Free Cosmetics – Commit to a Europe Without Animal Testing”⁴ and the 2021 European Parliament resolution to accelerate a transition to innovation without the use of animals in research, regulatory testing, and education,⁵ it is vital that the EU keep pace with scientific advancements and that its evolving policies reflect a commitment to ending animal use and supporting the development and adoption of advanced non-animal methods based on human biology.

We identify a number of strategic priorities and append further information regarding areas where there are opportunities for the immediate and near-future replacement of animal use. We have also included information outlining areas in which further development, validation, and implementation of non-animal methods are needed.



II. Limited Predictive Value of Research Using Animals

A great deal of scientific research shows that animal studies are flawed and divert both monetary and intellectual resources from more reliable and relevant methodologies. There are many factors at play in the failure of animal experimentation to predict human outcomes reliably, including reporting and publication bias, poor study design, and inadequate sample size.⁶ Critically, intrinsic biological and genetic differences among species contribute significantly to inescapable problems in extrapolating results from non-human animals to humans, even in the best-controlled and best-executed study designs



Lack of Validity

Problems with reproducibility (internal validity) and translation (external validity) contribute to the failure of animal experiments to progress biomedical research from bench to bedside. The internal validity of animal experiments is undermined by poor study design, including failure to implement processes to prevent bias, such as ensuring the individuals conducting the experiments and analysing the data do not know whether the animals or the samples belong to the treatment or the control group (blinding). Following a meta-analysis of systematic reviews of preclinical animal experiments across a wide variety of disease areas, University of Oxford scientists found that a lack of measures to reduce bias in animal experiments likely results in overestimation of the benefits of the treatment studied and may reduce trust in the result and waste scarce resources.⁷ They also advised, “Since human studies are often justified based on results from animal studies, our results suggest that unduly biased animal studies should not be allowed to constitute part of the rationale for human trials.”⁷

Poor internal validity means that many experiments on animals cannot be reproduced, a critical aspect of the scientific process that speaks to the potential validity of a finding. It can therefore be of little surprise that a 2015 investigation concluded that between 50% and 89% of all preclinical research, much of which involves animal testing, could not be reproduced.⁸

However, the weaknesses of animal experiments cannot be overcome by simply improving study design, because external validity, or the “extent to which research findings derived in one setting, population or species can be reliably applied to other settings, populations and species”,⁹ can never be achieved. Inherent species differences mean that non-human animals cannot serve as analogues for understanding the biological effects of drugs and chemicals on humans. As Wall and Shani write, even the “extrapolated results from studies using tens of millions of animals fail to accurately predict human responses”.¹⁰

In a 2018 review in the *Journal of Translational Medicine*, Pandora Pound and Merel Ritskes-Hoitinga discuss species differences as an insurmountable problem of external validity for preclinical animal models.⁹ Attempts to control for or correct species differences result in what the authors refer to as the “extrapolator’s circle”: “[I]f we want to determine whether a mechanism in animals is sufficiently similar to the mechanism in humans to justify extrapolation, we must know how the relevant mechanism in humans operates. But if we already know about the mechanism in humans then the initial animal study is likely to have been redundant.”⁹ They also discuss the concerning trend among those involved in animal experimentation to minimise the issue of species differences and the effects on external validity, a problem that is acknowledged by a number of researchers.^{11,12}



Pound and Ritskes-Hoitinga go on to state that it is unsurprising that the issue of species differences is downplayed, as not doing so would force experimenters to confront the “possibility that the preclinical animal research paradigm no longer has a great deal to offer”. There is growing scientific

consensus that far more is to be gained from non-animal research methods that are better suited to solving biomedical or environmental research and regulatory assessment questions. As a recent UK industry report emphasised, the time has come to humanise drug discovery and toxicology.¹³

Lost in Translation

Given the problem of poor validity and reproducibility inherent in studies using animals, it comes as no surprise that their results often fail to translate into clinical relevance for human patients. As mentioned above, NIH reports that novel drugs fail “in about 95 percent of human studies”.² This includes drugs that have been shown to be safe and effective in experiments using animals.

To assess whether or not the promises of basic biomedical research were being fulfilled, Stanford Professor of Medicine, Health Research, and Policy John Ioannidis and his colleagues identified 101 articles published in the most prestigious medical journals in which the authors explicitly stated that their research would lead to a new application with real potential for a clinical breakthrough. The majority of the articles analysed (63%) were for experiments on animals. Their investigation of the application of basic science to clinical applications found that fewer than 10% of these self-proclaimed highly promising basic science discoveries enter routine clinical use within 20 years.¹⁴

More recently, a 2014 analysis published in *The BMJ* found that studies using animals have not furthered knowledge in the field of human health or led to the development of treatments for conditions affecting humans.¹⁵ The authors note, “[I]f research conducted on animals continues to be unable to reasonably predict what can be

Fewer than 10% of highly promising basic science discoveries enter routine clinical use within 20 years.

expected in humans, the public’s continuing endorsement and funding of preclinical animal research seems misplaced.”¹⁵

The difficulties in applying data derived from one species to another are compounded by the confinement and unnatural conditions of laboratory life, which thwart animals’ ability to engage in natural behaviour.^{16,17} This deprivation contributes to their stress and alters their physiology and neurobiology, causing them to exhibit various psychopathologies.^{18–22}

Importantly, the fact that animals in laboratories have altered physiology and neurobiology means that they will not even be good models for their counterparts in the wild. A mouse in a laboratory will not respond to a drug in the same way that a mouse in a field would. One then has to ask, how does this biologically distinct mouse reliably represent the biology of human beings?

Complex gene-environment interactions and inherent methodological problems mean that genetically altering the mouse model or trying to “humanise” mice by inserting human genes will not solve the translatability issue – but it will continue to divert limited resources from modern, human-relevant research.²³



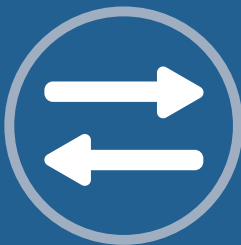
Evidence Box 1: Lack of Clinical Success

The failure of basic and applied scientific studies involving animals is perhaps most evident in the stark litany of seemingly promising treatments that have not worked in humans. For example, stroke experiments on animals have been an outright failure. Researchers at the Institute for Stroke and Dementia Research in Munich have described the shortcomings:

More than 1000 neuroprotective compounds have been tested in rodent models with the aim to improve stroke outcome. ... Indeed, many agents reduced brain damage (in most cases measured as decreased infarct volume) in rodent models of experimental stroke. Out of these candidates approximately 50 neuroprotective agents were tested in more than 100 clinical stroke trials, but none has improved outcome in clinical stroke patients.²⁴

Oncology drugs, which undergo extensive animal testing, have a success rate of only 3.4%.²⁵ This theme pervades many human disease areas. There is an abundance of literature documenting the failings of various animal models of neurodegenerative diseases – such as Alzheimer’s, for which the clinical failure rate for new drugs is 99.6%.²⁶

III. The Need for a Paradigm Shift



If our finite public funds are to be used responsibly, they must fund reliable research and test methods that lead to effective treatment of diseases and protection of human health and the environment.

In support of using an evidence-based approach to accelerating the delivery of useful drugs to the patients who need them, 15 Vanderbilt University researchers published a 2017 article calling for the elimination of experiments using animals where there is clear evidence that animals are not useful indicators or predictive of human disease:

“The literature is replete with examples of contradictions and discordance between animal and human effects, including many cases in which promising animal results have failed to translate to clinically significant efficacy in humans. This is particularly true in some therapeutic areas such as neurodegenerative, psychiatric, and central nervous system diseases, as well as sepsis and inflammatory diseases. [...]

Supported by several illustrative examples encountered in our drug repurposing program, we propose herein an approach for assessing when it is appropriate to conduct the ‘last experiment first’,

that is, progressing directly to human investigations when animal work would likely fail to provide data appropriate for translation into human applications of interest. This represents a significant – and we suggest, avoidable – barrier to drug introduction.”²⁷

The shifting scientific consensus away from the use of animals in experimentation can be observed in a number of arenas, including in publications documenting the limited predictive value of experiments on animals,¹⁵ in the increased awareness of animal cognition and sentience,²⁸ and in the fast-eroding public support for animal studies.²⁹ For example, *The Turkish Journal of Gastroenterology* – the journal of the Turkish Society of Gastroenterology – officially banned the publication of studies involving experiments on animals from its pages. Journal editor Dr Hakan Şentürk wrote that the policy represents “growing concern about the lack of applicability of animal research to humans”.³⁰ He further commented, “[w]hen we recognize that the reliance on inherently



flawed animal models of human disease are largely responsible for clinical failure [...] it does not make sense to continue to promote this practice.[...] Human-relevant approaches should be more aggressively developed and utilized instead.”

Significantly, a move away from research using animals will allow for substantial growth in the science and technology sectors and for faster return on investment in research and development, as seen after the cosmetics testing ban in the EU, despite

initial resistance from some corners of industry. An evolution of research funding priorities towards human-relevant methods, i.e. methods that recapitulate human physiology and biology without using other animals or their tissue, will get treatments to the patients who need them more safely and likely in less time.^{31,32} As public funding for research is limited, it should be focused on the development and implementation of scientifically sound non-animal research and testing methods.

IV. Opportunities for Economic Advancement



The high Cost of Drug Development

By mandating a move away from animal experimentation and towards advanced scientific methods, the EU has the opportunity to rapidly expand job growth in science and technology and reduce healthcare costs. As Meigs and colleagues report in their review, “Animal Testing and Its Alternatives – the Most Important Omics Is Economics”, “an economy of alternative approaches has developed that is outperforming traditional animal testing”.³³

Likewise, the UK funding body Innovate UK has identified non-animal technologies “as one of a series of emerging technologies with the potential to drive future UK economic growth” and, in doing so, proposed that British companies be able to take advantage of these “new commercial opportunities”.³⁴

Moving a new drug to market may cost up to US\$2 billion (approximately €1.9 billion) and take as long as 15 years.² One factor in the high cost of research and development is the substantial risk associated with developing a product that fails to result in a marketable drug because it does not succeed in human clinical trials. NIH states that for novel drugs, “failure rates occur in about 95 percent of human studies”.² This includes drugs that have been shown to be safe and effective in animals then failing in

humans. Conversely, drugs that could be effective in humans may be rejected without clinical trials because they were ineffective or unsafe in animals. Columbia University scientists Kacey Ronaldson-Bouchard and Gordana Vunjak-Novakovic, in advocating for the use of human tissues *in vitro* during drug development, also make the following observation:

“Equally damaging is the cautious elimination of potentially curative new drugs because their adverse effects in animals do not necessarily translate into humans. These false-positive and false-negative readouts create an enormous financial burden, resulting in decision-making in which the potential profitability of a drug is leveraged against the potential risks, rather than on the drug’s potential to improve disease outcomes.”⁴²



Evidence Box 2: The Dangers of Misleading Results

Many novel drugs don't simply fail, representing a huge loss in time and investment – they harm humans. In 2016, a Portuguese company developed a drug intended to help with mood, anxiety, and motor problems related to neurodegenerative disease. The drug was administered orally to volunteers as part of the Phase I clinical trial conducted by a French drug evaluation company. Six men, aged 28 to 49, experienced such adverse reactions that they had to be hospitalised. One participant was pronounced brain-dead and later died. A report on this incident reveals that “[n]o ill-effects were noted in the animals, despite doses 400 times stronger than those given to the human volunteers”.³⁵

In his 2010 article “TGN1412: From Discovery to Disaster”, Husain Attarwala of Northeastern University in the US recounts the tragic outcome of the 2006 clinical trial for Theralizumab, an immunomodulatory drug. He writes, “After [the] very first infusion of a dose 500 times smaller than that found safe in animal studies, all six human volunteers faced life-threatening conditions involving multiorgan failure for which they were moved to [the] intensive care unit.”³⁶ Five of the six participants had to remain hospitalised for three months after the initial dose, while the other was comatose. Even six months later, participants suffered from headaches and memory loss. One had to have toes and fingers amputated as a result of gangrene.³⁷ Studying this and other trials, Attarwala concluded, “Drugs showing safety and efficacy in preclinical animal models may show very different pharmacological properties when administered to humans.”³⁸

The opposite is also true: therapies that have not worked well in animals have sat useless on the shelf while patients have gone without life-saving treatment. For example, penicillin was first tested in rabbits in 1929, but as it had no apparent effect in this species, it was ignored for more than a decade – costing countless human lives. The first human clinical trials weren't conducted until the 1940s.^{39,40} Researchers later remarked on the good fortune that it was not first tested in guinea pigs, for whom the antibiotic is lethal. Had experimenters seen this result, penicillin may have never been tried in humans.⁴¹

Compounding the problem of effectively and efficiently bringing new drugs to market is the lack of reproducibility of preclinical trials. An investigation by the UK House of Commons Science and Technology Committee into the scientific integrity of government-funded research highlighted the current “reproducibility crisis” and indicated the continued upward trend in misconduct and mistakes in publishing.⁴³ At the most conservative US estimate, the abundant failure to reproduce preclinical research results in approximate annual spending of \$28 billion (about €26 billion) on potentially misleading experimentation.⁸ Additionally, even in journals that support the “Animal Research: Reporting of In Vivo Experiments” (ARRIVE) guidelines⁴⁴ – which aims to improve the reporting of research using animals – studies continue to demonstrate low reproducibility, poor value for money, and a waste of animals' lives.⁴⁵

Through the use of human-relevant technology in place of expensive, time-consuming, and inaccurate animal experiments, the cost of drug discovery has

the potential to decrease dramatically. Writing in the official journal of the American Society for Clinical Pharmacology & Therapeutics, Tal Burt and his co-authors made the following comments:

“Increasing costs of drug development and ethical concerns about the risks of exposing humans and animals to novel chemical entities favour limited exposure clinical trials such as microdosing and other phase 0 trials. An increasing body of research supports the validity of extrapolation from the limited drug exposure of phase 0 approaches to the full, therapeutic exposure. An increasing number of applications and design options demonstrate the versatility and flexibility these approaches offer to drug developers.”⁴⁶

To achieve the highest standards of rigour, reproducibility, and relevance in the study of human disease, it is critical that considerable financial support be made available for the implementation and further investigation of reliable non-animal approaches that focus on human tissue and biology.



Employment and Economic Growth in the Technology Sector

The market for human cell-based *in vitro* technology for biomedical research and testing is growing rapidly. A leading market research company estimated that “[t]he global cell-based assays market should reach \$47.3 billion by 2027 from \$29.2 billion in 2022”⁴⁷ (approximately €44.3 billion and €27.4 billion, respectively) and “[t]he global market for induced pluripotent stem cells should grow ... to \$4.4 billion by 2026”⁴⁸ (approximately €4.1 billion). Market researchers also projected that the global organ-on-a-chip market will reach a volume of \$815.6 million by 2028 (approximately €763.8 million).⁴⁹

Evidence Box 3: Improved Drug Development

High-tech animal-free models such as organs-on-a-chip are increasingly being used by industry for drug development, for instance to assess drug-induced liver toxicity.⁵⁰

A 2022 publication by U.S. manufacturer Emulate showed that the company’s human liver-on-a-chip is capable of predicting drug-induced liver injury from small molecules. In the study, a blinded set of drugs, known from human clinical data to be either damaging for the liver or not, was used to measure the chip’s predictive performance. All the drugs used in the study had been deemed through animal testing to be appropriate for treating human medical conditions without causing significant side effects, including those that later showed toxic effects on the liver in humans. The chip was able to identify 100% of the non-toxic compounds and correctly detect “nearly 7 out of 8” of these liver-damaging drugs. This could have huge economic as well as medical implications: the authors estimate that increased productivity in research and development could generate over \$3 billion annually for the pharmaceutical industry.⁵¹

“The results of this study show how incorporating predictive Organ-Chips into drug development workflows could substantially improve drug discovery and development, allowing manufacturers to bring safer, more effective medicines to market in less time and at lower costs..”⁵¹

New technology such as organs-on-a-chip will streamline drug development, making the process safer, cheaper, and more effective. Developing such techniques allows for the establishment of interdisciplinary research teams that will be fundamental to the creation of personalised disease models for precision medicine and the development of effective and precise systems for toxicological risk assessment.



V. Opportunities for Regulatory Toxicology

The past quarter-century has seen a revolution in the way in which chemicals are tested – non-animal tests are rapidly replacing animal tests. This is the result of our better understanding of biological processes and the emergence of new technology, which has allowed for the development of testing methods that can look directly at cellular mechanisms rather than at the crude, inscrutable results that come from using animals. It is also the result of public pressure and, as explained below, dissatisfaction among scientists with the results from animal tests. Cellular and genetic information about the potential toxicity of a chemical, such as the potential for receptor binding or gene or pathway activation, is obtained more readily with non-animal tests (using human cells *in vitro*) than with animal tests.⁵²



There is growing recognition among regulators and the regulated community that animal tests do not adequately protect either human health or the environment and that “the current approach is time-consuming and costly, resulting in an overburdened system that leaves many chemicals untested, despite potential human exposure to them”.⁵³

In 2007, the US National Academies of Sciences, Engineering, and Medicine published a landmark report titled “Toxicity Testing in the 21st Century: A Vision and a Strategy”.⁵⁴ The report states that advances in toxicogenomics, bioinformatics, systems biology, epigenetics, and computational toxicology could transform toxicity testing from a system based on whole-animal testing to one founded primarily on *in vitro* methods that evaluate changes in biologic processes using cells, cell lines, or cellular components, preferably of human origin. The proposed changes will generate better data on the potential risks humans face from environmental agents such as pesticides, building a stronger scientific foundation that can improve regulatory decisions to mitigate those risks, while reducing the time, money, and number of animals needed for testing.

The report recommends an approach that would take advantage of rapidly evolving scientific understanding of the way genes, proteins, and small molecules interact to maintain normal cell function and how some of these interactions can be perturbed in ways that could lead to adverse health outcomes. Specifically, testing could focus on relevant toxicity pathways – also known as adverse

outcome pathways (AOPs). These are cellular pathways that, when sufficiently perturbed, are expected to lead to adverse health effects. Robust *in vitro* toxicity tests can be designed to evaluate the effects of chemicals on specific events in these toxicity pathways and, therefore, an understanding of how and at what exposure level an adverse outcome may occur.⁵⁴

The current processes by which we validate new *in vitro* approaches must be adapted to account for their capacity to assess mechanisms of toxicity or specific events within an AOP.⁵⁵ The traditional approach to assessing the accuracy of a new method typically requires a one-to-one comparison of the new data with data from tests on animals. This is problematic not only because of the lack of reproducibility of many *in vivo* tests but also because *in vivo* tests often produce species-specific apical results that do not necessarily correlate with human biology, mechanisms of toxicity, or specific AOP events.

Furthermore, to keep up with the rapidly evolving field of non-animal toxicology testing, it is essential that research funds be dedicated to training opportunities for regulators and researchers. It is also critical to maintain databases of the number of animals used in each type of experiment so that efforts to replace animal tests can be prioritised and progress can be monitored.

By eliminating the use of tests on animals for regulatory purposes where replacements exist and by promoting further optimisation of methods currently in development, the EU has the



opportunity to better protect human health and the environment.

Opportunities to end the use of animals for regulatory testing immediately or within the

coming years are elaborated on in the appendices. These include tests for eye and skin irritation, skin sensitisation or the safety and efficacy of vaccines and biologics.

VI. Public Opinion and Animal Sentience

Public opposition to animal research is a major factor driving policy change. Indeed, the EU included cosmetics testing and marketing bans in the EU Cosmetics Regulation following decades of public and political support across Europe premised on the fundamental belief that the harm caused to animals used in testing cannot be outweighed by the potential benefits of new cosmetics products.⁵⁶ In support of a European citizens' initiative regarding animal experimentation, over 1.2 million Europeans asked the European Commission to protect and strengthen the cosmetics testing ban, reform chemical testing to focus on the implementation of non-animal methods, and commit to the establishment of a concrete plan to ultimately end animal testing.⁴ As a result, the European Commission is taking action to accelerate the transition to animal-free science, including committing to the development of a roadmap to end tests on animals for industrial chemicals, pesticides, biocides, and human and veterinary medicines. Specific action regarding cosmetics testing is pending the outcome of a case before the Court of Justice of the European Union.⁵⁷



Given the growing recognition of animal sentience, public opposition to animal experimentation is not surprising. In 2012, a prominent international group of neuroscientists issued *The Cambridge Declaration on Consciousness*, which definitively stated that “humans are not unique in possessing the neurological substrates that generate consciousness” and that, like humans, “[n]on-human animals have the [...] capacity to exhibit intentional behaviours”.²⁸ The declaration illustrates that recognition of animal sentience is growing within the scientific community, too. Statistics make clear that animals are not appropriate human surrogates in biomedical research, but when it comes to their ability to suffer, how much like humans need they be before a critical review of animal-based research is considered mandatory?

More than 150 academics, intellectuals, and writers have also backed a report by the Oxford Centre for Animal Ethics that condemns experiments on animals as both morally and scientifically indefensible.⁵⁸ “The deliberate and routine abuse of innocent, sentient animals involving harm, pain, suffering, stressful confinement, manipulation, trade, and death should be unthinkable. Yet animal experimentation is just that: the ‘normalisation of the unthinkable’,” write the report’s authors. They conclude that experimenting on animals contradicts what we now know about animals’ ability to experience not only pain but also shock, fear, foreboding, trauma, anxiety, stress, distress, anticipation, and terror.

Statistics make clear that animals are not appropriate human surrogates in biomedical research, but when it comes to their ability to suffer, how much like humans need they be before a critical review of animal-based research is considered mandatory?



VII. World Leadership



There is movement internationally that reflects the growing consensus in the scientific community that using animals in biomedical research, education and training or for regulatory assessment requirements is neither ethical nor efficacious. In many parts of the world, cosmetics tests on animals are now illegal. In addition, Israel⁵⁹ and India⁶⁰ have ended animal testing for household products and their ingredients, and the UK Home Office has placed strict limitations on the use of animals for such tests.⁶¹ The UK Health and Safety Executive has also significantly limited animal testing for plant-protection products.⁶²

In 2016, the Dutch government announced its plan to become the world leader in animal-free innovation by 2025. Soon after, the Netherlands National Committee for the protection of animals used for scientific purposes (NCad) published an advice report on the country's transition to animal-free innovation in which it concluded, among other things, that toxicity tests on animals for chemicals, food ingredients, pesticides, veterinary medicines, and vaccines could be phased out by 2025.⁶³

Subsequently, the government-led Transition Programme for Innovation without the use of animals (TPI) was established, aiming to bring together stakeholders and offer a platform for developing activities to speed up the transition towards animal-free innovation.⁶⁴

The US Environmental Protection Agency (EPA) released the first update to its New Approach Methods Work Plan for reducing the use of animals in testing in December 2021.⁶⁵ The plan lists concrete steps that the agency will take in the coming three years to reduce tests on vertebrates for pesticides and industrial chemicals, including establishing metrics to monitor the agency's progress in replacing animal use; developing, establishing confidence in, and accepting non-animal tests; offering educational opportunities on the use of non-animal methods; and engaging with stakeholders. The EPA work plan highlights that non-

animal methods have the potential to increase the "rigor and sophistication" of chemical assessment by the agency.⁶⁵ This is in addition to the Frank R. Lautenberg Chemical Safety for the 21st Century Act (2016)⁶⁶ that requires the use of reliable non-animal testing approaches for assessing the safety of industrial chemicals, when they exist.

Also in the US, the FDA Modernization Act 2.0 amended the Federal Food, Drug, and Cosmetic Act in 2022 to indicate that there is no compulsory requirement to test all new drugs on animals. The act specifies that the tests that are "most likely to predict human response based on scientific evidence" include cell-based assays, organ chips and microphysiological systems, computer modelling, and other human biology-based methods.⁶⁷

In 2021, members of the European Parliament almost unanimously passed a resolution calling on the European Commission to develop an action plan – with a timeline and milestones – to phase out experiments on animals and accelerate the transition to innovation without the use of animals in research, regulatory testing, and education.⁵

Such changes are necessary to improve the quality of biomedical research and regulatory assessment and for Europe to prove itself as a world leader in innovative and superior research and testing methods.

"The deliberate and routine abuse of innocent, sentient animals involving harm, pain, suffering, stressful confinement, manipulation, trade, and death should be unthinkable. Yet animal experimentation is just that: the 'normalisation of the unthinkable.'"

– Oxford Centre for Animal Ethics



VIII. Plan of Action: Recommendations to Modernise Scientific Research and Assessment

1. Immediately eliminate animal use in areas for which animals have already shown to be poor and unreliable predictors for humans and their use has impeded scientific progress.

Multiple reviews have documented the overwhelming failure of animal use to benefit human health in specific areas, including neurodegenerative diseases, neuropsychiatric disorders, cardiovascular disease and stroke, cancer, diabetes and obesity, inflammation and immune responses, HIV/AIDS research, addiction studies, trauma research, and medical training. As such, animal experiments in these research areas should be ended as soon as possible and replaced with more effective and efficient non-animal research methods. Please find appended further elaboration and recommendations on these areas.

2. Conduct critical scientific reviews to identify the areas in which the use of animals has failed to advance human or environmental health and should therefore be ended.

For those areas of investigation where there is still some question as to whether the use of animals is beneficial, a thorough systematic review should be conducted to determine the efficacy of using animals. Systematic reviews, which critically analyse multiple research studies, are the first step in assessing the effectiveness of biomedical research and toxicity testing and are included in the Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) Guidelines on what should be carried out when planning animal experiments.⁶⁸ Some countries recommend that systematic reviews be conducted before animal studies can receive funding. Scientists at Radboud University Medical Centre in the Netherlands published the following statement prior to this recommendation:

Making systematic reviews of animal studies a routine is our scientific and societal responsibility, just as with clinical studies in humans. [...] Funding agencies should stimulate and fund systematic reviews. [...] Systematic reviews disclose inadequacies in methodology of individual studies. [...] Specifically, funding agencies can mandate systematic reviews of animal experiments as part of a funding.”⁶⁹

Furthermore, Article 58 of Directive 2010/63/EU mandates that the European Commission conduct periodic thematic reviews concerning the use of animals in scientific procedures, thus providing a clear mechanism for advancing the replacement of animals in scientific procedures. To keep pace with scientific innovations, it is crucial that this process be focused and timely, and in order to maximise the process’s potential, it is vital that member states and other stakeholders such as animal welfare groups feed into it. To date, no such reviews have taken place.

3. Implement transparent, robust prospective and retrospective project evaluations, as required by Directive 2010/63/EU on the protection of animals used for scientific purposes, and allow a public commenting period so that external experts can contribute to them. This must apply to all projects.

Directive 2010/63/EU on the protection of animals used for scientific purposes requires that applications to conduct research using animals be evaluated to ensure full use of available alternative techniques and test methods as well as consideration of whether the expected outcome of the research can justify the level of pain, distress, and suffering likely to be experienced by animals.⁷⁰ While these project evaluations are generally conducted through government bodies, they at least provide a means by which ethical evaluations can take place. However, a recent retrospective analysis by Pandora Pound and Christine J Nicol concluded that “[t]he



regulatory systems in place [...] failed to safeguard animals from severe suffering or to ensure that only beneficial, scientifically rigorous research was conducted”.⁷¹ They compared the harms experienced by animals in preclinical studies for six treatment interventions to the benefits the studies offered to humans, concluding that fewer than 7 per cent of studies should have been permitted and that all the studies were of poor quality.

In addition to mandatory prospective project evaluations, Directive 2010/63/EU also requires retrospective reviews of procedures classified as “severe” and those involving non-human primates in order to assess harms retrospectively and to judge “whether the objectives of the project were achieved”.⁷⁰ The requirement, in place since 2013, is yet to be fully evaluated, but for retrospective project evaluation to be used as intended, it must be treated as more than a tick-box exercise. It is hoped that comparing the objectives of the experiment with the outcomes judged to have been achieved will prove useful in future decision-making, and as such, the retrospective evaluations must be publicly accessible and feed into the thematic reviews required under Article 58 of Directive 2010/63/EU.

Therefore, to increase scientific scrutiny of research proposals and to identify failing animal models we recommend that member states develop and implement a robust schedule of prospective and retrospective evaluations in line with the requirements of Directive 2010/63/EU.⁷⁰ To increase the transparency and accountability of the regulatory process further, project licence applications should be made available for a public commenting period, through which experts in non-animal methods have the opportunity to provide guidance, and associated retrospective evaluations should be published and linked to the original application. Such changes will help ensure the accuracy of the harm-benefit analysis process and its relevance to human clinical outcomes.

4. Work with organisations and agencies globally to harmonise and promote international acceptance of non-animal testing methods for regulatory testing requirements.

As described above, the regulatory acceptance of non-animal techniques in one region or country is an open door to international modernisation of testing requirements. Therefore, we advocate that national and international regulatory bodies and standards organisations liaise with industry, research agencies, and relevant non-governmental organisations worldwide to establish and promote clear paths to and streamlined frameworks for the validation and harmonisation of non-animal techniques for regulatory testing requirements.

Scientific confidence can be gained through transparent, peer-reviewed assessments of the fitness for purpose, technical reliability, and relevance of a new method. Implementing a streamlined framework for evaluating new toxicity testing methods that incorporates these key elements – and which is based on how well the methods reflect human biology rather than how well they align with traditional *in vivo* results – will allow faster implementation of the best science and replace the use of flawed animal tests.⁵⁵

To implement the vision of a more sophisticated approach to toxicity testing that will more adequately provide safety information on all chemicals in commerce, we further recommend that regulatory and government agencies *enforce* the existing EU legal requirement that a scientifically satisfactory method or testing strategy not entailing the use of live animals be used instead of a procedure involving animals wherever possible. In addition, we recommend that a public-private centre for predictive animal-free toxicology be coordinated through the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM). Such a centre would help transform the science of safety assessment, with new tools to guide industry, government, consumers, and international trade partners to adopt best practices.



5. Increase funds for non-animal studies and decrease funds for animal studies.

Poor predictivity of preclinical experiments on animals for toxicity and efficacy in humans has led to high attrition rates in the development of new therapies and is likely the cause of poor investment in the life sciences. The EU and individual member states should focus on driving economic growth by investment in and development of inventive, intelligent technology that can also encourage outside investment in the life sciences. As described above, non-animal techniques are one of the emerging fields with growing economic potential, and investment in them could increase returns and, in turn, encourage new investors and collaboration opportunities.

Not only does the development of this field make financial and scientific sense, EU member states are also legally bound to act by Article 47 of Directive 2010/63/EU, which mandates contribution to the development and validation of non-animal methods, the encouragement of further research in this field, and the promotion and dissemination of information about non-animal approaches.⁷⁰

National, European, and international institutes must now take the next step and end the funding of crude experiments on animals that have failed to provide effective treatments and cures. This will free up immense resources that when reinvested in exciting and innovative non-animal methods, career tracks and institutes together with bold policy initiatives, will boost the development of far more promising cures and treatments for humans. This will also alleviate the almost unimaginable suffering of millions of animals.

6. Educate and train researchers and regulators on the benefits of and how to use non-animal testing approaches.

As the fields of animal-free research and testing continue to expand, increased education and hands-on training will accelerate the transition to these methods. However, in deploying such initiatives, it is important to recognise that barriers can exist to adopting new technology, and therefore, efforts to build confidence are needed. For example, the UK's innovation agency, Innovate UK, has recognised that overcoming scepticism about the ability of non-animal methods to model biological processes will help remove a major barrier to the use of these methods. Furthermore, conservatism and inertia obstructing the move away from animal-based methods can be overcome by encouraging scientists "to think beyond their immediate research areas to how their skills, technology and 'know-how' can be leveraged and exploited to accelerate the development and adoption of" advanced non-animal methods.⁷² It is vital that such educational initiatives be adopted and given ample financial support across the whole research and testing sector, including academia, scientific and funding communities, industry, and regulators, from future scientists to established professionals.

There is a need for additional education and hands-on training in non-animal methods. Students and early career scientists must be provided with opportunities to develop the skills necessary to contribute to this research field so that the EU can compete with international developments. Because many study programmes lack sufficient courses about animal-free methods, supplemental training programmes have been developed. For example, the European Commission's Joint Research Centre (JRC) hosts a summer school on non-animal approaches.⁷³ Similar programmes could be replicated at a national level. In Canada, the University of British Columbia has accepted a new undergraduate module offered by the Society for Humane Science on "Non-Animal Methods in Biomedical Science", which focuses on training students in animal-free methods for research and testing.⁷⁴ Many online resources by experts in the field also exist, including those offered by PETA Science Consortium International e.V. and the Physicians Committee for Responsible Medicine.^{75,76} Thus, information about animal-free research and testing is available and should be a component of all biomedical education.

Awareness among scientists of animal-free methods may be increased through the creation of a national centre of competences for animal-free research and testing, tenure tracks and professorships based on non-animal methods, and animal-free research officer positions to advise professors, staff, and students. Universities and other academic institutions could also be encouraged to develop a departmental body with



regard to the transition to animal-free research and testing that can work and advise across different departments. Such bodies could help organise PhD and other postgraduate programmes that use only non-animal methods as well as workshops, seminars, and summer schools on *in vitro* and *in silico* methods.

Because non-animal science and technology are rapidly evolving, it is not only education and training at universities that is needed. The curriculum for registered professions such as the European Registered Toxicologist should also include mandatory courses on new approach methodologies, *in vitro* to *in vivo* extrapolation, systematic reviews, and AOPs. Furthermore, established researchers and regulators using animal-based methods should be provided with retraining opportunities and encouraged to forge multidisciplinary collaborations to evolve their skills and establish new and innovative ways of asking research questions and methods for answering them. For example, the Dutch Transition Programme for Innovation created a series of “helpathons”, action-orientated workshops built around a specific question that encourages researchers through a community forum to think creatively and harness the power of coincidence in the discovery of new opportunities with regard to non-animal approaches.

Funders may also require intermittent training to identify the most promising advanced animal-free methods that could have commercial potential. Similarly, regulators responsible for authorising experiments on animals – and those requiring testing data to meet legislative requirements, such as for medicinal and veterinary products, chemicals, biocides, and pesticides – should partake in compulsory training in advances in animal-free science as part of their continuing professional development.

As the field of animal-free testing methods continues to expand, researchers and regulators must keep pace with these pivotal developments. Increased education and training initiatives are urgently required to build confidence in reliable and relevant non-animal methods that can best protect human health and the environment.



References

1. Harris R. *Rigor Mortis: How Sloppy Science Creates Worthless Cures, Crushes Hope, and Wastes Billions*. Basic Books; 2017.
2. National Center for Advancing Translational Sciences (NCATS). About NCATS. Published July 3, 2018. Accessed July 3, 2018. <https://ncats.nih.gov/about>
3. EURL ECVAM. Reviewing the use of alternative methods in biomedical research. Published April 18, 2018. Accessed November 15, 2018. <https://ec.europa.eu/jrc/en/science-update/alternative-methods-biomedical-research>
4. European Union. European Citizens' Initiative: Save Cruelty Free Cosmetics - Commit to a Europe Without Animal Testing. Published 2023. Accessed August 9, 2023. https://europa.eu/citizens-initiative/initiatives/details/2021/00006_en
5. European Parliament. Plans and actions to accelerate a transition to innovation without the use of animals in research, regulatory testing and education. 2021;(2021):P9_TA(2021)0387. https://www.europarl.europa.eu/doceo/document/TA-9-2021-0387_EN.pdf
6. Macleod MR, Lawson McLean A, Kyriakopoulou A, et al. Risk of Bias in Reports of In Vivo Research: A Focus for Improvement. *PLOS Biol*. 2015;13(10):e1002273. doi:10.1371/journal.pbio.1002273
7. Hirst JA, Howick J, Aronson JK, et al. The Need for Randomization in Animal Trials: An Overview of Systematic Reviews. Thoms B, ed. *PLoS One*. 2014;9(6):e98856. doi:10.1371/journal.pone.0098856
8. Freedman LP, Cockburn IM, Simcoe TS. The Economics of Reproducibility in Preclinical Research. *PLOS Biol*. 2015;13(6):e1002165. doi:10.1371/journal.pbio.1002165
9. Pound P, Ritskes-Hoitinga M. Is it possible to overcome issues of external validity in preclinical animal research? Why most animal models are bound to fail. *J Transl Med*. 2018;16(1):304. doi:10.1186/s12967-018-1678-1
10. Wall RJ, Shani M. Are animal models as good as we think? *Theriogenology*. 2008;69(1):2-9. doi:10.1016/j.theriogenology.2007.09.030
11. van der Worp HB, Howells DW, Sena ES, et al. Can Animal Models of Disease Reliably Inform Human Studies? *PLoS Med*. 2010;7(3):e1000245. doi:10.1371/journal.pmed.1000245
12. Bailoo JD, Reichlin TS, Würbel H. Refinement of Experimental Design and Conduct in Laboratory Animal Research. *ILAR J*. 2014;55(3):383-391. doi:10.1093/ilar/ilu037
13. BioIndustry Association, Medicine Discovery Catapult. State of the discovery nation 2018 and the role of the Medicines Discovery Catapult. Published January 2018. Accessed August 27, 2021. <https://md.catapult.org.uk/resources/report-state-of-the-discovery-nation-2018/>
14. Contopoulos-Ioannidis DG, Ntzani EE, Ioannidis JPA. Translation of highly promising basic science research into clinical applications. *Am J Med*. 2003;114(6):477-484. doi:10.1016/S0002-9343(03)00013-5
15. Pound P, Bracken MB. Is animal research sufficiently evidence based to be a cornerstone of biomedical research? *BMJ*. 2014;348(may30 1):g3387-g3387. doi:10.1136/bmj.g3387
16. Lahvis GP. Point of View: Unbridle biomedical research from the laboratory cage. *Elife*. 2017;6. doi:10.7554/eLife.27438
17. Latham N, Mason G. From house mouse to mouse house: the behavioural biology of free-living *Mus musculus* and its implications in the laboratory. *Appl Anim Behav Sci*. 2004;86(3-4):261-289. doi:10.1016/j.applanim.2004.02.006
18. Garner JP. Stereotypies and Other Abnormal Repetitive Behaviors: Potential Impact on Validity, Reliability, and Replicability of Scientific Outcomes. *ILAR J*. 2005;46(2):106-117. doi:10.1093/ilar.46.2.106
19. Bayne K, Würbel H. The impact of environmental enrichment on the outcome variability and scientific validity of laboratory animal studies. *Rev Sci Tech l'OIE*. 2014;33(1):273-280. doi:10.20506/rst.33.1.2282
20. Wolfer DP, Litvin O, Morf S, Nitsch RM, Lipp H-P, Würbel H. Laboratory animal welfare: Cage enrichment and mouse behaviour. *Nature*. 2004;432(7019):821-822. doi:10.1038/432821a
21. Gross AN, Richter SH, Engel AKJ, Würbel H. Cage-induced stereotypies, perseveration and the effects of environmental enrichment in laboratory mice. *Behav Brain Res*. 2012;234(1):61-68. doi:10.1016/j.bbr.2012.06.007
22. Balcombe JP. Laboratory environments and rodents' behavioural needs: a review. *Lab Anim*. 2006;40(3):217-235. doi:10.1258/002367706777611488
23. Robinson V, Jennings M. Refinement and Reduction in the Production of Genetically Modified Mice. *Altern to Lab Anim*. 2004;32(1_suppl):373-375. doi:10.1177/026119290403201s61
24. Roth S, Liesz A. Stroke research at the crossroads - where are we heading? *Swiss Med Wkly*. 2016;146:w14329. doi:10.4414/smww.2016.14329
25. Wong CH, Siah KW, Lo AW. Estimation of clinical trial success rates and related parameters. *Biostatistics*. 2019;20(2):273-286. doi:10.1093/biostatistics/kxx069
26. Cummings JL, Morstorf T, Zhong K. Alzheimer's disease drug-development pipeline: Few candidates, frequent failures. *Alzheimer's Res Ther*. 2014;6(4):1-7. doi:10.1186/alzrt269



27. Pulley JM, Jerome RN, Zaleski NM, et al. When Enough Is Enough: Decision Criteria for Moving a Known Drug into Clinical Testing for a New Indication in the Absence of Preclinical Efficacy Data. *Assay Drug Dev Technol.* 2017;15(8):354-361. doi:10.1089/adt.2017.821
28. Low P. The Cambridge Declaration on Consciousness. Published July 7, 2012. Accessed July 16, 2018. <http://fcmconference.org/img/CambridgeDeclarationOnConsciousness.pdf>
29. Ipsos MORI. Attitudes to animal research in 2016. Published September 15, 2016. Accessed August 27, 2021. <https://www.ipsos.com/ipsos-mori/en-uk/attitudes-animal-research-2016>
30. Şentürk H. Moving Beyond Animal Models. *Turkish J Gastroenterol.* 2015;26(5):A-XI.
31. Kramer LA, Greek R. Human Stakeholders and the Use of Animals in Drug Development. *Bus Soc Rev.* 2018;123(1):3-58. doi:10.1111/basr.12134
32. Piesing M. How tech could spell the end of animals in drugs testing. *The Guardian.* Published August 23, 2014. Accessed August 2, 2018. <https://www.theguardian.com/science/2014/aug/23/tech-end-animals-drugs-testing>
33. Meigs L. Animal testing and its alternatives – the most important omics is economics. *ALTEX.* 2018;35(3):275-305. doi:10.14573/altex.1807041
34. Johnston N, Innovate UK. New kids on the emerging block. Published March 23, 2016. Accessed August 26, 2021. <https://webarchive.gov.uk/ukgwa/20210728212457/https://innovateuk.blog.gov.uk/2016/03/23/new-kids-on-the-emerging-block/>
35. AFP in Paris. Man who died in French drug trial had “unprecedented” reaction, say experts. *The Guardian.* Published March 7, 2016. Accessed September 20, 2018. <https://www.theguardian.com//2016/mar/07/french-drug-trial-man-dead-expert-report-unprecedented-reaction>
36. Attarwala H. TGN1412: From Discovery to Disaster. *J Young Pharm.* 2010;2(3):332-336. doi:10.4103/0975-1483.66810
37. Ferguson PR. The TGN1412 drug disaster. *Am Bar Assoc.* 2009;5(4):12-13.
38. Fleming A. On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to Their Use in the Isolation of B. influenzae. *Br J Exp Pathol.* 1929;10(3):226-236.
39. Greek R, Hansen LA. The Strengths and Limits of Animal Models as Illustrated by the Discovery and Development of Antibacterials. *Biol Syst.* 2016;02(02). doi:10.4172/2329-6577.1000109
40. Florey H. The advance of chemotherapy by animal experiment. *Conquest.* 1953;41(12).
41. Koppányi T, Avery MA. Species differences and the clinical trial of new drugs: A review. *Clin Pharmacol Ther.* 1966;7(2):250-270. doi:10.1002/cpt196672250
42. Ronaldson-Bouchard K, Vunjak-Novakovic G. Organs-on-a-Chip: A Fast Track for Engineered Human Tissues in Drug Development. *Cell Stem Cell.* 2018;22(3):310-324. doi:10.1016/j.stem.2018.02.011
43. House of Commons Science and Technology Committee. Research integrity: Sixth report of session 2017-19. Published June 26, 2018. Accessed August 27, 2021. <https://publications.parliament.uk/pa/cm201719/cmselect/cmsctech/350/350.pdf>
44. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol.* 2010;8(6):e1000412. doi:10.1371/journal.pbio.1000412
45. Leung V, Rousseau-Blass F, Beauchamp G, Pang DSJ. ARRIVE has not ARRIVED: Support for the ARRIVE (Animal Research: Reporting of in vivo Experiments) guidelines does not improve the reporting quality of papers in animal welfare, analgesia or anesthesia. Pritchett-Corning KR, ed. *PLoS One.* 2018;13(5):e0197882. doi:10.1371/journal.pone.0197882
46. Burt T, Yoshida K, Lappin G, et al. Microdosing and Other Phase 0 Clinical Trials: Facilitating Translation in Drug Development. *Clin Transl Sci.* 2016;9(2):74-88. doi:10.1111/cts.12390
47. BCC Research. Cell-based Assays: Technologies and Global Markets. Published August 2022. Accessed August 9, 2023. <https://www.bccresearch.com/market-research/biotechnology/cell-based-assays-technologies-markets-report.html>
48. BCC Research. Induced Pluripotent Stem Cells: Global Markets. Published July 2021. Accessed October 16, 2023. <https://www.bccresearch.com/market-research/biotechnology/induced-pluripotent-stem-cells-report.html>
49. KBV Research. *Global Lipid Market Size, Share & Industry Trends Analysis Report By Application, By Source, By Regional Outlook and Forecast, 2022 – 2028.*; 2022. Accessed August 9, 2023. <https://www.kbvresearch.com/orga n-on-chip-market/>
50. Cox B, Barton P, Class R, et al. Setup of human liver-chips integrating 3D models, microwells and a standardized microfluidic platform as proof-of-concept study to support drug evaluation. *Biomater Biosyst.* 2022;7(May):100054. doi:10.1016/j.bbiosy.2022.100054
51. Ewart L, Apostolou A, Briggs SA, et al. Performance assessment and economic analysis of a human Liver-Chip for predictive toxicology. *Commun Med.* 2022;2(1):154. doi:10.1038/s43856-022-00209-1
52. Hartung T, FitzGerald RE, Jennings P, et al. Systems Toxicology: Real World Applications and Opportunities. *Chem Res Toxicol.* 2017;30(4):870-882. doi:10.1021/acs.chemrestox.7b00003
53. The National Academies. Report Calls for New Directions, Innovative Approaches in Testing Chemicals for Toxicity To Humans. *ScienceDaily.* Published July 4, 2007. Accessed October 16, 2023. <https://www.sciencedaily.com/releases/2007/06/070628071625.htm>



54. NRC. *Toxicity Testing in the 21st Century: A Vision and a Strategy*. National Research Council. The National Academies Press.; 2007. doi:10.17226/11970
55. van der Zalm AJ, Barroso J, Browne P, et al. A framework for establishing scientific confidence in new approach methodologies. *Arch Toxicol*. 2022;96(11):2865-2879. doi:10.1007/s00204-022-03365-4
56. European Commission. Staff working document: Impact assessment on the animal testing provisions in Regulation (EC) 1223/2009 on cosmetics. COM(2013) 135 final. Published March 11, 2013. Accessed March 13, 2013. <http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52013SC0066&from=EN>
57. European Commission. C(2023) 5041 - Communication from the Commission on the European Citizens' Initiative (ECI) 'Save cruelty-free cosmetics – Commit to a Europe without animal testing.' Accessed October 24, 2023. https://single-market-economy.ec.europa.eu/publications/communication-commission-european-citizens-initiative-eci-save-cruelty-free-cosmetics-commit-europe_en
58. Working Group of the Oxford Centre for Animal Ethics. Normalising the unthinkable: The ethics of using animals in research. Executive Summary. Accessed October 18, 2021. [https://www.crueltyfreeinternational.org/sites/default/files/Oxford summary final.pdf](https://www.crueltyfreeinternational.org/sites/default/files/Oxford%20summary%20final.pdf)
59. Fisher G. Import ban on animal-tested products goes into effect. Published January 1, 2013. Accessed October 16, 2023. <https://www.timesofisrael.com/import-ban-on-animal-tested-products-goes-into-effect/>
60. Library of Congress. India: No Import of Cosmetics Tested on Animals. Published October 17, 2014. Accessed October 16, 2023. <https://www.loc.gov/item/global-legal-monitor/2014-10-17/india-no-import-of-cosmetics-tested-on-animals/>
61. UK Home Office. Ban will end testing of household products on animals. Published March 12, 2015. Accessed August 27, 2021. <https://www.gov.uk/government/news/ban-will-end-testing-of-household-products-on-animals>
62. UK Health and Safety Executive (HSE). Vertebrate testing (toxicology). Accessed October 18, 2021. <https://www.hse.gov.uk/pesticides/pesticides-registration/applicant-guide/vertebrate-testing.htm>
63. Netherlands National Committee for the protection of animals used for scientific purposes (NCad). Transition to non-animal research: On opportunities for the phasing out of animal procedures and the stimulation of innovation without laboratory animals. Published online December 2016:76. Accessed November 15, 2018. <https://www.ncadierproevenbeleid.nl/binaries/ncadierproevenbeleid/documenten/rapport/2016/12/15/ncad-opinion-transition-to-non-animal-research/NCad+Opinion+Transition+to+non-animal+research.pdf>
64. Transition Programme for Innovation without the use of animals (TPI). The TPI's aim. Accessed September 15, 2020. <https://www.transitieproefdiervrijeiinnovatie.nl/english/tpi>
65. EPA. *New Approach Methods Work Plan (V2)*. U.S. Environmental Protection Agency. EPA/600/X-21/209.; 2021. Accessed January 3, 2022. https://www.epa.gov/system/files/documents/2021-11/nams-work-plan_11_15_21_508-tagged.pdf
66. Frank R. Lautenberg Chemical Safety for the 21st Century Act, HR 2576, 114th Cong (2016). Pub L No. 114-84. Accessed August 9, 2023. <https://www.congress.gov/114/plaw%20As/publ182/PLAW-114publ182.pdf>
67. FDA Modernization Act 2.0. S 5002, 117th Cong (2021–2022). Accessed August 9, 2023. <https://www.congress.gov/bill/117th-congress/senate-bill/5002>
68. Smith AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T. PREPARE: guidelines for planning animal research and testing. *Lab Anim*. 2018;52(2):135-141. doi:10.1177/0023677217724823
69. Hooijmans CR, Ritskes-Hoitinga M. Progress in Using Systematic Reviews of Animal Studies to Improve Translational Research. *PLoS Med*. 2013;10(7):e1001482. doi:10.1371/journal.pmed.1001482
70. EU. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. *OJ*. 2010;L 276:33-79. <https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32010L0063>
71. Pound P, Nicol CJ. Retrospective harm benefit analysis of pre-clinical animal research for six treatment interventions. Olsson IAS, ed. *PLoS One*. 2018;13(3):e0193758. doi:10.1371/journal.pone.0193758
72. Innovate UK, NC3Rs, BBSRC, DSTL, EPSRC, MRC. A non-animal technologies roadmap for the UK. Published November 10, 2015. Accessed August 9, 2023. <https://www.ukri.org/publications/non-animal-technologies-in-the-uk-a-roadmap-strategy-and-vision/>
73. EU Science Hub. JRC Virtual Summer School on "Non-animal approaches in science: the three R...evolution". Accessed August 9, 2023. https://joint-research-centre.ec.europa.eu/events/jrc-summer-school-non-animal-approaches-science-3-2021-05-17_en
74. Society for Humane Science. University Education. Accessed August 9, 2023. <https://www.forhumanescience.org/influencing-science-culture/university-education/>
75. PETA Science Consortium International e.V. Training Opportunities. Accessed August 9, 2023. <https://www.thepsci.eu/our-work/training/>
76. Physicians Committee for Responsible Medicine. NAM Use for Regulatory Application. Accessed August 9, 2023. <https://www.pcrm.org/ethical-science/animal-testing-and-alternatives/nura>



Appendices

Please find in the following pages further details on opportunities to replace animals in the following areas of biomedical research and training, forensic sciences, toxicity assessment, and laboratory production methods. Also included is information regarding the expertise of PETA scientists. The appendices feature several examples of the implementation of non-animal methods. However, they do not represent a complete collection of the scientific literature or regulations worldwide.

Any mention of PETA Science Consortium International e.V. prior to December 2020 refers to PETA International Science Consortium Ltd.

Table of Contents

Glossary	23	Toxicity Assessment	47
Basic and Applied Biomedical Research	24	Approaches to Toxicity Assessment	47
Cancer	24	Ecotoxicity	48
Cardiovascular Disease	25	Aquatic Toxicity	48
Diabetes	27	Avian Toxicity	48
Inflammation and Immunology	28	Endocrine Disruption	49
HIV/AIDS	28	Eye Irritation/Corrosion	50
Mouse Immunology	29	Genotoxicity and Carcinogenicity	51
Sepsis	31	Genotoxicity	51
Nerve Regeneration	32	Carcinogenicity	53
Neurodegenerative Diseases	34	Phototoxicity	54
Neuropsychiatric Disorders and Neurodivergence	36	Pyrogenicity	54
Stroke	38	Reproductive and Developmental Toxicity	55
Substance Abuse	39	Skin Irritation/Corrosion	56
Trauma	41	Skin Sensitisation	57
Training and Forensic Enquiries	43	Systemic Toxicity	57
Forensic Sciences	43	Acute Systemic Toxicity	57
Medical Training	44	Repeat-Dose Systemic Toxicity	58
Microsurgery Training	44	Oral Route	58
Trauma Training	45	Dermal Route	59
		Inhalation Route	59
		Tobacco and E-Cigarette Testing	59
		Laboratory Production Methods	61
		Antibody Production	61
		Biologic Drugs	62
		Foetal Bovine Serum	63
		Scientific Advisory Capabilities of PETA	65



Glossary

3Rs	replacement, reduction, and refinement (of animal use)	ISO	International Organization for Standardization
AD	Alzheimer's disease	JaCVAM	Japanese Center for the Validation of Alternative Methods
ADHD	attention-deficit/hyperactivity disorder	JRC	European Commission Joint Research Centre
AIDS	acquired immune deficiency syndrome	LAL	Limulus amebocyte lysate
ALS	amyotrophic lateral sclerosis	LTT	live tissue training
AOP	adverse outcome pathway	MAT	monocyte activation test
ATLS	advanced trauma life support	NICEATM	US NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
BCOP	bovine corneal opacity and permeability	NIH	US National Institutes of Health
CTA	cell transformation assay	NOS	nitric oxide synthase
DPRA	direct peptide reactivity assay	NRU	neutral red uptake
ECHA	European Chemicals Agency	NTP	US National Toxicology Program
EDQM	European Directorate for the Quality of Medicines & HealthCare	OECD	Organisation for Economic Co-operation and Development
EDSP	Endocrine Disruptor Screening Program	PD	Parkinson's disease
EMA	European Medicines Agency	PDAC	pancreatic ductal adenocarcinoma
EPA	US Environmental Protection Agency	Ph. Eur.	European Pharmacopoeia
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing	REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
FBS	foetal bovine serum	RhCE	reconstructed human cornea-like epithelium
GEMM	genetically engineered mouse model	RHE	reconstructed human epidermis
GHS	Globally Harmonized System of Classification and Labelling of Chemicals	RPT	rabbit pyrogen test
h-CLAT	human cell line activation test	SA	structural alert
HD	Huntington's disease	SCCS	Scientific Committee on Consumer Safety
HIV	human immunodeficiency virus	SCHEER	European Commission Scientific Committee on Health, Environmental and Emerging Risks
hPL	human platelet lysate	SCI	spinal cord injury
IATA	integrated approach to testing and assessment	SIV	simian immunodeficiency virus
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods	STAIR	Stroke Therapy Academic Industry Roundtable
IET	Institution of Engineering and Technology	STE	short time exposure
IFV	influenza virus	T2DM	type 2 diabetes mellitus
		TER	transcutaneous electrical resistance
		TZD	thiazolidinedione
		VR	virtual reality
		WoE	weight of evidence



Basic and Applied Biomedical Research

Detailed below are opportunities to end the non-regulatory use of animals immediately in a number of specific areas of biomedical research.

Cancer

Recommendation: End the use of animals

Cancer is one of the leading causes of death worldwide.¹ Even after significant investment in research for cancer therapies, the success rate for oncology drugs is only 3.4%,² despite those drugs having been successful in preclinical animal testing. Decreases in cancer rates over the past two decades are attributed primarily to personal preventive measures, including refraining from cigarette smoking, eating more fruits and vegetables, and having regular check-ups for screening,^{3,4} rather than to the results of biomedical research.

The scientific community is aware that the use of animals, particularly mice, for human cancer research is problematic. For one, published results from the Reproducibility Project: Cancer Biology show that cancer experiments on animals have smaller effect sizes and are less likely to be replicated than non-animal cancer experiments.⁵ Even though study design and other logistical issues in research can create problems, cancer physicians at McMaster University in Ontario stated the following:

“[M]ost futilities in fact originate from molecular mechanisms of the drug(s) tested. ... Crucial genetic, molecular, immunologic and cellular differences between humans and mice prevent animal models from serving as effective means to seek for a cancer cure.”⁶

There are several methods by which rodents – predominantly mice – are used in basic and translational cancer experimentation, including xenotransplantation, genetic engineering, and, less frequently, environmental induction, which involves exposing animals to known cancer-causing agents.

In xenograft modelling, human or animal cancer cells are transplanted either under the skin or into an organ of immunocompromised rodents, who may then be treated with a chemical or test substance of interest.⁷ Following an analysis of 1,110 mouse xenograft tumour models, scientists and physicians from Harvard University, Massachusetts Institute of Technology, the Dana-Farber Cancer Institute, and other respected institutions reached a conclusion that fundamentally challenged the ability of xenograft models to predict human patients’ response to therapy. They found that transplanting human cancer cells into these mice altered the genetic composition of those cells in ways that would be unlikely to happen in humans. That, in turn, altered the responses that the cells had to chemotherapy drugs.⁸ Essentially, when human tumour cells are transplanted into mice, they develop characteristics of mouse cells, which are not relevant to human biology.

Experimenters create genetically modified (transgenic) mice by inducing the expression of oncogenes or by inactivating tumour-suppressing genes.⁹ However, with these methods, researchers are often unable to control the level and pattern of the gene expression or gene inactivation, thus failing to mimic the sporadic and multistep nature of tumour growth seen in natural tumour development.⁹ In addition, random integration of the oncogenes can result in unexpected outcomes that would not be present in human patients.⁹ These models are also time-consuming and costly to create, and they use large numbers of animals because of the extensive breeding requirements.^{10,11}

Given the many shortcomings of cancer modelling in animals as well as the astonishingly low translational success rate of such models, it is clear that they are not suitable for human cancer experimentation. In light of



this and the pain and suffering experienced by the animals who are used, it should be a priority to move away from animal models and focus instead on human-relevant methods.

In August 2021, the European Commission's Joint Research Centre (JRC) published a report on immuno-oncology and highlighted important publications that describe promising, advanced non-animal models. These studies employed human-based, non-animal methods for developing immunotherapies, studying cancer initiation and development, exploring anti-cancer therapies, studying immunomodulation of cancer physiology or potentially effective strategies for enhancing the anti-tumour immune response, determining molecular features that can represent biomarkers in specific cancer pathogenesis, exploring adoptive cell therapies and virotherapies, and more.¹²

Some examples of recent human-relevant cancer research include vascular human tumour models – created using three-dimensional bio-printing – that mimic key steps of cancer metastases,¹³ patient-specific human lung-on-a-chip models for precision medicine,¹⁴ sophisticated analyses of human mammary tumour organoids¹⁵ and breast cancer cell lines,¹⁶ genomics to improve understanding of uniquely human aspects of cancer,^{17,18} artificial intelligence for faster diagnoses¹⁹ and for predicting individual drug responses,²⁰ and wearable bionic chips to collect real-time data from patients.²¹

Former US National Cancer Institute Director Dr Richard Klausner stated:

“The history of cancer research has been a history of curing cancer in the mouse. We have cured mice of cancer for decades – and it simply didn't work in humans.”²²

Cancer is a highly variable, individualised disease that will require individualised treatment to overcome.²³ Scientists using non-animal methods for cancer research are faced with a smaller translational hurdle, since they are able to use patients' own cancer cells and because all human-relevant methods are grounded in human, not rodent, biology.

Cardiovascular Disease

Recommendation: End the use of animals

Cardiovascular disease is the number one cause of death in several countries worldwide, yet the development and approval of new drug candidates for treating it have declined over the past two decades.²⁴

Species differences in resting heart rate, action potentials, myofilament protein isoforms, excitation-contraction (E-C coupling), and force-frequency relations limit the translatability to humans of many animal models of cardiovascular function.^{25,26} A meta-analysis evaluating 11 measured functional parameters of the heart, comparing rodents with humans, concluded that only one (systolic pressure) was within an acceptable range for comparison between the two species.²⁷ The properties of calcium-handling proteins and their composition differ in the hearts of rats, mice, rabbits, dogs, and humans, and rodents and humans do not have the same profiles or functions of contractile proteins.²⁷ This makes the profile of ventricular repolarisation and susceptibility to arrhythmia different, leading to varied drug responses. Rodents are also resistant to atherosclerosis, a major cause of many cardiovascular diseases, owing to their lack of cholesteryl ester transfer protein.²⁸ Rat and mouse models of heart failure do not exhibit the same miRNA expression profiles as patients with acute heart failure.²⁹ Additionally, most animal models do not mimic the complex genetic and environmental contributors associated with cardiovascular health or the progressive nature of human cardiovascular disease.³⁰

In the field of heart failure, “insights gleaned from animal based research efforts have shown poor translation in terms of deciphering human heart failure and developing effective therapies”, and “lack of concordance



between animal models and human disease state has been acknowledged as a major contributing factor [to this translational failure]”.³¹

The continued reliance on inadequate animal models affects not only cardiovascular disease research but also drug development for all other disease areas. In a recent review article, Dartmouth College scientists noted, “The majority of phase I drug failures and post-approval withdrawal of medicinal products are attributed to cardiovascular toxicity. Almost half of the drugs in the pharmacology market since the 1990s have been retracted due to cardiovascular complications.”³² Experts point out the “lack of concordance between the effects of compounds in animals (or animal-derived tissues) and those in humans”³³ and the many known species-related differences in cardiac contractile function and calcium handling and that “substantial differences in drug responsiveness between species can limit the effectiveness of predicting clinical outcome from animal toxicity testing”.³⁴ In a coauthored review, scientists from Stanford University, the US Food and Drug Administration (FDA), and the biopharmaceutical company AbbVie refer to testing cardiotoxicity in animal models as a “black box” approach.³³ It is clear that human-relevant *in vitro* and *in silico* methods are much more suitable for cardiotoxicity testing and cardiovascular research in general.

The global stem cell biotechnology company Novoheart is using a platform called MyHeart™ composed of engineered human cardiac tissues, which has been able to “detect the devastating arrhythmogenic hazards of certain ‘anti-arrhythmic’ drugs that had previously caused fatalities in human patients despite passing through the flawed process of animal testing for FDA approval”.³⁵ Worcester Polytechnic Institute’s Marsha Rolle, a tissue engineer, has created functional blood vessels from human cells to “replicate what happens when [human blood vessels are] diseased”.³⁶ In a news release, she noted that the 10-year average timescale for developing new medications is “exacerbated by the fact that animal testing, which is the way most new drugs are tested, is not always an accurate indicator of how human blood vessels will respond to the same drugs”.³⁶ Investigators at the University of California–Los Angeles and Sharif University of Technology in Tehran recently designed a heart-on-a-chip platform that incorporated microgrooves and electrical pulse stimulations to recapitulate the well-aligned structure and synchronous beating of cardiomyocytes and can be utilised for high-throughput screening for cardiotoxicity.³⁷

Other recent advancements in human tissue engineering for cardiovascular research include the ability of scientists to control the electrical pace of laboratory-grown heart cells using light,³⁸ the use of a plant-derived cellulose framework as scaffolding to build networks of human veins,³⁹ and the development of an *in vitro* three-dimensional model of early heart development in humans that “could serve as an embryotoxicity screening assay in drug discovery, regulation, and prescription for healthy fetal development”.⁴⁰ This three-dimensional “organogenesis-in-a-dish” model could provide a way to determine drug safety in pregnant women.

Using microfluidic tissue chips with multiple pulmonary arterial cell types from male and female patients, researchers at Texas Tech University Health Sciences Center identified cell-specific differences in response to hormones that may contribute to the complex sex disparities of pulmonary arterial hypertension (PAH), a progressive and life-threatening disease impossible to recapitulate fully in animal models.⁴¹ This sex-specific PAH chip design was noted for being a “useful model for studying mechanism of sex disparity to advance sex-specific treatment for PAH patients”.⁴² Researchers at the Medical University of South Carolina, Clemson University, and Janssen Research and Development have recently designed a human cardiac organoid disease model of the acute post-myocardial infarction cardiac state at a transcriptomic, structural, and functional level.⁴³

Computer modelling is also rapidly advancing human cardiovascular and cardiotoxicity research. Recently, an international team of researchers developed a machine learning–based tool to predict progression of hypertrophic cardiomyopathy, a disease that effects one in 500 young adults and can cause sudden death.⁴⁴ Clemson University Assistant Professor Ethan Kung was given a prestigious National Science Foundation grant for his work “aimed at reducing human and animal testing and addressing concerns that the skyrocketing cost



of developing new devices and surgeries is unsustainable”.⁴⁵ His research merges numerical computer models with experimental data to create modern cardiovascular biochemical models. University of Oxford researchers have demonstrated that *in silico* methods are more accurate than animal models at predicting the cardiotoxicity of certain drugs.⁴⁶

Diabetes

Recommendation: End the use of animals

From 1984 to 2014, more than 50 papers were published per month describing experiments on rodent models of type 2 diabetes mellitus (T2DM).⁴⁷ Considering these numbers, we now know a great deal about diabetes, or metabolic disturbances that look like diabetes, in rodents, but “many details of human T2DM pathogenesis remain unclear, and means of preventing disease progression remain elusive”.⁴⁷ Rodent studies were used to identify thiazolidinedione (TZD) drugs as possible therapeutics for humans with T2DM or insulin dysfunction. Unfortunately, the studies did not predict that TZDs would increase the risk of cardiovascular death in these patients by 64%; in fact, they provided contradictory evidence.⁴⁸

T2DM is a disease of glucose misregulation resulting from impaired insulin secretion action and pancreatic β -cell dysfunction that leads to broad physiological effects. Rodents differ from humans on every tier of glucose regulation, from the level of nucleic acids to differences in proteins, pathways, cells, tissues, and organs. The two species also differ in terms of disease progression at the organism level and, dramatically, in environmental exposure and autonomy of lifestyle.^{47,48} “Because mice rely principally on the liver for glucose homeostasis, while humans rely on skeletal muscle where transport mechanisms and biochemical pathways differ, mice may not be expected to be analogous to [T2DM] patients in regards to mechanisms of glucose metabolism or its dysfunction.”⁴⁸ And as Joan Mir-Coll and colleagues point out, “[R]odent β -cells differ from human β -cells in parameters such as response to different stressors, proliferative capacity under insulin resistance, glucose uptake, kinetics of insulin secretion, cellular composition and architectural distribution, and transcriptional profile.”⁴⁹ Despite these clear discrepancies, diabetes research in animals continues while more relevant, human-based methods are often ignored.

Many genetic models of T2DM are based on leptin or leptin receptor deficiency, even though neither of these represents an important contributor to T2DM in humans.⁵⁰ Mice who have been genetically modified to lack select insulin-signalling genes are also poor models. For example, mice with a complete deletion of the insulin receptor die within a few days of birth, while humans with this rare condition can survive until age 2.⁴⁸ Overall, observed phenotypes in these and similar animal models of diabetes are only “secondary to genetic mutations that do not reflect disease etiology in humans”.⁵⁰

In their 2018 publication, Ali, Chandrasekera, and Pippin discuss a wealth of relevant methods for studying diabetes, stressing the need to focus on human biology for human diabetes research:

As we continue to uncover major species differences in factors affecting glucose biology – such as cell division, stimulus-secretion coupling and autocrine-paracrine interactions ... it is now becoming unquestionable that **new information should be derived solely from human primary cells, tissues and organs**, obtained from nonpatient controls and patients in the various progressive stages of T2DM. ... If the ultimate goal of the diabetes research community is to understand disease mechanisms that will lead to better T2DM prevention and therapeutic outcomes for patients, then the best way to achieve that goal is by prioritising human-centred research.⁵¹ [*Emphasis added*]



Human-relevant alternatives to the use of animals in diabetes research include human imaging, *in vitro* technology using human heterologous cell lines, human induced pluripotent stem cells, organotypic three-dimensional cell culture, the use of human organs *ex vivo*, post-mortem human tissue, non-invasive human imaging, epidemiological and human genetic studies – including nutrigenomics and nutrigenetics – and *in silico* modelling.^{47,51} For example, scientists at Glasgow Caledonian University used human cells from a tissue bank to generate wound-healing models for diabetic patients, who have difficulty with wound healing and controlling skin infections.⁵² Additionally, the FDA has approved a closed-loop insulin pump developed using *in silico* modelling as a substitute for animal testing, providing just one example of how “[r]ealistic computer simulation is capable of providing invaluable information about the safety and the limitations of closed-loop control algorithms, guiding clinical studies, and out-ruling ineffective control scenarios in a cost-effective manner”.⁵³ *In silico* models are being used to rapidly assess potential natural and pharmaceutical interventions for T2DM.^{54,55} Numerous investigators are using islet-on-a-chip microfluidic systems to study disease mechanisms and test therapeutic agents.^{56–58}

Inflammation and Immunology

Recommendation: End the use of animals

The use of animals in research to study human inflammation and immunology encompasses a great deal of basic and disease-related research. We will briefly discuss three main areas: the use of animals for HIV/AIDS research, the use of mice for human immune research, and the use of animals to study human sepsis.

HIV/AIDS

The failure to translate experiments on animals into the useful human application of HIV/AIDS vaccines was recognised more than 20 years ago when, in 1995, the US National Institutes of Health (NIH) instituted a moratorium on the breeding of chimpanzees, the most commonly used animal in HIV/AIDS research at the time, acknowledging the failure of studies using the species to produce clinically useful data in this field. Following NIH’s acknowledgement that chimpanzees aren’t human-relevant surrogates for this research, experimenters began to use other non-human primate (NHP) species, notably macaques.

Because humans are the only primates who contract HIV and develop AIDS, experimenters instead infect monkeys with simian immunodeficiency virus (SIV), a virus unique to African primates. The genetic homology between HIV and SIV is only 55%, and SIV is less genetically diverse than HIV.^{59,60} Owing to differences in surface proteins and other molecular markers, antibodies that neutralise SIV have no effect on HIV, and vice versa,⁶¹ making them useless in HIV research. Importantly, the dose of SIV administered to non-human primates in experiments is often much higher than the typical amount of HIV-1 to which a human is exposed during sexual transmission.⁶² Sometimes, experimenters use an engineered SIV/HIV concoction. AIDS researcher Mark Girard has stressed, “One should realize that we still do not know how the SIV or SHIV model compares to HIV infection in humans. Extrapolating from vaccine protection results in non-human primate studies to efficacy in man may be misleading.”⁶³

In a peer-reviewed journal, an animal experimenter at the Washington National Primate Research Center admitted that non-human primate models of HIV “do not allow direct testing of HIV vaccines” and that “because of the complexity and limitations of the NHP models, it remains difficult to extrapolate data from these models to inform the development of HIV vaccines”.⁶⁴ Experimenters have developed dozens of vaccine candidates using monkeys. Only five have reached as far as human trials, and all of them have failed.⁶⁵ One of them even increased the likelihood of HIV infection in humans.⁶⁶ After one of the human vaccine trials failed in 2018, Anthony Fauci, director of the US National Institute of Allergy and Infectious Diseases, acknowledged that the original positive results of a macaque study “might be a fluke”.⁶⁷



Because of broad failures in non-human primate HIV/AIDS research, experimenters have shifted some focus to mice – a species even more genetically removed from humans. The “humanised” mouse model for HIV/AIDS research is a mouse who has been partially repopulated by human immune cells, allowing the animal to be infected with HIV-1. However, humanised mice are limited in their longevity with the disease and retain parts of their murine immune systems, “complicating immune response interpretations”.⁶¹ Not surprisingly, the use of humanised mice has also failed to generate useful results for clinical HIV/AIDS treatment.

Considering the differences between a laboratory environment and human society, it is clear that experiments on animals will never capture the complexity of this human disease. Mice and rats used in experiments are kept in conditions in which the primary pathogens present are those in their own faeces, and cofactors that may be present in human patients, such as other microbial infections, are absent, significantly altering the acquisition and course of the virus.⁵⁹ Non-human primates used in HIV research, on the other hand, have been found to be harbouring confounding infections like valley fever, which compromises findings when they are used in HIV studies.⁶⁸

Researchers at Emory University in Atlanta stated, “HIV persistence is a very complex virological and immunological phenomenon, with infection of several cell types in a wide array of anatomic tissues that are all regulated differently,”⁶⁹ and they recognised that human *in vitro* models are needed to replicate this human disease and develop treatment. Thinking progressively about non-animal methods, UK scientists have said, “Existing animal models predicting clinical translations are simplistic, highly reductionist and, therefore, not fit for purpose,” and they reported that clinical attrition data “focusses the attention back on to early target selection/lead generation, but it also questions the suitability of current animal models with respect to congruency with and extrapolation of findings for human hosts”.⁷⁰

Scientists admit that even after costly and unreliable experiments on animals, human data are still needed to determine whether a drug is fit for the clinical setting. Rao and Alving of the US Military HIV Research Program stated that “human clinical trials still appear to be the only reliable way to determine whether an HIV vaccine candidate will have activity or efficacy in humans”.⁷¹ Scientists from Australia, France, Italy, and the UK have been studying the immune cells of individuals called “HIV controllers”, who can become infected with HIV but are able to control the spread of the virus without any intervening therapy.⁷² The hope is that immune cells from HIV controllers can be transferred to HIV-infected patients to help them fight the virus. This promising research is human-specific and requires human-specific testing methods.

Other recent examples of non-animal HIV research include the use of interactive molecular dynamics simulations in virtual reality to predict exactly how drug molecules will bind to HIV proteins,⁷³ novel imaging techniques to discover previously unknown aspects of HIV structure that open up the potential for new therapies,⁷⁴ and bioinformatics analysis of specimens from individuals with viremia and *in vitro*-infected cells from healthy donors to construct an atlas of the phenotypes of HIV-susceptible cells.⁷⁵

Nobel laureate Sydney Brenner declared, “We don’t have to look for model organisms anymore because we are the model organism.”⁷⁶ Similarly, in 2007, the associate editor of *The BMJ* stated, “When it comes to testing HIV vaccines, only humans will do.”⁷⁷

Mouse Immunology

Because of the development of tools allowing for manipulation of the mouse genome, the mouse is the most commonly used research subject worldwide. However, it should be no surprise that with this rampant use comes substantial evidence that mice are not the same as humans and that there are certain fields, in particular, in which the dramatic differences in physiology between the two species disqualify the use of mice as research subjects. One of the most noted fields in this category is immunology.

In 2004, a compelling review was published in *The Journal of Immunology* outlining the many differences between mouse and human immune systems, including in the anatomy of lymphoid tissue, ratios of white



blood cell types, antimicrobial peptide profiles, cytokine profiles and functions, mechanisms for crosstalk between the adaptive and innate immune systems, antibody subtypes, development and regulation of lymphocytes, and activation of clotting factors.⁷⁸ Since then, several other analyses have been published detailing the many differences between human and mouse immunology.

A 2014 study found fundamental differences between the species in the innate immune response, stating, “[W]hile in human blood mechanisms of immune resistance are highly prevailed, tolerance mechanisms dominate for the defense against pathogenic microorganisms in mouse blood.”⁷⁹ Logically, these differences make sense: we humans “do not live with our heads a half-inch off the ground”,⁷⁸ and we have considerably longer life spans and a larger body size than mice do.^{78,79} As concisely stated by Leist and Hartung, “[H]umans are definitely no 70-kg mice.”⁸⁰ Despite the glaring contrast, mice continue to be used for immunological research.

The use of mice as a model of influenza (IFV) infection has been heavily criticised: “There are ... a number of drawbacks of the [mouse] model that make it unsuitable for addressing certain virological questions and can render data obtained in mice difficult to translate to the human situation.”⁸¹ Viral infection is species-specific, and mice cannot naturally catch human IFV. To bypass this problem, experimenters have altered the strain of the mice and the strain of the viruses used. The BALB/c mouse, for example, is highly susceptible to viral infection because of the lack of MX1 gene, which codes for Mx1 protein that can selectively inhibit IFV replication.⁸² The lethal dose of a deadly IFV strain (H5N1) is about 100 times lower in BALB/c mice compared to their wild cousins.⁸³ BALB/c mice do not possess genetic heterogeneity or proper immune function for virology research.

The viruses used in animal studies are often adapted through serial passage in target hosts (mice, in this case) for easy infection.⁸¹ This is because human IFV receptors ($\alpha 2,6$ -linked sialic acids) are not abundant in the upper airways of mice, who have a different receptor ($\alpha 2,3$ -linked sialic acids).⁸⁴ Through serial passage, the virus can adapt to the new host and become distinct from the kind that predominantly affects humans.

There are many more differences between mice and humans in terms of IFV disease progression. For example, mice get hypothermia rather than fever following infection.⁸⁵ They do not cough or sneeze.⁸¹ Moreover, the virus does not transmit between mice.⁸⁶ Additionally, we now know that gut microbiota are intimately linked to the immune system,⁸⁷ and studies have demonstrated drastic differences between the microbiomes of humans and mice. For example, 85% of bacterial species in mice don’t exist in humans.⁸⁸ The aforementioned evidence supports the inapplicability of mouse immunity to human immunity.

Considering the obvious failure of mice as surrogates in the study of human immune systems, investment in human-relevant *in vitro* and *in silico* models is needed. Advances in data collection and computer analyses have allowed for the development of human-relevant multiscale models that “can consistently integrate immunological data generated at several scales, and can be used to describe and optimize therapeutic treatments of complex immune diseases”.⁸⁹

Vanderbilt University researchers have used a dual-chamber blood-brain barrier microfluidic device called the NeuroVascular Unit to study the human blood-brain barrier’s response to neuroinflammation.⁹⁰ German scientists developed a computer model that gives them the capability to assess, for the first time, the electrophysiological consequence of the acidosis in human immune cells accompanying most forms of inflammation.⁹¹ Additionally, a University of Tennessee–Knoxville mathematician, along with surgical and immunological specialists at the University of Pittsburgh, used a mechanistic mathematical model to characterise human immune responses during organ transplantation.⁹²

A review summarising the progress of immune-competent human skin disease models recognises the failures of animal studies to translate into effective treatments for diseases such as fibrosis, psoriasis, cancer, contact allergy, and autoimmune diseases, due, in part, to the immunological nature of these conditions. The authors go on to describe how co-culture, three-dimensional organotype systems, and organ-on-a-chip technology will



“enable human models of well-controlled complexity, yielding detailed, reliable data; thus providing a fitting solution for the drug development process”.⁹³

Sepsis

Sepsis is a life-threatening condition caused by the body’s response to infection. The most recent global incidence data show that sepsis affected an estimated 48.9 million people worldwide and resulted in 11 million deaths in 2017.⁹⁴ It is a leading cause of death in US hospitals and is one of the most expensive conditions to treat.^{95,96}

Mice are the animals most commonly used in sepsis research – not because they make good models of human sepsis but because they’re cheap, plentiful, small, and docile.⁹⁷ The difficulty in reliably translating results from mice to humans is believed to be a primary cause of the failure of practically all human trials of sepsis therapies.

In 2013, *Proceedings of the National Academy of Sciences of the United States of America (PNAS)* published a landmark study that had been 10 years in the making and involved the collaboration of 39 researchers from institutions across North America, including Stanford University and Harvard Medical School. Dr Junhee Seok and his colleagues compared data obtained from hundreds of human clinical patients with results from experiments on animals to demonstrate that when it comes to serious inflammatory conditions such as sepsis, burns, and trauma, humans and mice are not similar in their genetic responses.⁹⁸

Former NIH Director Dr Francis Collins authored an article about these results, lamenting the time and resources spent developing 150 drugs that had successfully treated sepsis in mice but failed in human clinical trials. He called this disaster “a heartbreaking loss of decades of research and billions of dollars”.⁹⁹ The *PNAS* paper reveals that in humans, many of the same genes are involved in recovery from sepsis, burns, and trauma but that it was “close to random” which mouse genes might match these profiles. Collins explains it as follows:

Mice, however, apparently use distinct sets of genes to tackle trauma, burns, and bacterial toxins – when the authors compared the activity of the human sepsis-trauma-burn genes with that of the equivalent mouse genes, there was very little overlap. No wonder drugs designed for the mice failed in humans: they were, in fact, treating different conditions!⁹⁹

Even before this landmark study, the criticism of mouse models had been documented in more than 20 peer-reviewed scientific papers. The mice used in sepsis experiments are young, inbred, and of the same age and weight, and they live in mostly germ-free settings. In contrast, it is mostly infant and elderly humans, who live in a variety of unsterilised, unpredictable environments, who develop sepsis.^{100,101} When experimenters induce the condition in mice, the onset of symptoms occurs within hours to days, whereas it takes place within days to weeks in humans. Mice are not typically provided with the supportive therapy that human patients receive, such as fluids, vasopressors, and ventilators.¹⁰² Unlike humans, mice are rarely given pain relief,¹⁰³ another difference that undermines data of already questionable value, as pain affects other physiological processes.

The “gold standard” method of inducing sepsis in mice is through cecal ligation and puncture, a procedure in which experimenters cut open a mouse’s abdomen and puncture their intestines with a needle before sewing the animal back up. However, mice’s responses to this procedure vary depending on age, sex, strain, laboratory, the size of needle used, and the size of the incision, which makes results incomparable between laboratories.¹⁰⁴ In addition, the procedure causes the formation of an abscess, whose effects may disguise or be disguised by the effects of the sepsis itself.¹⁰² This means that an intervention that appears to be beneficial for sepsis may actually be beneficial only because of its effects on the abscess.

Rats, dogs, cats, pigs, sheep, rabbits, horses, and non-human primates, including baboons and macaques, have also been used in sepsis experimentation. None of these species reproduce all the physiologic features of



human sepsis. The pulmonary artery pressure responses of pigs and sheep differ from those of humans, so this aspect of sepsis cannot be compared between these species.¹⁰⁵ Furthermore, baboons and mice are less sensitive to a species of bacteria commonly used to induce sepsis in experimental settings.¹⁰⁶ A recent study found that rhesus macaques and baboons differ markedly in their innate immune response to pathogens compared to humans.¹⁰⁷

A 2019 report from the US National Advisory General Medical Sciences Council (NAGMSC) Working Group on Sepsis states, “Despite decades of intensive study of the underlying mechanisms of this condition, no new drug or significantly new diagnostic technology has emerged. Dozens of prospective trials of agents or strategies targeting the inflammatory basis of sepsis have failed.”¹⁰⁸ In its report, the NAGMSC Working Group on Sepsis recommended that the US National Institute of General Medical Sciences (NIGMS), under NIH, “rebalance” its sepsis research–funding portfolio to “include a more clinical focus”.¹⁰⁸ In a “Notice of Information” issued by NIGMS following the NAGMSC report, the institute indicated its intention to support more sepsis research that “uses new and emerging approaches, such as clinical informatics, computational analyses, and predictive modeling in patients, and new applications of high-resolution and high-throughput bioanalytical techniques to materials obtained from septic patients” and called the support of “[s]tudies using rodent models of sepsis” a “low priority”.¹⁰⁹ In other words, NIGMS intends to prioritise funding human-relevant sepsis research over sepsis experiments on animals.

In 2015, an expert working group consisting of veterinarians, animal technologists, and scientists issued a report on the implementation of the 3Rs (the replacement, reduction, and refinement of animal use) in sepsis research.¹¹⁰ The group noted several methods that could be used instead of animal models, such as *in vitro* cell culture models for studying sepsis mechanisms, systems and computation biology for laying out the inflammatory processes occurring during sepsis, three-dimensional cell culture models for exploring human disease progression and infectious disease mechanisms, synthetic human models to recreate human disease–related cell types and tissues, and human genomic information to discover how sepsis affects individuals differently and which groups may be more at risk. The authors stated that genomic information “will complement or even replace the need for mouse models in disease discovery and drug development”.¹¹⁰

The following are examples of recent developments in human-relevant sepsis research:

- Critical care physicians at Brigham and Women’s Hospital and Harvard Medical School teamed up with mechanical engineers in the Republic of Korea to create a sophisticated analysis platform that can be used to monitor a sepsis patient’s white blood cell function hourly at their bedside, a “critical yet unmet need for managing many critical care patients”.¹¹¹
- Researchers in Jena, Germany, used a human liver–on-a-chip model to discover a new biomarker that plays a role in sepsis pathophysiology and, potentially, subsequent liver dysfunction.¹¹²
- Physicians from Cincinnati Children’s Hospital support using microfluidic devices to study sepsis in infants, whose cells could be captured from a very small amount of blood.¹¹³
- Because early detection of sepsis is likely the most important factor in reducing mortality from this condition,¹¹⁴ researchers around the globe are exploring different artificial intelligence and machine learning tools to aid in sepsis early prediction and diagnosis.^{115–117}

Nerve Regeneration

Recommendation: End the use of animals

Many neuroprotective agents have been developed that are successful in treating spinal cord injury (SCI) in animal models, but clinical trials have been disappointing. Neurologist Aysha Akhtar has described three major reasons for this failure: “[D]ifferences in injury type between laboratory-induced SCI and clinical SCI, difficulties in interpreting functional outcome in animals, and inter-species and interstrain differences in pathophysiology



of SCI.”¹¹⁸ In their systematic review of the use of animal models to study nerve regeneration in tissue-engineered scaffolds, Angius and colleagues noted, “The large majority of biomaterials used in animal models have not progressed for approval to be tested in clinical trials in spite of the almost uniform benefit described in the experimental papers.”¹¹⁹ The authors lamented the low quality of described experiments on animals, as necessary detail and rationale had been omitted, making it difficult to compare data.

For example, methylprednisolone, a routinely used treatment for acute SCI, has generated inconsistent results in animal models. A systematic review examining 62 studies of the drug on a wide variety of species, from rodents to monkeys, found that 34% of the studies reported beneficial results, 58% no effect, and 8% mixed findings.¹²⁰ The results were inconsistent both among and within species, even within strains. Furthermore, the variability in results remained even when many of the study design and procedure variables were controlled. The authors pointed out numerous intrinsic differences between, and limitations of, each species/model and suggested that as a result of these immutable inter- and intra-species differences, no human-relevant animal model can be developed. They concluded that the “research emphasis should be on the development and use of validated human-based methods”.¹²⁰

Among species, rats are particularly unsuitable for nerve repair or regeneration research. Experts have pointed out three major problems with rat models in this field:

1. The majority of nerve regeneration data is now being generated in the rat, which is likely to skew treatment outcomes and lead to inappropriate evaluation of risks and benefits.
2. The rat is a particularly poor model for the repair of human critical gap defects due to both its small size and its species-specific neurobiological regenerative profile.
3. Translation from rat to human has proven unreliable for nerve regeneration, as for many other applications.¹²¹

More specifically, the inconsistencies between animal models and the clinical situation include the following:

1. Healthy animals versus sick patients;
2. short versus long gap lengths (the clinical need for *large* gap repairs, while 90% of *in vivo* studies are in rats and rabbits where gap lengths are usually ≤ 3 cm);
3. animal models that almost always employ *mixed sensory-motor* autografts for repairing mixed defects, versus clinical repairs that almost always involve *sensory* autografts (usually sural nerve) for repairing mixed defects;
4. protected anatomical sites in animal models, versus repairs that must often cross articulating joints in humans; and
5. inbred, highly homogeneous animal strains and ages, versus diverse patient populations and ages: It is well recognized that animal models fail to mimic the human condition in terms of the *uniformity* of animal subjects used.¹²¹

University of Florida biomedical engineers Mobini and colleagues add, “We are incapable of truly mimicking human neural injuries in animal models because of the extensive anatomical, functional, molecular, immunological, and pathological differences between humans and frequently studied animals.”¹²² Human-relevant methods such as human stem cells and clinical research can bypass these limitations and should be the focus.

Human-relevant methods for studying nerve injury and regeneration have been reviewed by a number of research groups and include human organoids, microfluidics, engineered human tissue scaffold moulds, bioprinting, and other *in vitro* uses of human cells. *Ex vivo* models, such as those that use three-dimensional engineered scaffolds, bioreactors, neurospheres, and organoids, allow for more controlled studies on specific parameters than do animal experiments.¹²² Bioprinting can use bioinks containing human cells and materials to



construct heterogeneous tissue models in a single step and with great consistency,¹²³ an aspect of nerve regeneration research that has been particularly lacking in animal models.¹¹⁹

Shrirao and colleagues at Rutgers University recommend microfluidic devices, which are “adaptable for modeling a wide range of injuries” and provide advantages over traditional *in vivo* and *in vitro* experiments by “allowing researchers to (1) examine the effect of injury on specific neural components, (2) fluidically isolate neuronal regions to examine specific effects on subcellular components, and (3) reproducibly create a variety of injuries to model TBI and SCI”.¹²⁴ For example, scientists from the biotechnology company MIMETAS collaborating with scientists from Leiden University and Utrecht University developed a three-dimensional motor neuron model using iPSC-derived motor neurons that allows for directed neurite growth and separation of axons from soma and dendrites to advance the study of motor neuron disease and nerve regeneration mechanisms.¹²⁵ Researchers at the University of Texas Health Science Center have developed cerebral organoids that can be used to study human-specific pathological changes induced by traumatic brain injury (TBI). Their model is being used to simulate the controlled cortical impact procedures commonly used to create TBIs in rodents and other animals.^{126,127} Mobini and colleagues note that microfluidics offer advantages in precision, scalability, and cost-effectiveness when compared to traditional cell culture or experiments on animals and that these are currently on the market and available for neural regenerative medicine research.¹²²

Neurodegenerative Diseases

Recommendation: End the use of animals

There is sufficient literature documenting the failings of various animal models of neurodegenerative diseases, including Alzheimer’s (AD), Parkinson’s (PD), Huntington’s (HD), and amyotrophic lateral sclerosis (ALS), to write a lengthy appendix for each disease. However, since many of the same limitations of animal models prohibit translation across these conditions, they will be discussed briefly as a whole. For one, all these diseases are human-specific, meaning that none of them occurs naturally in other animals. No animal model has been developed that recapitulates all aspects of a particular neurodegenerative disease.¹²⁸ For AD research, the clinical failure rate for new drugs is 99.6%.¹²⁹ This includes the 2018 failure of AstraZeneca and Eli Lilly’s lanabecestat, which was hailed as extremely promising, due to futility.¹³⁰

In a bioinformatic analysis comparing transcriptional signatures of human AD, PD, HD, and ALS with mouse models of these diseases, Stanford scientists made the following findings:

[M]ost available mouse models of neurodegenerative disease fail to recapitulate the salient transcriptional alterations of human neurodegeneration and ... even the best available models show significant and reproducible differences compared to human neurodegeneration. Although the reasons for the poor transcriptional performance of mouse models varied, the unifying theme was the failure of mouse models to exhibit the variety and severity of diverse defects observed in human neurodegeneration.¹³¹

These molecular discrepancies underscore the artificial ways in which such models are created. Physical and chemical lesioning and systemic administration of toxins are often used. These are acute stressors, not long-term degenerative processes, and as such, they initiate events in animal models that are not present in human patients. The acute and immediate nature of particular disease models, such as the 6-OHDA and MPTP models of PD and the 3-NP model of HD, fail to capture the progressive nature of the disorders that they aim to mimic. In addition, it is commonplace for scientists to use young animals, both rodents and primates, to “model” diseases associated with aging,¹³² further reducing the likelihood that their observations will be of use to humans.



Genetically modified mouse models of neurodegenerative disease exhibit an inconsistent range of pathological and behavioural phenotypes, in part because of the transgenes used, inconsistencies in transgene insertion and expression, and mouse background strains.¹³³ The most commonly used genetic mouse model of ALS, the SOD1 model, is based on a gene that accounts for only 3% of ALS cases in the human population.¹³³ Literature reviews have concluded that findings from this model have not translated into any effective human therapy for ALS, that “a biased estimation of treatment efficacy in animals may lead to unnecessary (and possibly harmful) clinical trials in humans”,¹³⁴ and that “animal models are not an ideal system for studying ALS or for developing drug therapies”.¹³⁵ In PD, even non-human primate studies do not “constitute a valid scientific modality for the complete understanding of PD and for the development of future neuromodulation therapeutic strategies”.¹³⁶

As in much of biomedical research, animal subjects suffer greatly when they are used to mimic neurodegenerative disease. In an analysis of published research on animal models of HD, 51 studies referenced experiments “in which animals were expected to develop motor deficits so severe that they would have difficulty eating and drinking normally”.¹³⁷ However, only three out of 51 reported making adaptations to the animals’ housing to facilitate food and water intake. The authors of this analysis concluded that experimenters are not following the 3Rs principle and, in their failure to do so, are compromising not only animal welfare but also the relevance of their studies to HD.¹³⁷

As animal studies fall short, scientists and policymakers are realising that research strategies should be more human-relevant. Following a review of AD research, an interdisciplinary panel recommended that funding be allocated away from animal studies and towards more promising techniques involving patient-derived induced pluripotent stem cell models, “omic” technology (genomics, proteomics, etc.), *in silico* models, neuroimaging, and epidemiological studies.¹³⁸ For advancements in human blood-brain barrier research, which will greatly benefit scientific progress in developing treatments for human neurodegenerative disease, please see the section on [Stroke](#).

The following are highlights in cutting-edge, human-relevant AD research:

- Scientists at the University of Texas Southwestern Medical Center have discovered a “Big Bang” of AD, identifying the genesis of tau pathology in the disease, not by experimenting on animals but by extracting proteins from human brains and isolating single molecules.¹³⁹
- Collaborators from numerous medical schools in China, using resources from the Chinese National Human Brain Bank for Development and Function, recently analysed the protein profiles of hippocampal subfields in post-mortem brain tissues from individuals at varying stages of cognitive and neuropathological decline and determined that myelin- and oligodendrocyte-related protein expression changes in some of these subfields may contribute to myelin loss and subsequent cognitive decline in AD.¹⁴⁰
- Thanks to developments in human brain imaging, scientists at the University of Cambridge were able to trace tau protein in human brains.¹⁴¹
- Patient-derived stem cells were used by Hungarian and Danish scientists to compare neurons from the brains of patients with sporadic AD to those with the familial form of the disease, discovering key similarities and differences between the two pathologies and concluding that stem cell technology is suitable for modelling both forms of the disease.¹⁴²
- At the Karolinska Institute in Sweden, researchers identified a molecular fingerprint for dementia present in the synapses of brains collected post-mortem from patients and subjected to proteomic analyses.¹⁴³
- Researchers at the University of Southern California, the University of California—Los Angeles, and the University of California—Irvine recently used 2-[¹⁸F]fluoro-3(2(S) azetidylmethoxy) pyridine (2FA) PET imaging to compare nicotinic cholinergic receptor binding in brain regions of patients with AD, individuals with mild cognitive impairment, and healthy age-matched controls and investigate how binding differences related to cognitive abilities in these groups.¹⁴⁴

Biological engineering is also transforming ALS research. A team of researchers in the Hickman Hybrid Systems Lab at the University of Central Florida have developed a human neuromuscular junction—on-a-chip, the first of



its kind, which can be used for toxicity testing of drugs designed to treat neuromuscular diseases, such as ALS and spinal muscular atrophy.¹⁴⁵ When the researchers tested three known drugs on this model, the results matched live human data. Scientists at Harvard University and Lawrence Livermore National Laboratory are also using brain-on-a-chip technology to study how neurons communicate and how exposure to certain chemicals may affect the human brain over time.^{146,147}

Human-based *in vitro* tools are also significantly advancing understanding of PD. For example, researchers at Dongguk University in Seoul and the University of Pennsylvania have created three-dimensional midbrain organoids of LRRK2-associated PD that exhibit increased α -synuclein, a pathological signature of LRRK2 patients absent in animal models.¹⁴⁸

For many years, experimenters have tormented monkeys, mice, dogs, and other animals in an effort to create drugs to treat these devastating diseases. However, since other animals don't contract these human diseases naturally, experimenters have manipulated their genomes in order to force certain symptoms. The results, after decades of tests, include more than 100 failed drugs, an untold number of animal deaths, and the continued suffering of human victims of the disease. For these patients, a switch to human-relevant methods is long overdue.

Neuropsychiatric Disorders and Neurodivergence

Recommendation: End the use of animals

Animal models of neuropsychiatric disorders and neurodivergence lack the following critical aspects of model validity: (1) construct validity, meaning that the mechanistic underpinnings creating the observed symptoms in animals are different from those that lead to the disorder in humans; (2) face validity, meaning that animals lack the ability to “recapitulate important anatomical, biochemical, neuropathological, or behavioural features of a human disease”¹⁴⁹; and (3) predictive validity, meaning that results from experiments on animals don't reliably translate into similar results in humans. No single animal model is able to replicate all aspects of a particular condition, and features of human behaviour representing hallmarks of these disorders cannot be produced or properly assessed in animals.

Human depressive disorders, for example, are characterised, in part, by a generalised feeling of sadness, hopelessness, and despair. In an effort to measure “despair” in rodents, the most commonly used behavioural test is the forced swim test, in which a rat or mouse is placed in a container of water with no way to escape and no place to rest out of the water. Naturally, the animal will spend some time swimming and trying to find a way out of the stressful situation but will eventually become immobile and float. The time spent swimming may be extended by giving the animal some forms of human antidepressant drugs, a finding that led some scientists to assert that less time spent immobile was a sign that animals were less “depressed” and that more time spent immobile meant they were more “depressed”, as if they had “given up” and were in despair.

However, as has now been widely discussed in the scientific literature, immobility in the forced swim test may simply be an animal's adaptation to their situation and should not be used to determine their mood.¹⁵⁰

Individual animals who are quicker to float save their energy and are less likely to sink, meaning that those who pick up on this sooner and spend less time struggling may simply be learning this adaptive behaviour more readily. Time spent swimming versus floating is also influenced by an animal's strain as well as experimental variances, such as water depth and temperature.^{151–153}

In August 2021, a PETA neuroscientist and her psychologist collaborator published a paper that discredited the use of the forced swim test as a screen for antidepressant drugs. In the study, they examined the use of this test by the world's top 15 pharmaceutical companies and found that for 109 compounds used in forced swim



test experiments, most of which purportedly showed “antidepressant-like effects” in the test, none are currently approved for market.¹⁵⁴

In a series of citation analyses, researchers have demonstrated that human medical papers in the field of major depressive disorder rarely cite results from experiments on rats or monkeys, two of the most common species used in this field, and more frequently relied on the results of research using human cells and human biological data.^{155–157} A similar failure of animal studies to contribute to clinical knowledge has been noted with bipolar depression research,¹⁵⁸ and animal studies have been cited as the primary source of attrition (failure of drugs) in neurobehavioural clinical trials.¹⁵⁹ Nevertheless, thousands of published papers ignore these warnings and use the forced swim test to draw erroneous conclusions about an animal’s mood¹⁵⁰ or the potential effects of compounds on human depressive disorders.

Significant differences in physiology between humans and other animals likely account for a large percentage of failed translation. For example, the gene encoding tyrosine hydroxylase, the enzyme involved in the formation of dopamine, was found to be regulated in an entirely different manner in humans than it is in mice.¹⁶⁰ Misregulation of tyrosine hydroxylase has been implicated in several psychiatric illnesses, such as bipolar disorder and schizophrenia. In a 2019 study published in *Nature*, 64 researchers analysed the brains of mice and humans and found substantial species differences in types of brain cells and the ways they produce proteins critical to neuropsychiatric function. The authors noted numerous “failures in the use of [the] mouse for preclinical studies” because of “so many [species] differences in the cellular patterning of genes”.¹⁶¹

In addition to the lack of applicability of animal neuropsychiatric models to the human condition, animals used in these experiments suffer immensely. To induce “depression”, experimenters subject them to uncontrollable pain through electric shocks or chronic stressors such as restraining them for extended periods of time, starving them or denying them water, tilting their cages, forcing them to live in wet bedding, shaking them, or disrupting their circadian rhythms. Animals are often made to live in complete isolation from other members of their species, bullied and physically assaulted by other animals, deprived of parental care, and subjected to genetic or surgical manipulations in an effort to induce a depressed or altered mental state. To quote Dutch animal behaviourists van der Staay, Arndt, and Nordquist,

“If evidence accumulates that the intended goal/purpose cannot be reached, then one should consider abandoning further development of the model.”¹⁶²

This group also points out that in all cases, “benefits must outweigh the ethical costs of the animals. These costs include pain and suffering, distress and death”.¹⁶²

Funds should be allocated to more relevant, human-based experimental models, such as computational modelling using already well-defined biomarkers¹⁶³ and the use of patient-specific stem cells for personalised medicine, which “affords the ability to generate neuronal cell-based models that recapitulate key aspects of human disease”¹⁶⁴ and can be used in drug discovery. Complex diseases like schizophrenia are ideal disorders “to model through stem cell approaches due to ... heterogeneous, complex genetics that are hard to recapitulate in animal models”.¹⁶⁵

Recent developments in the field of human neuropsychiatric research include the following:

- A research group at Johns Hopkins Bloomberg School of Medicine used stem cell–derived “mini-brains” to study the effects of an antidepressant drug on neurons in the developing human brain.¹⁶⁶
- University of California–San Diego scientists created organoids using reprogrammed cells from patients with a specific genetic mutation strongly linked to autism to study early brain development.¹⁶⁷ The authors noted that mouse models of this genetic mutation have phenotypes that are the opposite of what is observed in humans¹⁶⁷ and that a “patient-derived model will be ideal and more beneficial than looking at the mouse”.¹⁶⁸
- At Brown University, neuroscientists and engineers conducted the first-ever study of electrical activity in the brains of people with obsessive-compulsive disorder over an extended period of time while the participants



were in their homes, going about daily living.¹⁶⁹ Along with behavioural biomarkers, the team used machine learning to examine correlations between real-life behavioural measures and brain signals. This research can be used to help guide adaptive deep brain stimulation treatments for this population.

- Scientists in Tokyo used a combination of brain imaging and machine learning to create a diagnostic algorithm for autism, schizophrenia, and psychosis based on brain scans.¹⁷⁰
- A team of Indian and Canadian researchers used artificial intelligence and functional magnetic resonance imaging data to develop a diagnostic tool that can predict schizotypy in first-degree relatives of patients with schizophrenia with 87% accuracy.¹⁷¹

Owing to the psychological distress inherent in animals provoked to display neuropsychiatric disease tendencies and the inapplicability of the results to humans, we recommend that the use of animals in such studies be ended.

Stroke

Recommendation: End the use of animals

According to researchers at the Institute for Stroke and Dementia Research in Munich, “More than 1000 neuroprotective compounds have been tested in rodent models with the aim to improve stroke outcome. ... Indeed, many agents reduced brain damage (in most cases measured as decreased infarct volume) in rodent models of experimental stroke. Out of these candidates approximately 50 neuroprotective agents were tested in more than 100 clinical stroke trials, but none has improved outcome in clinical stroke patients.”¹⁷²

Many factors contribute to this failure, such as flaws in experimental design, publication bias, disease-management inconsistencies between animal models and clinical populations, and physiological differences between species. Experts in the field admit that “animal models of stroke mimic at best less than 25 percent of all strokes”.¹⁷³ The Stroke Therapy Academic Industry Roundtable (STAIR) published its first recommendations in 1999, but the success rate of clinical trials has not improved. One drug, NXY-059, which fulfilled the STAIR criteria, failed in clinical trials.¹⁷³ These realities illustrate the need to shift away from animal models and focus on human-centred methods.

In a 2017 review,¹⁷⁴ Clemens Sommer, managing director of the Institute of Neuropathology at the University Medical Center of Johannes Gutenberg University Mainz, details the following aspects of animal experimentation that limit the translatability of animal-based stroke research to the clinical setting:

- Most animals studied in stroke research have lissencephalic, or smooth, brains, unlike the gyrencephalic brains of humans.
- The expression of certain signalling molecules differs between rodents and humans in three types of brain cells – neurons, astrocytes, and microglia – both at baseline and in response to oxygen deprivation.
- In humans, ischemic damage to the white matter of the brain is important in the prognosis of stroke, but white matter content in humans is much higher than in other animals. “While in humans the percentage of white matter accounts for 60%, it decreases to about 35% in dogs, 20% in rabbits, 15% in rats and is as low as 10% in mice,”¹⁷⁵ meaning that a major factor in stroke outcomes for humans cannot be accurately compared in animal models.
- Blood vessels in the brain have a different anatomy in humans compared to other animals; even strains of rodents differ in their vascular framework. These “functional differences may have deeper implications concerning the pathophysiology of the ischemic cascade”.¹⁷⁴
- In humans, the gene for the neurotransmitter nitric oxide synthase 2 (NOS2) is regulated differently than it is in mice. NOS is important, since nitric oxide may be an essential gas-signalling molecule during stroke.¹⁷⁶



- As discussed elsewhere in this report, immune system differences between humans and other species are drastic. Sommer describes this as follows:
[T]he percentage of neutrophils in mice and rats is about 10–20% compared to 50–70% in humans, while the opposite situation is seen for lymphocytes, which comprise about 50–100% in rodents compared to 20–40% in humans, respectively. Moreover, there is only a minimal intersection of whole-genome mRNA and microRNA expression in leukocytes from rodents versus humans at both baseline and after stroke, raising the question whether rodents are acceptable models at all for the human immune system after stroke.¹⁷⁴
- The RNA profile of a mouse brain is more similar to that of other tissues in a mouse's body, such as the lungs, liver, and heart, than it is to that of a human brain.¹⁷⁷
- Ischemic stroke typically occurs in heterogeneous elderly patients with comorbid conditions, whereas animal stroke experiments are predominantly carried out in young, healthy, male, inbred animals.

On the other hand, human-based models of stroke do not suffer from these species-inherent deficiencies. Scientists from the Department of Molecular and Cellular Physiology at Louisiana State University have written that a “key benefit of *in vitro* systems is the opportunity to work with human cells, as such Werth *et al.*, utilized the brain slice method in human cortical slices to provide the first direct evidence of glutamate receptor involvement in ischemic injury in the human brain”.^{178,179}

Thanks to technological advances, including accurate three-dimensional representations of multiple neuronal cell types and structures of the human brain, researchers are able to overcome some of the previously limiting factors of human *in vitro* brain research. For example, physicians and chemists at the University of Duisburg–Essen, in Germany, are cultivating six different human cell types to create mini-brains for use in stroke research and drug discovery.¹⁸⁰ At the Wake Forest Institute for Regenerative Medicine, a brain organoid of this type has already been created and was validated in stroke experiments after the model showed clinically accurate responses to known drugs.¹⁸¹ Neurosurgeons and biomedical engineers at Stanford University and Johns Hopkins University teamed up to create a neurovascular unit on a microfluidic chip that they are using to assess the restorative potential of stem cell therapies for use in ischemic stroke recovery.¹⁸² In the Netherlands, the company MIMETAS has also created a neurovascular unit–on-a-chip that can be used for basic stroke research and drug discovery¹⁸³ and computational scientists at the University of Amsterdam have developed an *in silico* trial platform that can be used to assess treatment of acute ischemic stroke using clinical parameters of virtual patients.¹⁸⁴ Clinical researchers are now utilising artificial intelligence to improve stroke prevention, detection, and care.^{185–187}

A report authored by 42 scientists following a workshop by the US National Institute of Neurological Disorders and Stroke on translational stroke research concluded, “With increased availability of human cell lines/tissues, organoids, and inducible pluripotent stem cell technologies and high-throughput assays, *in vitro* strategies, in combination with data from animal models, may hold increasing prominence in future drug development strategies.”¹⁸⁸ Animal models will never be able to recapitulate the nature of human stroke nor the human-specific inflammatory response that follows. Considering that every 40 seconds, someone in the US suffers from a stroke and that every four minutes, someone dies of one,¹⁸⁹ we cannot afford to spend our limited resources on substandard, animal-based research.

Substance Abuse

Recommendation: End the use of animals

Fundamental aspects of non-human animals make them inappropriate for the study of human addiction. First, the use of and addiction to drugs of abuse in humans is a vastly complex experience, one that has been impossible to mimic using animals in a laboratory setting.¹⁹⁰ It has been argued that attempts to model human disorders such as addiction in non-human animals, especially rodents, are “overambitious” and that the



“‘validity’ of such models is often limited to superficial similarities, referred to as ‘face validity’ that reflect quite different underlying phenomena and biological processes from the clinical situation”.¹⁹¹

Second, the pharmacokinetic actions of drugs are different among species. For example, “the rate of metabolism of MDMA [street name: Ecstasy, E, or Molly] and its major metabolites is slower in humans than rats or monkeys, potentially allowing endogenous neuroprotective mechanisms to function in a species specific manner”.¹⁹² Pharmacokinetic differences between humans and “model” animals likely explain why the neurotoxicity seen in rodents after MDMA administration has not been observed in the clinical setting.¹⁹² Since MDMA is being explored not only because of its illegal use as a recreational drug but also for its potential use as a therapeutic, accurate knowledge regarding its safety in humans is paramount.

Third, serious flaws in experimental design of addiction experiments greatly skew interpretation of their results. In the human experience with drugs, the user chooses to consume the addictive substance. They choose it over other substances or activities that they may find rewarding. Animals in laboratories are typically not given this option. When they are, the vast majority of them will choose an alternative reward, such as sugar, over the drug of abuse.¹⁹³ This holds true for primates as well as mice and rats. Even in animals with very heavy previous drug use, only about 10% would continue to give themselves a drug when they had the option to make another rewarding choice.¹⁹³ In a review on the “validation crisis” in animal models of drug addiction, French neuroscientist and addiction researcher Serge Ahmed asserts that the lack of choice offered to animals in these experiments elicits “serious doubt” about “the interpretation of drug use in experimental animals”.¹⁹³

The non-human animal has been called a “most reluctant collaborator” in studying alcohol addiction and has been noted to have a “determined sobriety” that the experimenter must fight against in order to overcome “their consistent failure to replicate the volitional consumption of ethanol to the point of physical dependency”.¹⁹⁴ Researchers from the US National Institute of Mental Health reason that “it is difficult to argue that [drug self-administration by rodents] truly models compulsion, when the alternative to self-administration is solitude in a shoebox cage”.¹⁹⁵

Despite the prevalence of addiction research conducted on animals, “drugs that effectively curb opioid or psychostimulant addiction by promoting abstinence and preventing relapse have yet to be developed” and “very little clinical development is currently ongoing”.¹⁹⁰ The data from animal studies were promising in certain drug classes, but these have either failed to be effective in human trials or not been tolerated well by humans, a negative outcome that was not predicted by animal trials.¹⁹⁰

Non-invasive human research methods can provide us with answers to the questions that non-human animals, in their distaste for drugs of abuse, are fundamentally unable to answer. Rutgers University Robert Wood Johnson Medical School researchers recently authored a review article describing how the use of human induced pluripotent stem cells (iPSC) can provide a “unique opportunity to model neuropsychiatric disorders like [alcohol use disorders] in a manner that ... maintains fidelity with complex human genetic contexts. Patient-specific neuronal cells derived from [induced pluripotent stem] cells can then be used for drug discovery and precision medicine”.¹⁹⁶

Human-relevant, non-animal research on alcohol use disorder is being carried out by scientists at the University of Connecticut, who recently used stem cells donated by alcoholic and non-alcoholic subjects to study the effects of alcohol on a specific receptor in the brain that is targeted by alcohol. Their results were at odds with some of the findings from animal experiments.¹⁹⁷ At Rutgers, scientists used patient-derived cells to generate neural cell types specific to individuals in which they could study alcohol’s effects on various aspects of cell physiology. Their results demonstrated a role for neuronal inflammation in the pathophysiology of alcohol use disorder.¹⁹⁸ Researchers at the US National Institute on Drug Abuse are using three-dimensional neocortical organoids to study the effects of prenatal cocaine exposure on the developing human brain.¹⁹⁹ Scientists at the Medical College of Wisconsin are using human iPSC-derived organoids to study the mechanisms of ethanol-



induced gene dysregulation on the development of foetal alcohol spectrum disorders.²⁰⁰ Other investigators are using human iPSCs to study the effects of alcohol on the human liver.²⁰¹

In addition, the funds used to support ineffective and wasteful substance abuse studies in animals could instead be used to aid effective and directly human-relevant drug prevention, rehabilitation, and mental health programmes.

Trauma

Recommendation: End the use of animals

After rodents, pigs are the species most commonly used in trauma experiments. However, notable species-specific differences between pigs and humans render results from this research unintelligible. For example, pigs' coagulation activity differs from that of humans, making it difficult to achieve a state of coagulopathy, or the inability to clot, in pigs. In instances of human trauma, coagulopathy represents part of the "lethal triad" for patients and is a great concern for researchers and physicians.²⁰² In addition, there are differences in the administration of mechanical ventilation and drugs such as vasopressin and heparin in research.^{202,203} Importantly, as with mice and humans, immune responses are different between pigs and humans.

Trauma is extremely heterogeneous: patients differ in age, gender, ethnicity, medical history, alcohol and drug use, and the presence of other injuries, making the production of an appropriate animal model difficult,²⁰⁴ if not impossible. In studies of traumatic brain injury, all promising therapeutics identified in animals have failed in human clinical trials.²⁰⁵ There is a significant amount of discussion regarding the limitations of animal models of trauma and haemorrhagic shock, which is summarised in this excerpt from a review by Combes:

Scientific problems with the animal models include the use of crude, uncontrolled and non-standardised methods for traumatising, an inability to model all key trauma mechanisms, and complex modulating effects of general anaesthesia on target organ physiology. Such effects depend on the anaesthetic and influence the cardiovascular system, respiration, breathing, cerebral haemodynamics, neuroprotection, and the integrity of the blood-brain barrier. Some anaesthetics also bind to the NMDA brain receptor with possible differential consequences in control and anaesthetised animals. There is also some evidence for gender-specific effects. Despite the fact that these issues are widely known, there is little published information on their potential, at best, to complicate data interpretation and, at worst, to invalidate animal models. There is also a paucity of detail on the anaesthesiology used in studies, and this can hinder correct data evaluation.²⁰⁶

Fortunately, it has been shown that computer simulation can accurately replicate real-life trauma and predict patient outcomes.²⁰⁷ For example, scientists at the University of Pittsburgh used a computer model to examine the relationship between spinal cord injury and pressure ulcers in human patients and found that a certain treatment was effective at reducing inflammation and tissue damage.²⁰⁸ This Pittsburgh group also used data-driven and mechanistic modelling to discover that patients who survive traumatic brain injury have a different inflammatory response than individuals who do not survive, information that "may point to both novel mechanistic insights and clinically translational applications".²⁰⁹

In addition, clinical research remains invaluable in this field and both informs and benefits from mathematical and computer modelling. A study conducted at the US Army Institute of Surgical Research used data from more than 250 human experiments to model mechanistically the physiology that underlies blood loss and shock in humans suffering from haemorrhage. The authors describe the study as follows:



Unlike an animal model, we introduce the utilization of lower body negative pressure as a noninvasive model that allows for the study of progressive reductions in central blood volume similar to those reported during actual hemorrhage in conscious humans to the onset of hemodynamic decompensation (i.e. early phase of decompensatory shock), and is repeatable in the same subject. Understanding the fundamental underlying physiology of human hemorrhage helps to test paradigms of critical care medicine, and identify and develop novel clinical practices and technologies for advanced diagnostics and therapeutics in patients with life-threatening blood loss.²¹⁰

Artificial intelligence is being used to improve care over the course of a traumatic event, from field triage to treatment in the emergency room and beyond, to improve outcomes for patients after they are discharged.^{211–213} In molecular studies at Wayne State University, critical care surgeon Dr Lawrence Diebel and his team are using *in vitro* microfluidic models to study human endothelial function during trauma and shock.^{214,215} As a result of the heterogeneity of the causes and outcomes of trauma and because of physiological and immunological differences among species, only human-relevant research methods are suitable for informing human trauma research.



Training and Forensic Enquiries

Detailed below are opportunities to end the use of animals immediately in forensic research and biomedical education.

Forensic Sciences

Recommendation: End the use of animals

Forensic science is a unique research area and deserves serious ethical scrutiny, as its goal is to understand crime-related issues, rather than improving human health or life conditions, and the experimental methods are often horrific and conducted without anaesthesia. Italian scientists Cattaneo and colleagues explain that there is a “moral obligation to pursue and respect this [responsibility to take care of other animal species], especially where mankind’s actual survival is not at risk”.²¹⁶

The use of animals in forensic research was heavily criticised as early as 1992, when Bernard Knight asserted that “painful, sometimes mutilating experiments on conscious animals” in order to obtain “tenuous potential benefit to some medico-legal problem” cannot be condoned, particularly when one considers that such works “are not regularly used in routine forensic practice” and just “gather dust in university libraries”.²¹⁷ He also observed that “a vast amount of published material using animal experimentation seems to have little practical relevance, other than to expand the curriculum vitae and the career prospects of the researcher”.²¹⁷

In 2015, Cattaneo and colleagues published a meta-analysis and review examining 404 forensic science articles and found that 69.1% “concerned studies involving animals sacrificed exclusively for the sake of the experiment” and that “killing still frequently includes painful methods such as blunt trauma, electrocution, mechanical asphyxia, hypothermia, and even exsanguination; of all these animals, apparently only 60.8% were anaesthetised”.²¹⁶ In 2018, another meta-analysis was conducted by South African researchers Calvin Gerald Mole and Marise Heyns, who examined 204 original forensic science studies, using 5,050 animals, which were conducted between 2012 and 2018.²¹⁸ In these, animals – including rats, pigs, mice, rabbits, sheep, and cows – were drowned, electrocuted, cut, beaten, and made to ingest acid, among other cruel procedures. Mole and Heyns conclude that not enough is being done in forensic science research to uphold basic ethical principles of research and to adhere to the 3Rs. They suggest that “much of the reported animal tissue use in the traumatic research articles in the current study could be minimized using human tissue obtained at medico-legal autopsy” and that “[m]edico-legal autopsies may be an underutilized resource for scientific research specimens”.²¹⁸

Cruelty aside, Cattaneo and colleagues stress, “[T]he history of forensic sciences has provided us with much evidence of the inapplicability of data obtained from studies performed on animal models”,²¹⁶ given the anatomical, physiological, and genetic differences between species. For example, recent research funded by the US National Institute of Justice and conducted at the Forensic Anthropology Center at the University of Tennessee indicates that decomposition data from non-human animals varies considerably from humans and is not recommended for use in forensic casework.²¹⁹

In addition, there is a plethora of alternative methods, such as manikins, simulators, artificial materials, and *in vitro* technology, and it has been recognised that “applying alternative methods rather than using animals has provided, in the forensic field, important and reproducible results”.²¹⁶ Taken together, the ethical problems and scientific and practical issues associated with animal experimentation as well as the abundance of readily available alternative methods signify that forensic research is a prime area for animal use to end.



Medical Training

Recommendation: End the use of animals

Animals have traditionally been used in biomedical education to teach human physiology and pharmaceutical principles, study human anatomical form and function, and practice human surgical procedures. Yet numerous developments have contributed to a paradigm shift in this field. They include improvements in human-patient simulation and computer-assisted learning technology that teaches biomedical education as well as or better than animal dissection and experimentation,²²⁰ rising public opposition to animal use in laboratories,²²¹ increasing animal laboratory cost burdens,²²² and a renewed focus by the medical community on improving patient safety and reducing clinical errors through simulation-based training.²²³

Human simulation-based teaching has become the gold standard. Now, medical students in Canada, India, and the US learn without using animals throughout the undergraduate curricula.^{224,225} Medical experts have recommended a transition away from an animal-based pedagogy and towards “a robust curriculum composed of didactics, task trainers, virtual reality, cadavers, computer software, high-fidelity patient simulators, and supervised clinical work”.²²⁶ Unlike animal-based approaches, these non-animal training methods accurately model human anatomy and physiology, allow students to repeat medical procedures until proficiency is achieved, improve provider confidence and transference of learned skills to clinical practice, and allow educators to receive real-time objective performance feedback.²²⁷

The benefits of animal-free training methods have been demonstrated across a variety of medical disciplines and techniques. For example, a meta-analysis on the efficacy of virtual reality (VR) training in laparoscopic surgery found it to be as effective as or superior to traditional, video, or box trainers in training performance and in the operating room.²²⁸ Another meta-analysis found that time efficiencies and improvements in technical surgical performance on robot-assisted surgery VR simulators were transferable to the operating room and that performance on the simulators was predictive of performance in the operating room.²²⁹ Improvement in technical skills was found in a meta-analysis of obstetric VR simulation studies, and the authors note “that consideration ought to be given to integrate simulation training into the clinical curriculum”.²³⁰ Other evidence supports using simulations to improve skills and/or clinical performance in lumbar punctures,²³¹ suturing,²³² myringotomy,²³³ and many other procedures.

There is no scientific or ethical justification for continuing to use animals for medical training, and as such, we recommend ending the use of animals for this purpose.

Microsurgery Training

There now exists an array of low- and high-fidelity non-animal methods that researchers have developed for the effective teaching of a wide variety of basic and advanced microsurgical skills to novice and expert physicians, and these have been endorsed as replacements for live-animal use. They include task trainers and ethically sourced perfused human cadavers that can be used to teach procedures such as anastomoses, resection of artificial tumours, bypasses, and aneurysm creation, dissection, and clipping.

For example, a study from the University of Toronto comparing the microsurgical anastomosis skills of surgical residents trained on live rats to those trained on a silicone model found that, following identical initial training on inanimate models, the latter group was as proficient at performing single-layer, microsurgical anastomoses as those trained on live animals. The authors concluded, “[T]raining with low-fidelity bench models is as effective as training with high-fidelity, live animal models for the acquisition of technical skill among surgical trainees.”²³⁴

A systematic review of microsurgical training methods supported these findings:



It would appear from the best available evidence that simulated microsurgery training on low fidelity models can be as effective as on high fidelity models. ... In the UK and elsewhere, the mainstay of microsurgical simulated training has historically been exposure to an *in vivo* rat microsurgery course, but generally this [is] at a far too early stage in training where the bridge with clinical hands-on exposure to relevant cases cannot be made, and without repetition.²³⁵

A study by a team of researchers in London evaluated the validity of a three-in-one silicone model, Surgitate, to reduce reliance on the use of animals in microsurgery training and to abide by the 3Rs. The participants performed end-to-end anastomosis on arteries, veins, and nerves and rated the model favourably for acquiring basic microsurgical skills. The authors stated that the Surgitate model “could be particularly useful in enhancing suturing skills as a replacement or reduction in the use of chicken models”.²³⁶ Given that plastic surgery is a subspecialty that often uses microsurgical techniques,²³⁷ a comprehensive review concluded that “prosthetic simulators are set to play a larger role in the development of a standardized, ethical, accessible, and objectively measurable microsurgery training curriculum for the modern-day plastic and reconstructive surgery resident”.²³⁸

A three-dimensional, animal-free neurosurgical simulator developed for aneurysm microsurgery training by a team in Bern, Switzerland, was touted as “reliable and potentially useful for training neurosurgical residents and board-certified neurosurgeons”, and a majority of the study participants reported that this simulator was superior to conventional neurosurgical training using animal models.²³⁹

VR technology also presents a promising training tool that bypasses the use of animals in microsurgical training. In a study in which authors sought to evaluate the impact of VR in microsurgical clipping of the middle cerebral artery, the team reported that training with VR technology improved the participants’ surgical efficiency, speed, and safety, regardless of complexity of the procedure.²⁴⁰

Given the myriad validated, animal-free training methods already available, we recommend ending the use of animals for microsurgery training.

Trauma Training

A study published by a US Air Force team compared the self-efficacy reported by military trainees taught emergency procedures on human simulators versus those taught using live animals – otherwise known as live tissue training (LTT) – and found equivalent results in both groups, concluding that “the belief in the superiority of animal training may just be a bias” and that “if the goal for trainers is to produce individuals with high self-efficacy, artificial simulation is an adequate modality compared with the historical standard of live animal models”.²⁴¹ The lead author published a separate letter in the same medical journal stating, “We have entered into an age where artificial simulator models are at least equivalent to, if not superior to, animal models. ... [T]he military should make the move away from all animal simulation when effective equivalent artificial simulators exist for a specific task. For emergency procedures, this day has arrived.”²⁴²

Non-animal methods are used exclusively instead of animals for military medical education by more than 70% of NATO member states,²⁴³ and the US Coast Guard has become the first branch of the US Armed Forces to end the use of animals for this practice.²⁴⁴ These developments confirm that animal use for trauma training is neither necessary nor justified.

Efforts to replace the use of animals with human simulators in military trauma training have gained many prominent supporters, including *The New York Times* Editorial Board²⁴⁵ as well as numerous medical and veterans organisations representing more than 255,000 physicians and doctors-in-training, which have former US surgeons general among their leadership.²⁴⁶



A 2018 study found that “[h]igh-fidelity simulation offers many advantages, including broad exposure to procedures, their complications, and the opportunity for repetitious learning in a non-clinical setting” and that “[s]ynthetic models can produce a stress response equivalent to that of live tissue during simulation training” and “produce a sufficient immersive and realistic experience for trainees”.²⁴⁷

One study examined the training of US Navy and US Army surgical teams involving live human role players wearing a surgical simulator known as a “cut suit” and using film industry special effects. The authors found that simulation training enhances team performance and “improves surgical procedures and processes”, concluding, “High fidelity surgical simulation equipment such as the ... ‘Cut Suit’ combined with highly realistic replicated settings will allow surgical trauma teams to improve their life-saving skills and teamwork communication to maximize successful patient outcomes. High fidelity, highly realistic, immersive and stress-provoking surgical trauma training is now an option to improve the readiness and capabilities of trauma teams.”²⁴⁸

In addition, a 2019 study in the *Journal of Surgical Education* states that the purported benefits of LTT to patient outcomes are unsubstantiated: “[N]o published evidence from prospective controlled trials exists suggesting that surgical skills training courses change trauma patient outcome, or improve performance of the skills taught, when performed in the real-world operating room. ... Published evidence of course training benefit was not identified for many established courses including: Definitive Surgical Trauma Skills, Emergency Management of Battlefield Injuries, Endovascular Skills for Trauma and Resuscitative Surgery, Emergency War Surgery Course (EWSC), Military Operational Surgical Training, Specialty Skills in Emergency Surgery and Trauma, Surgical Training for Austere Environments, or Surgical Trauma Response Techniques” – all of which, according to the paper, “used live tissue (usually porcine).”²⁴⁹

Furthermore, an independent, peer-reviewed study published by German scientists has shown that the use of animals in such LTT is ethically unacceptable. The researchers conclude, “A close examination of the evidence base for the presumed advantages of LTT showed that it is not superior to simulation-based methods in terms of educational benefit. Since credible alternatives that do not cause harm to animals are available, we conclude that LTT on animal models is ethically unjustified.”²⁵⁰

In the civilian sector, the American College of Surgeons has affirmed that human simulators can replace the use of animals in Advanced Trauma Life Support (ATLS) training,²⁵¹ and national ATLS programmes in numerous countries have made the transition to ending animal use for this purpose.²⁴⁸

Based on the evidence supporting the efficacy of non-animal training methods, we recommend ending the use of animals for military and civilian trauma training.



Toxicity Assessment

Detailed below are opportunities to end or significantly reduce the use of animals for the toxicity assessment of substances in the context of regulatory toxicity requirements. Also described are areas in which greater support is required to develop innovative methods that are relevant for the assessment of human health and environmental endpoints.

Please note that where tests are required for regulatory purposes, the direct sources (such as the websites of the OECD, ICH, and EPA) should be consulted for the most recent versions of test guidelines and guidance documents.

Approaches to Toxicity Assessment

Recommendation: Immediately promote the use of integrated approaches to testing and assessment to dramatically reduce the use of animals

Regulatory decision-making is facilitated by making use of all the relevant information available on a substance. One way to evaluate all the lines of evidence is to use an integrated approach to testing and assessment (IATA)²⁵² that considers all information in a weight of evidence (WoE) approach. Information to consider includes any existing data on the substance (e.g. from *in chemico*, *in vitro*, *in vivo* human, or *in vivo* animal studies), the physiochemical properties of the substance, data from non-testing approaches (e.g. QSARs and read-across), newly generated data (preferably from reliable and relevant non-animal methods), and use patterns or exposure scenarios. Data that are considered more reliable, relevant, and/or useful for the regulatory question have a greater influence on the final conclusion of the assessment. By assessing the available data together, it may be possible to conduct a robust risk assessment of the substance without generating new data through additional *in vivo* studies (for an example, see the Carcinogenicity section). Additionally, a holistic assessment of the data will ensure that existing *in vivo* studies are not duplicated.

IATAs and WoE assessments often require expert judgement, making these approaches unavailable to applicants who don't yet have the necessary expertise. Defined approaches (DA) consist of a fixed data interpretation procedure (e.g. a mathematical model or a rule-based approach) applied to data generated with a defined set of information sources to derive a prediction without the need for expert judgement.²⁵³ For examples of DAs, see the Skin Sensitisation section.

Unlike animal tests, non-animal methods have the ability to reflect human-relevant biology and mechanisms of toxicity, for example by assessing key events in adverse outcome pathways (AOP). AOPs comprise causally linked key events that connect chemical exposure to an adverse outcome. Non-animal tests that query specific key events in an AOP allow for a mechanistic understanding of whether an adverse outcome will occur following chemical exposure in humans.

As mentioned above, consideration of exposure should be part of an integrated approach. When human and environmental exposures to a substance are low, or when the physicochemical properties of a substance dictate that specific routes of exposure are not relevant, it may not be scientifically justified (or possible) to conduct toxicity tests for certain data requirements. When exposure is considered, the focus of regulatory decision-making can shift from a hazard-based "tick box" approach to a risk-centric approach that allows for the minimisation of tests on animals.²⁵⁴



Ecotoxicity

Recommendation: In light of existing non-animal methods and WoE approaches, the use of animals in ecotoxicity testing can be dramatically reduced

Aquatic Toxicity

Aquatic toxicity tests are conducted to measure the effects of chemicals on the environment and wildlife. In 2019, nearly 100,000 fish were used for toxicological and other safety assessments in the EU.²⁵⁵ As assessment of aquatic toxicity is required in various regulatory frameworks, strategies to replace testing using aquatic animals are urgently needed.

Several non-animal methods are now available. In 2018, two assays for the assessment of *in vitro* intrinsic clearance using cryopreserved rainbow trout hepatocytes²⁵⁶ and rainbow trout liver S9 subcellular fraction²⁵⁷ and an associated guidance document²⁵⁸ were adopted by the OECD. Liver intrinsic clearance values can be used either for physiologically based toxicokinetic models for fish bioaccumulation or for extrapolation to an *in vivo* biotransformation rate. The latter can be used with *in silico* models for the prediction of bioconcentration factors. Thus, although these test guidelines require the use of fish to obtain primary cells, they can contribute to replacing the use of live fish in OECD Test No 305 on bioaccumulation in fish.²⁵⁹

To reduce the number of juvenile and adult fish used in acute aquatic toxicity testing, the European Chemicals Agency (ECHA) will accept data from the fish embryo acute toxicity test²⁶⁰ in a WoE approach²⁶¹ on a case-by-case basis.

A promising cytotoxicity assay using the RTgill-W1 cell line has been developed for the determination of acute aquatic toxicity testing,²⁶² and the respective OECD test guideline was adopted in 2021.²⁶³ This *in vitro* assay has the potential to reduce or even replace the use of fish in the acute fish toxicity test.²⁶⁴

To enhance the prediction of acute fish toxicity, a Cefic Long-Range Research Initiative—funded project entitled “Strengthening Weight of evidence for FET data to replace acute Fish Toxicity (SWIFT)” is centred around a probabilistic Bayesian network approach.²⁶⁵ The outcomes of this project will be taken into account in project 2.54 in the OECD Test Guidelines Programme work plan to develop a guidance document on IATAs for acute fish toxicity testing. This project is co-led by Austria and the International Council on Animal Protection in OECD Programmes (ICAPO), represented by PETA Science Consortium International.

Furthermore, when testing on animals is still required, the number of animals used and the need to repeat studies can be reduced by careful application of OECD guidance document 23 on Aquatic Toxicity Testing of Difficult Substances and Mixtures.²⁶⁶ This guidance document was updated in 2019 to provide information on approaches to aquatic toxicity testing of difficult-to-test chemicals. Particular attention was paid to updating the methods available for testing poorly water soluble test chemicals while avoiding the use of solvents. Thus, the need for a solvent control group is eliminated, reducing the number of animals used for testing. In addition, the US and ICAPO (represented by PETA Science Consortium International) are co-leading Project 2.55 in the OECD Test Guidelines Programme work plan on the use and analysis of control fish in toxicity studies. In this project, statistical analyses of existing data and statistical simulations are being used to investigate whether it is possible to conduct aquatic toxicity studies using only one control when a solvent is used, further reducing the number of animals used.

Avian Toxicity

Avian toxicity tests are currently required by most regulatory authorities to assess the potential ecological effects of chemicals on terrestrial birds. Three avian toxicity tests, including acute oral, dietary, and reproduction tests, are commonly required to fulfil regulatory requirements. In the acute oral and dietary tests, up to 120 birds are used. In the oral test, they are dosed with a chemical through gavage for one day, followed



by a 14-day observation period, and in the dietary test, they are fed the chemical for five days, followed by a three-day observation period. For reproduction tests, more than 120 adult birds are fed the chemical for eight to 10 weeks, and several hundreds to thousands of offspring are killed in order to examine potential adverse reproductive outcomes.

Scientists have raised concerns about the utility of the avian tests to protect terrestrial species. The results of these tests, often conducted on two species, are used to extrapolate the potential effects on thousands of species of regional birds. Additionally, food avoidance, regurgitation, and other issues caused by the methods used for dosing the birds have led to inaccurate toxicity estimates.

To address these concerns, PETA Science Consortium International collaborated with the US EPA to retrospectively assess the use of avian oral and dietary tests in risk management decision-making.²⁶⁷ The retrospective review examined 20 years' worth of risk assessment data and found that the dietary test is generally not used for risk management. This study was used to support the EPA's 2020 policy entitled "Final Guidance for Waiving Sub-Acute Avian Dietary Tests for Pesticide Registration and Supporting Retrospective Analysis", which has the ability to prevent more than 700 birds from being subjected to toxicity tests each year and save resources that can be better spent developing fit-for-purpose non-animal methods for terrestrial toxicity testing.²⁶⁸

PETA Science Consortium International is undertaking a similar initiative to examine the use of two species in the avian reproduction tests. This retrospective review will examine hundreds of pesticide active ingredients to analyse trends in species differences used to support decision-making. The aim of the initiative is to identify any potential information that is not being used in regulatory decision-making. In addition to these projects, initiatives such as Sequence alignment to predict across-species susceptibility (SeqAPASS) aim to modernise ecological testing using predictive computational methods that have the potential to reduce testing on terrestrial animals while improving ecological protection.²⁶⁹

Global harmonisation is needed to end testing requirements that do not provide information used to maintain ecological protections. For example, the European Commission and the Central Insecticides Board and Registration Committee (CIB&RC) in India require the use of a single test species for the avian reproduction test, while the US EPA and Canada's Pest Management Regulatory Agency require two test species. Furthermore, the EPA allows waivers for the avian dietary test, and the dietary test is not required by the European Commission or in Japan, but it is still required by the CIB&RC and in China. Thus, alignment is necessary to end globally the requirement for tests that have been shown not to provide useful information or that are affecting the quality of regulatory decision-making.

Endocrine Disruption

Recommendation: In light of existing non-animal methods and WoE approaches, the use of animals in endocrine testing can be dramatically reduced

Endocrine disruptors are natural or synthetic chemicals that interfere with the body's endocrine system,²⁷⁰ triggering a wide array of responses in biological pathways responsible for regulating fundamental biological functions, such as growth, development, reproduction, energy balance, metabolism, or body weight regulation. The most investigated endocrine pathways from a regulatory chemical safety perspective are the oestrogen, androgen, thyroid, and steroidogenesis (EATS) systems and, to a lesser degree, the retinoid pathway.²⁷¹

Much is understood about the complex mechanisms through which chemicals can interfere with endocrine pathways in humans²⁷² and wildlife.^{273,274} Numerous AOPs related to endocrine disruption are included in the AOP-Wiki,²⁷⁵ and the OECD has published several case studies on IATAs.²⁷⁶ Due to the complexity and sensitivity of endocrine mechanisms, *in vivo* tests show high variability (e.g. stress experienced by the animal can



significantly influence the outcome of the study).²⁷⁷ Classical endpoint studies are not appropriate in this area and need to be replaced by *in vitro* studies in which the multiple factors that could affect test results can be more effectively controlled.

Since 2019, eight projects under the European Cluster to Improve Identification of Endocrine Disruptors (EURION), with €50 million of funding from the European Commission, focused on the development of tools aiming to improve regulatory assessment of endocrine effects and reduce the reliance on animal testing. For example, the SCREENED project²⁷⁸ aims to develop three-dimensional *in vitro* tools to screen for the influence of endocrine disruptors on the thyroid gland.

The US EPA's Office of Research and Development (ORD) is developing *in silico* and *in vitro* assays as well as AOPs to support the robust assessment of chemicals for effects on the endocrine system. For example, the EPA's Toxicity Forecaster (ToxCast) ranks and prioritises chemicals using more than 700 high-throughput screening assays and computational toxicology approaches, which cover a variety of relevant cellular responses and signalling pathways.

The ToxCast assays are being used successfully in the US and the EU. Following a comparative study of ToxCast oestrogen pathway assay results and uterotrophic assay results,²⁷⁹ the EPA announced that it will accept the data from the ToxCast ER Bioactivity Model as an alternative to at least one animal test^{276,280,281} – the uterotrophic assay – that screens for effects on the oestrogen pathway.²⁸² In the EU, the ER Bioactivity Model is currently accepted as a source of *in vitro* mechanistic mode of action information required as part of identification of substances as endocrine disruptors under the current regulatory framework for biocides and plant protection products. Its use as an alternative for the uterotrophic assay is currently being debated.

The thyroid pathway is more complex than either the oestrogen or the androgen pathways. In collaboration with other organisations, the EU Joint Research Centre and the EPA ORD are developing and assessing the validity of sets of relevant assays based on the thyroid AOP.²⁸³

Eye Irritation/Corrosion

Recommendation: Immediately eliminate the use of animals for eye irritation/corrosion testing

To assess eye irritation and corrosion using the Draize eye irritancy test, a chemical substance is applied to rabbits' eyes and the degree of damage is monitored over a 14-day period. Rabbits may endure eye swelling, discharge, ulceration, haemorrhaging, cloudiness, or blindness. The Draize test was developed in 1944, and advanced replacements have since been developed and shown to be as or more reliable and relevant than the rabbit test. For example, an analysis of 491 chemicals with at least two rabbit eye tests showed that there was a 73% (for category 1), 32.9% (for category 2A), 15.5% (for category 2B), and 93.9% (for no category) probability of obtaining the same GHS classification more than once.²⁸⁴ Importantly, these results showed that there was a 10.4% chance that a chemical once identified as category 1 would later be identified as no category.

There are opportunities available to avoid animal tests based on criteria described in OECD guidance document 237.²⁸⁵ An OECD guidance document on an IATA of serious eye damage and irritation was published in 2017,²⁸⁶ and the available *in vitro* methods are listed below:

- **OECD Test No 491: Short Time Exposure (STE) In Vitro Test Method** – This may be used to identify chemicals causing serious eye damage (GHS category 1) or not requiring classification (GHS no category).
- **OECD Test No 492: Reconstructed Human Cornea-Like Epithelium (RhCE) Test Method** – This may be used to identify chemicals not classified for eye irritation or causing serious eye damage (GHS no category).



- **OECD Test No 492B: Reconstructed Human Cornea-Like Epithelium (RhCE) Test Method for Eye Hazard Identification** – This may be used to identify chemicals not requiring classification (GHS no category) or those requiring eye irritation classification (GHS category 2) and serious eye damage classification (GHS category 1).
- **OECD Test No 494: Vitrigel-Eye Irritancy Test Method** – This may be used to identify chemicals not classified for eye irritation or causing serious eye damage (GHS no category).
- **OECD Test No 496: *In Vitro* Macromolecular Test Method** – This may be used to identify chemicals causing serious eye damage (GHS category 1) or not requiring classification.
- **OECD Test No 460: Fluorescein Leakage Test Method** – This may be used to identify chemicals causing serious eye damage (GHS category 1). It is recommended as an initial step within a top-down approach to identifying ocular corrosives or severe irritants.
- **OECD Test No 437: Bovine Corneal Opacity and Permeability (BCOP) Test Method** – This may be used to identify chemicals causing serious eye damage (GHS category 1) or not requiring classification.
- **OECD Test No 438: Isolated Chicken Eye Test Method** – This may be used to identify chemicals causing serious eye damage (GHS category 1) or not requiring classification. It is recommended as the first step within a top-down or bottom-up testing strategy.

Furthermore, **OECD Test No 467: Defined Approaches for Serious Eye Damage and Eye Irritation** describes approaches based on both a) physicochemical properties and *in vitro* data from Test No 492 and No 437 for neat non-surfactant liquids and b) *in vitro* data from Test No 491 and No 437 for neat and/or diluted non-surfactant liquids or solids dissolved in water. The defined approaches may be used to identify chemicals not requiring classification (GHS no category) and those requiring eye irritation classification (GHS category 2) and serious eye damage classification (GHS category 1).

These methods are generally validated for use with cosmetics and industrial chemicals. Certain methods will be more appropriate than others, depending on the applicability domain of the method, purpose of testing, and type of test chemical (e.g. surfactants or solids).

The EPA currently accepts the use of *in vitro* and *ex vivo* methods for the determination of eye irritation and corrosion when classifying antimicrobial cleaning products and, on a case-by-case basis, other pesticide products, and it has published a guidance document describing the testing framework that industry can use for this endpoint.²⁸⁷ Also, the EPA, in collaboration with PETA Science Consortium International, the US National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and industry members, published a paper showing that the *in chemico*, *in vitro*, and *ex vivo* methods are as good as or better than the rabbit test when considering reproducibility and human relevance, and that these methods should be used today for the assessment of chemicals, including agrochemical formulations.²⁸⁸

Genotoxicity and Carcinogenicity

Recommendation: In light of existing non-animal methods and WoE approaches, the use of animals in genotoxicity and carcinogenicity testing can be dramatically reduced.

Genotoxicity

The major genotoxicity endpoints to be evaluated for regulatory purposes are gene mutation, structural chromosomal aberrations (clastogenicity), and numerical chromosomal aberrations (aneuploidy). OECD test guidelines for assessing genotoxicity *in vitro* cover one or two endpoints simultaneously:



- **OECD Test No 471: Bacterial Reverse Mutation Test** – This test, commonly known as the Ames test, uses amino acid–requiring *Salmonella typhimurium* and *Escherichia coli* to detect point mutations by base substitutions or frameshifts.
- **OECD Test No 487: In Vitro Micronucleus Test** – This test can be used to detect micronuclei in the cytoplasm of interphase cells that have undergone cell division during or after exposure to the test substance. This assay detects structural and numerical chromosomal aberrations.
- **OECD Test No 490: In Vitro Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene** – Two distinct assays can be used to detect gene mutations induced by chemical substances.
- **OECD Test No 473: In Vitro Mammalian Chromosomal Aberration Test** – This test identifies chemical substances that cause structural chromosomal aberrations.
- **OECD Test No 476: In Vitro Mammalian Cell Gene Mutation Test Using the *Hrpt* and *xrpt* Genes** – These tests can detect gene mutations induced by chemicals.

The assessment of genotoxicity for regulatory purposes typically follows a step-wise approach starting with a core battery of *in vitro* tests (e.g. the Ames test, micronucleus test, and chromosome aberration test). The need to follow up *in vitro* tests with *in vivo* tests depends on the results and regulatory requirements. For example, in the case of the EU’s industrial chemicals and biocides regulations, a positive result in any of the required *in vitro* tests must be followed up with an appropriate *in vivo* test.^{289,290} However, if a substance produces negative results in the *in vitro* tests, it can be categorised as having no genotoxic potential and no further genotoxicity testing is required. Conversely, for some chemical classes, *in vivo* testing is required regardless of the *in vitro* test results (e.g. plant protection products and pharmaceuticals).^{291,292}

Appropriate data from *in silico* studies (e.g. QSARs and read-across) can help reduce the requirement to conduct *in vivo* tests. The EURL ECVAM—consolidated genotoxicity and carcinogenicity database published in the EURL ECVAM collection of the Joint Research Centre (JRC) data catalogue, for example, provides substantial resources for read-across.²⁹³

Furthermore, advanced *in vitro* methods can provide follow-up and de-risking options for use in a WoE approach. For example, the *in vitro* transcriptomic biomarker responsive to DNA-damage-inducing (DDI) agents, TGx-DDI,^{294,295} and the ToxTracker assay^{296–298} can provide information on the mode of action of potential genotoxicants and have been submitted to formal regulatory “qualification” programmes.^{299,300} Data generated using the ToxTracker assay and read-across have been used in the EU’s Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) dossiers.³⁰¹

The three-dimensional reconstructed skin micronucleus and comet assays for following up positive results from standard *in vitro* genotoxicity assays for dermally applied compounds offer additional animal-free methods and important opportunities to avoid the use of animals for genotoxicity testing.^{302,303} The information requirements for genotoxicity assessment on cosmetics³⁰⁴ already invoke the micronucleus test using three-dimensional reconstructed human skin or a comet test using either mammalian cells or three-dimensional reconstructed human skin. Rapid progress in the development of three-dimensional liver and airway models holds the prospect of animal-free assessment of genotoxicity of compounds administered by the oral or inhalation route in the near future.³⁰⁵

Non-animal methods are gaining ground internationally. Generating comprehensive data based on these methods and developing case studies, such as the one on coumarin in cosmetics products, is an important component of supporting the adoption of next generation risk assessment.^{296,306}

The genotoxicity³⁰⁷ and mutagenicity³⁰⁸ case studies on IATA, under the OECD IATA case studies project,³⁰⁹ illustrate feasible approaches for the development of adequate safety assessment guidelines for systemic genotoxicity risk assessment without animal testing.



Carcinogenicity

The assessment of carcinogenicity often requires that testing be conducted on rats and/or mice for the majority of their life (up to two years). The test requires a minimum of 400 rats and/or mice per chemical assessment (OECD Test No 451 and No 453).

While carcinogenicity studies in animals are still routinely conducted, the test has been under scientific scrutiny since the early 1970s for its lack of reproducibility³¹⁰ and its inability to predict human outcomes.³¹¹ Namely, there are two flawed assumptions that underlie these bioassays: (1) rodent carcinogens are human carcinogens, and (2) high-dose chemical exposure in rodents is indicative of an environmentally relevant dose. Both have been proved incorrect by 50 years' worth of carcinogenicity data. Decades of scientific reviews highlight the overall lack of reliability in the rodent cancer bioassays to predict human cancers.^{311–316}

For example, in an assessment of 202 pesticide evaluations from the EU review programme, it has been demonstrated that the mouse carcinogenicity study contributed little or nothing to either derivation of an acceptable daily intake for assessment of chronic risk to humans or hazard classification for labelling purposes.³¹⁷ In terms of pesticide approvals, the authors showed that the mouse study did not influence a single outcome. An additional study reported that data collected from 182 pharmaceutical chemicals show that little value is gained from the carcinogenicity study when compounds lack certain histopathologic risk factors, hormonal perturbation, and positive genetic toxicity results.³¹⁸ This study was used to support an international collaboration that developed a WoE approach to fulfil some of the carcinogenicity test requirements without the two-year test on rats.^{319,320} The collaboration resulted in an addendum to the guideline for carcinogenicity assessment of pharmaceuticals (ICH S1B) – thus providing an opportunity to spare 400 animals per pharmaceutical regulatory evaluation.³²¹ A similar effort called Rethinking chronic toxicity and Carcinogenicity Assessment for Agrochemicals Project (ReCAAP), led by PETA Science Consortium International, developed a framework to support a WoE-based assessment of agrochemicals without long-term carcinogenicity testing on rats and mice.³²²

Additionally, *in vitro* cell transformation assays (CTA) recapitulate a multistage process that models some aspects of *in vivo* carcinogenesis, and they have the potential to detect both genotoxic and non-genotoxic carcinogens. In its recommendation on the CTA based on the Bhas 42 cell line, EURL ECVAM notes that information on the transforming potential of substances generated by CTAs may be sufficient for decision-making.³²³ Following a study in which the Bhas 42 CTA was tested with 98 substances – including known human carcinogens – the OECD has recommended this assay be used as part of a testing strategy to help assess potentially cancer-causing substances.^{324,325} When combined with other information, such as genotoxicity data, structure-activity analysis, and toxicokinetic information, CTAs in general – and the Bhas 42 CTA specifically – can contribute to the assessment of carcinogenic potential and may provide an alternative to *in vivo* testing.^{326,327}

Several computational tools and models further help to assess carcinogenicity potential. Structural alerts (SA) flagging potential non-genotoxic carcinogens have been incorporated into the OECD QSAR Toolbox.³²⁸ Additionally, the EPA has published a computer model, OncoLogic™, to evaluate chemicals for carcinogenic potential,³²⁹ and commercial options are also available, such as those from Lhasa Limited, MultiCASE, UL Cheminformatics, and Instem. Ultimately, the identification of DNA-reactive chemicals with the Ames test or genotoxic SAs can potentially be combined with the identification of non-genotoxic carcinogens using SAs, leaving CTAs to model most of what is left unexplained in a WoE approach. An OECD expert group is working to generate an IATA for non-genotoxic carcinogens.³³⁰

Given the complexity of carcinogenesis, experts recognise that there needs to be an integration of new approaches (e.g. *in silico* or *in vitro*) to support a fit-for-purpose WoE-based safety assessment.³³¹ Fortunately, there are ongoing initiatives facilitating the integration of methods to ultimately achieve an animal-free, rapid, and human-relevant carcinogenicity assessment for chemical and pharmaceutical regulation.^{322,330,332,333}



Phototoxicity

Recommendation: Immediately eliminate the use of animals for phototoxicity assessments

Substances that absorb light in the UV and visible range (290 to 700 nm) and can reach the skin or eyes may require testing for potential phototoxicity. Phototoxicity is the toxic response to a topically or systemically administered substance that occurs after exposure to light. Phototoxicity can cause symptoms ranging from first-degree burns (redness, itching, and pain) to full thickness third-degree burns. Phototoxicity, often also called photosensitivity, is a well-known adverse effect of many drugs, including antimicrobials, nonsteroidal anti-inflammatory drugs, diuretics, and chemotherapeutic agents.³³⁴

Phototoxicity testing for systemically or topically administered compounds has been conducted in a variety of species, including guinea pigs, mice, and rats. However, no standardized *in vivo* study design has been established.³³⁵ By contrast, so far, three OECD test guidelines have been developed using *in chemico* and *in vitro* methods to assess phototoxicity:

- **OECD Test No 495: Ros (Reactive Oxygen Species) Assay for Photoreactivity** – This is an *in chemico* method that measures a substance's ability to create reactive oxygen species under exposure to artificial sunlight.
- **OECD Test No 432: *In Vitro* 3T3 NRU Phototoxicity Test** – This test measures the viability of a mouse cell line incubated with a potential phototoxicant and exposed to light.
- **OECD Test No 498: *In Vitro* Phototoxicity – Reconstructed Human Epidermis Phototoxicity Test Method** – A three-dimensional reconstructed human epidermis model is incubated with the potential phototoxicant and exposed to light.

OECD Test No 498 is based on a similar principle as **OECD Test No 432** but uses a three-dimensional reconstructed human skin model instead of the mouse cell line, which expands the applicability domain to a wider selection of substances including final formulations, complex mixtures, or dermatological patches.³³⁶ Substances with an extreme pH can also be tested using the three-dimensional skin models. In 2018, France and the Netherlands were the only EU member states to conduct any *in vivo* phototoxicity tests, which emphasises the relevance of OECD Test No 432.³³⁷

Pyrogenicity

Recommendation: Immediately eliminate the use of animals for pyrogenicity assessment

Before drugs and medical devices can be marketed, regulators require testing to demonstrate that they are not contaminated with substances that trigger a fever response. These substances, collectively termed pyrogens, are chemically and structurally diverse but incite fever in humans through a common mechanism: peripheral blood monocytes and macrophages detect pyrogens and release pro-inflammatory cytokines that induce a rise in body temperature. Two *in vitro* methods are available that detect pyrogens:

- **Monocyte activation test (MAT)**, defined in *European Pharmacopoeia (Ph Eur)* general chapter 2.6.30
- **Recombinant Factor C (rFC) assay**, defined in *Ph Eur* general chapter 2.6.32

Even though the mechanism of the human fever response is well understood, two animal-based tests are still commonly required by almost all global regulators to assess pyrogen contamination. The rabbit pyrogen test (RPT) requires that rabbits be injected with a test substance and subsequently restrained for three hours, during which changes in their body temperature are monitored rectally. In Europe alone, more than 200,000 rabbits were used between 2015 and 2019 in the RPT,³³⁸ even though it has never been formally validated for



its relevance to humans and its results can vary depending on the animal's stress level. There are also differences in pyrogen sensitivity among species, and the test is incompatible with certain drug classes.³³⁹

The Limulus amoebocyte lysate test (LAL), also called the bacterial endotoxins test, requires the use of haemolymph from captured horseshoe crabs and detects only bacterial endotoxins and no other pyrogens. After the bleeding process, up to 30% of the crabs die. Those who recover are less likely to survive in nature.³⁴⁰ A synthetic version of the LAL, in which the haemolymph is replaced by a recombinant reagent (the rFC assay), is available to test for bacterial endotoxins. The rFC assay is a very reliable and animal-friendly test with equal or superior performance to LAL.³⁴¹

Since 2010, the *in vitro* monocyte activation test (MAT), capable of detecting both endotoxin and non-endotoxin pyrogens, has been validated and included in the *Ph Eur* as a test for assessing pyrogen contamination.³⁴² In the MAT, drugs and medical devices are incubated with human whole blood or isolated human monocytes. After this exposure period, tests measure pro-inflammatory cytokines released by monocytes to determine the degree of contamination with pyrogenic substances.³⁴³ It avoids the aforementioned problems with the RPT and LAL tests, and case studies document instances in which the MAT detected pyrogen contamination in products that had passed the RPT and LAL but caused fever in human patients.³⁴⁴

Regulators in the EU, India, the UK, and the US accept the MAT, and the pharmacopoeias used in these regions all allow its use following product-specific validation. Nevertheless, animal tests are still used despite their well-documented limitations.³⁴⁵ To eliminate the use of animals in pyrogen tests, regulatory authorities and standards organisations must make an increased effort to integrate and harmonise a preference for the non-animal tests in international testing requirements and to encourage drug and device manufacturers to use and submit data from these tests in their product dossiers. In September 2018, participants at a workshop organised by PETA Science Consortium International and NICEATM discussed non-animal approaches to medical device pyrogen testing and called for more opportunities for training and education to increase the use of the MAT for regulatory purposes.³⁴⁶

Following a survey of pyrogen test users, the European Directorate for the Quality of Medicines & HealthCare (EDQM) revised the *Ph Eur* general chapter on the MAT to improve the usability of the method and to emphasise that it is considered a replacement for animal-based pyrogen tests.^{347,348} This endorsement is repeated in statements from the European Medicines Agency^{349,350} and, in 2021, **the *Ph Eur* Commission announced that it intends to completely replace the RPT in its guidance before 2026**. The International Organization for Standardization (ISO) is revising its guidance to allow use of the MAT when evaluating medical device pyrogen contamination, but the revision process has moved slowly.³⁴³ In the 8th edition of *Indian Pharmacopoeia*, the Indian Pharmacopoeia Commission revised the pyrogen testing general chapter, introduced the monograph on the MAT, and replaced the RPT with LAL.³⁵¹ However, due to unclear guidance and regulatory ambiguity about the applicability of the MAT as a stand-alone pyrogen test, the RPT and LAL are still being used.

Reproductive and Developmental Toxicity

Recommendation: Immediately fund and support the development of innovative non-animal methods for assessing reproductive and developmental toxicity

Reproductive toxicity studies measure the effect of a chemical on reproductive organs and fertility, while developmental toxicity studies measure a chemical's effect on developing offspring during pregnancy.

Developmental toxicity studies for chemical and pharmaceutical human safety assessment are primarily performed using rats. However, many regulatory frameworks – including the Biocidal Products and Plant



Protection Product Regulations and, in some circumstances, REACH in the EU – require registrants to submit test results using a second species, usually rabbits, under the assumption of interspecies differences in sensitivity to developmental effects. These studies use a large number of animals. For example, a prenatal developmental toxicity study conducted according to OECD Test Guideline 414 uses approximately 560 rabbits or 784 rats.³⁵²

None of the *in vivo* methods used for testing reproductive and developmental toxicity have been formally validated for their relevance to humans.³⁵³ Therefore, significant investment is required to develop human-relevant non-animal methods. EURL ECVAM has investigated the validation of *in vitro* reproductive toxicity test methods and is leading the development of an AOP for an aspect of reproductive toxicity, i.e. PPAR γ activation leading to impaired fertility.^{354,355} The EU FP6 project ReProTest has also investigated possible strategies to cover the entire mammalian reproductive cycle, resulting in a series of published works.³⁵⁶ Furthermore, the ChemScreen FP7 project has been designed to generate a rapid screening system that is relatively simple and cost-effective.³⁵⁷

In addition, the EU-ToxRisk project integrates advancements in cell biology, “omic” technology, systems biology, and computational modelling to define the complex chains of events that link chemical exposure to toxic outcome. The project focuses on repeat-dose systemic toxicity and developmental and reproductive toxicity. The EPA’s National Center for Computational Toxicology is also exploring the potential for chemicals to disrupt prenatal development through the use of its virtual embryo model, v-Embryo™, which integrates *in vitro* and *in silico* modelling approaches.³⁵⁸ The OECD, JRC, European Food Safety Authority (EFSA), and the EPA are developing guidance to demonstrate how the integration of a battery of *in vitro* assays can be used to determine the potential of chemical developmental neurotoxicity, and the partner agencies are working on case studies that apply to different chemical classes.³⁵⁹ In 2021, Health Canada³⁶⁰ compared *in vitro* bioactivity-based points of departure (POD_{Bioactivity}) with points of departure from oral repeat-dose, developmental, and reproductive studies (POD_{Traditional}) used in risk assessment. For 43 out of 46 of the examined chemicals, POD_{Bioactivity} was more conservative than the lowest POD_{Traditional}, demonstrating confidence in using *in vitro* bioactivity as a surrogate lower bound estimate of *in vivo* adverse effect levels – a strong indication that using POD_{Bioactivity} would be equally or more protective than using POD_{Traditional}.³⁶⁰

While the field is gradually moving towards a range of integrative strategies in order to cover the majority of possible mechanisms, much more research is required.

Skin Irritation/Corrosion

Recommendation: Immediately eliminate the use of animals for skin irritation/corrosion testing

Skin irritation and corrosion tests for chemicals are required or recommended by several regulatory agencies. In the animal test, a test substance is applied to the shaved skin of a rabbit, and they are observed for up to 14 days to assess the degree of skin damage. The tests can cause permanent skin damage, ulcers, bleeding, bloody scabs, and scarring.

Despite years of use, animal-based skin irritation studies have been shown to be generally poor predictors of human skin reactions and are highly variable.³⁶¹ For example, a comparison of data from rabbit tests and four-hour human skin patch tests for 65 substances found that 45% of classifications of chemical irritation potential based on animal tests were incorrect.³⁶²

There are opportunities to avoid the animal test based on criteria described in OECD guidance document No 237.²⁸⁵ Furthermore, the OECD has developed an IATA for skin irritation using *in vitro* skin irritation and corrosion methods that avoids or minimises animal use.³⁶³



- **OECD Test No 439: *In Vitro* Skin Irritation: Reconstructed Human Epidermis (RHE) Test Method** – This may be used for the hazard identification of irritant chemicals (substances and mixtures), in accordance with the UN Globally Harmonized System of Classification and Labelling of Chemicals (GHS), as category 2, or non-classified chemicals. It may be used as a stand-alone test or in a tiered testing strategy.
- **OECD Test No 431: *In Vitro* Skin Corrosion: RHE Test Method** – This may be used for the identification of corrosive chemical substances and mixtures. It may also distinguish between severe and less severe skin corrosives.
- **OECD Test No 435: *In Vitro* Membrane Barrier Test Method for Skin Corrosion** – This allows for the subcategorisation of corrosive chemicals into the three GHS subcategories of corrosivity.

Recently, **OECD Test Guideline No. 439** was validated for use in assessing the ability of medical device extracts to cause skin irritation, and the ISO 10993 guidance has been updated to include this test.³⁶⁴

Skin Sensitisation

Recommendation: Immediately eliminate the use of animals for skin sensitisation testing

The assessment of skin sensitisation involves measuring the likelihood that a substance will cause an allergic reaction if applied to the skin. In animals, such assessments have previously been based on applying a test substance to the shaved skin of guinea pigs in the guinea pig maximisation test or to the ears of mice in the local lymph node assay.

The regulatory requirement to test for skin sensitisation can be met with a defined approach, as described in **OECD Test No 497: Defined Approaches on Skin Sensitisation**, using a combination of *in chemico* and *in vitro* assays that each address a different key event in the AOP.²⁵³ The “2 out of 3” defined approach provides sufficient information for hazard identification, and the integrated testing strategies (ITSv1 and ITSv2) collate information from two of the *in vitro* assays below, along with *in silico* predictions, to predict hazard and potency.

- **OECD Test No 442C: Key Event–Based Test Guideline for *In Chemico* Skin Sensitisation Assays Addressing the Adverse Outcome Pathway Key Event on Covalent Binding to Proteins** – This test guideline addresses the molecular initiating event of the skin sensitisation AOP.
- **OECD Test No 442D: *In Vitro* Skin Sensitisation Assays Addressing the AOP Key Event on Keratinocyte Activation** – This test guideline addresses the second key event of the skin sensitisation AOP.
- **OECD Test No 442E: *In Vitro* Skin Sensitisation Assays Addressing Key Event on Activation of Dendritic Cells** – This method addresses the third key event of the skin sensitisation AOP.

The non-animal approaches to predicting skin sensitisation are as good as or better than the local lymph node assay when compared to human data.³⁶⁵

Systemic Toxicity

Recommendation: In light of existing non-animal methods and WoE approaches, the use of animals for systemic toxicity testing can be dramatically reduced

Acute Systemic Toxicity

To determine the danger of exposure to a product or chemical, a substance is administered to animals through the oral, dermal, or inhalation routes. Acute toxicity refers to adverse effects observed following one high level of exposure to a substance for a short duration (up to 24 hours). In these tests, the dose at which half the



animals would be killed – called the lethal dose 50 (LD₅₀) or lethal concentration 50 (LC₅₀) for inhalation testing – is determined. The LD₅₀ test and its adaptations have never been scientifically validated, and their accuracy in predicting chemical effects in humans remains in question. An analysis of the variability of the acute oral toxicity animal test showed that there is 78% or 74% accuracy in obtaining the same EPA or GHS classification, respectively, if the same chemical is tested more than once,³⁶⁶ while another analysis of existing acute oral LD₅₀ data demonstrated that replicate studies result in the same hazard categorisation on average 60% of the time.³⁶⁷ This second study demonstrated that inherent biological or protocol variability most likely underlies the variance in the results.

When scientific justification is provided, regulatory authorities may allow acute toxicity assessment without testing on animals. The OECD has published guidance for waiving or bridging acute toxicity testing,²⁸⁵ and the EPA has published similar guidance for pesticides and pesticide products.³⁶⁸ This includes the use of existing data for read-across and the consideration of the physicochemical properties of the test substance.

Repeat-Dose Systemic Toxicity

In repeat-dose toxicity studies, animals are exposed repeatedly to substances for up to one month (sub-acute), up to three months (sub-chronic), or up to several years (chronic) in order to measure the effects of multiple chemical exposures. Chemicals are usually administered to animals using oral gavage unless another route of exposure is more likely. Like other endpoints, there is evidence that regulatory studies using animals to assess repeat-dose toxicity are not fit for purpose, and there is a clear need to develop new approaches. In 2020, Pham and colleagues evaluated the sources of variability in the values used to derive safe exposure levels from a variety of repeat-dose studies in rodents and found that approximately one-third of the total variance could not be accounted for through considerations of study differences, e.g. administration route or study type.^{369,370}

While the assessment of repeat-dose toxicity is a standard requirement in human safety evaluation, no non-animal methods are currently accepted for regulatory purposes. To address this gap in the use of non-animal methods, the European Commission's Detection of Endpoints and Biomarkers of Repeated Dose Toxicity Using *In Vitro* Systems (DETECTIVE) project was funded as one of six research projects under the Safety Evaluation Ultimately Replacing Animal Testing (SEURAT-1) cluster umbrella. The aim of the project was to set up a screening pipeline of high-content, high-throughput, and "omic" technology to identify and investigate human biomarkers in cellular models for repeat dose *in vitro* testing. In addition, the EU-ToxRisk project integrates advancements in cell biology, "omic" technology, systems biology, and computational modelling to define the complex chains of events that link chemical exposure to toxic outcome. The project focuses on repeat-dose systemic toxicity and developmental and reproductive toxicity.

While the development and regulatory implementation of repeat-dose toxicity *in vitro* testing systems advances, the number of animals used for repeat-dose toxicity testing under various regulatory frameworks may be immediately reduced by the extrapolation of points of departure, from sub-chronic to chronic studies.³⁷⁰ A recent review of points of departure (NOAELs or LOAELs) determined from *in vivo* studies with food additives showed that the chronic values may be extrapolated with high confidence from sub-chronic studies, supporting previous analyses of other types of substances, including industrial chemicals and pesticides. The risk assessment and derivation of health-based guidance values may be further strengthened by a precautionary application of an additional uncertainty factor of 2 to account for any outlying values – an approach recommended by EFSA and supported by data from a number of recent studies.³⁷¹

Oral Route

NICEATM and ICCVAM organised a project to develop predictive models for acute oral systemic toxicity.³⁶⁶ The outcome was a Collaborative Acute Toxicity Modelling Suite (CATMoS) tool for predicting acute oral toxicity to meet various regulatory needs, which were presented at an April 2018 workshop.³⁷² CATMoS is implemented through Open Structure-Activity/Property Relationship App (OPERA), a freely available and open-source QSAR tool.³⁷³ This model is routinely optimised, and updates are available on the NICEATM Integrated Chemical Environment (ICE) and EPA websites.³⁷⁴ PETA Science Consortium International, the Physicians Committee for



Responsible Medicine, and the EPA developed webinars to provide overviews of both the CATMoS tool and the ICE database (ThePSCI.eu/training-videos-webinars).

EURL ECVAM recommends the use of an *in vitro* 3T3 neutral red uptake (NRU) cytotoxicity assay, which can be used in a WoE approach to support the identification of non-classified substances.³⁷⁵ *In vitro* tests, such as the 3T3 NRU and normal human keratinocyte assays that measure basal cytotoxicity, can also be useful in determining starting doses in animal tests. EURL ECVAM is currently working to improve confidence in the 3T3 NRU through the use of QSARs and by accounting for target organ information and the lack of metabolism in 3T3 cells.^{376–378}

In its “Guidance on Information Requirements and Chemical Safety Assessment”, ECHA advises that an *in vivo* acute oral toxicity study can potentially be avoided if a registrant has relevant data, which are used in a WoE approach.²⁸⁹ In cases in which the WoE adaptation leads to the assumption of low/no expected acute oral toxicity (>2000 mg/kg bw/d), the registrant can avoid animal testing pursuant to REACH Articles 13(1) and 25(1).³⁷⁹ More information about ways to reduce the number of animals used to assess acute oral toxicity for REACH can be found at ThePSCI.eu/training-videos-webinars.

Dermal Route

The EPA and NICEATM analysed the relative contributions of data from acute oral and dermal toxicity tests to pesticide hazard classification and labelling. Finding that the dermal data provided little to no added value in regulatory decision-making, the EPA published guidance allowing registrants to submit scientifically sound justification for why the acute oral test results are protective for potential acute dermal effects.^{380,381} In addition, dermal studies are not required for substances that are non-classified by the oral route and not absorbed dermally.²⁸⁵ Furthermore, substances that are not classified by the oral route do not require dermal data under REACH Annex VIII.

Inhalation Route

Testing by the inhalation route can be avoided based on physicochemical parameters (e.g. low volatility) or if exposure through inhalation is unlikely (e.g. in cases in which the substance is not aerosolised or otherwise made respirable under conditions of use). However, in instances in which testing is required, non-animal methods can be applied to fulfil the informational requirements. For example, to fulfil an informational need, the EPA accepted the use of an *in chemico* biosolubility test, which showed that a polymer, initially classified as a poorly soluble, low toxicity substance, was soluble in simulated epithelial lung fluid and, therefore, was not a hazard concern from lung overload.³⁸² In another example, the EPA is considering data from *in silico* computational fluid dynamic modelling and *in vitro* testing using three-dimensional reconstructed human lung tissues to fulfil the re-registration requirements for a pesticide.³⁸³ Several other promising research efforts are underway to develop non-animal methods for inhalation toxicity.³⁸⁴

PETA Science Consortium International has hosted numerous webinars (ThePSCI.eu/inhalation-webinars) and workshops, at which several approaches were presented that could eventually replace animal testing for this endpoint.^{385,386} Additionally, the Science Consortium has funded method development and organised several awards to provide researchers with equipment and *in vitro* respiratory tissues to conduct inhalation toxicity studies.³⁸⁷ More information on inhalation toxicity testing can be found at ThePSCI.eu/our-work/inhalation.

Tobacco and E-Cigarette Testing

Recommendation: Immediately eliminate the use of animals for the development and testing of tobacco and e-cigarette products

Around the world, animals are used to test existing tobacco products and for the development of new ones, such as electronic nicotine delivery systems (ENDS, or e-cigarettes) or tobacco heating products. In such tests,



rats may be confined to narrow tubes and forced to inhale toxic substances for up to six hours each day for several years.

The European Commission Scientific Committee on Health, Environmental and Emerging Risks (SCHEER) stated that, in light of the EU policy banning animal studies for chemicals to be used in voluntary products such as cosmetics, animal studies are not endorsed to assess the safety of tobacco additives.³⁸⁸ In addition, Belgium, Estonia, Germany, Slovakia, and the UK already prohibit the use of animals for the development and testing of tobacco products because of ethical concerns.^{389–393}

The hazard assessment of tobacco products increasingly employs innovative non-animal methods, including the exposure of cell and tissue cultures to whole cigarette smoke or e-cigarette vapour at the air–liquid interface, CTAs, and genomic analyses.^{386,394,395} These techniques have been used to investigate cytotoxicity, genotoxicity, inflammation, and gene expression and are more relevant to actual human exposure than are animal tests that have historically under-predicted the hazards of tobacco. To facilitate the uptake and use of such *in vitro* techniques to assess tobacco products and other inhaled chemicals, PETA Science Consortium International has donated VITROCELL *in vitro* exposure systems to the Institute for *In Vitro* Sciences (IIVS) to allow it to expand its testing of tobacco products. Most of the Science Consortium’s extensive work on inhalation toxicity testing (ThePSCI.eu/our-work/inhalation) is also applicable to the testing of tobacco and tobacco-derived products.



Laboratory Production Methods

Detailed below are opportunities to end the use of animal-derived products for scientific or medical purposes and to reduce significantly the use of animals for the production of drugs and vaccines.

Antibody Production

Recommendation: Immediately eliminate the production of animal-derived antibodies for scientific applications

Affinity reagents such as antibodies are essential tools used in research to bind to a molecule to identify it or influence its activity. Every year, tens of thousands of animals are injected with viruses, bacteria, or other foreign substances and then killed for the antibodies that their bodies produce in response. Animals used in antibody production are subjected to a number of invasive and painful procedures, including antigen injection and repeated blood or ascites collection, before being killed. In the ascites method of antibody production, animals have been reported to be unable to eat, walk, or breathe properly. A number of countries, including Australia, Canada, Germany, the Netherlands, Switzerland, and the UK, restricted or banned the production of antibodies obtained via the ascites method because of animal welfare concerns.^{396,397}

Growing concern about the lack of quality and reproducibility of animal-derived antibodies, which often show poor specificity or fail to recognise their targets, is also evident in the literature. In a 2015 *Nature* commentary, 111 academic and industry scientists called for an international shift to the use of recombinant antibodies for reasons that include increased reliability and reduced batch-to-batch variability in affinity reagents.³⁹⁸ In addition, a 2015 *Nature* news feature reported that antibodies may be the laboratory tool most commonly contributing to the “reproducibility crisis”.³⁹⁹ In fact, poorly characterised and ill-defined antibodies were considered a primary cause of irreproducible research in a survey of preclinical studies that found that the results of 47 out of 53 studies could not be replicated. Furthermore, a systematic analysis of 185 commercially available hybridoma monoclonal antibodies found that one-third were not reliably monospecific, and the authors recommended replacing the use of animal-derived monoclonal antibodies with sequence-defined recombinant antibodies as a straightforward and cost-effective solution to this serious problem.⁴⁰⁰ This issue is not limited to monoclonal antibodies. Polyclonal antibodies, which are dependent on the animal used to produce the antibodies and vary in their composition by definition, cannot be consistently reproduced, leading to calls within the scientific community to phase them out of research completely.³⁹⁸

In addition to the lack of scientific reliability and the animal welfare concerns, there are significant economic issues related to using animal-derived antibodies. It is estimated that \$800 million is wasted annually worldwide on unreliable antibodies.³⁹⁸ Thus, there are potential cost savings associated with the more reproducible research that would result from using higher-quality affinity reagents.

Non-animal affinity reagents, such as recombinant antibodies and aptamers, can be used in all applications in which traditional antibodies are used, including in basic research, regulatory testing, and clinical applications. They are commercially available and, with appropriate resources, can be developed by researchers in their own laboratories.^{396,401} The numerous scientific advantages of non-animal affinity reagents over animal-derived antibodies include high affinity and specificity, shorter generation time, reduced immunogenicity, the ability to control selection conditions, and the ability to be generated against unstable, toxic, immunosuppressant, and non-immunogenic antigens.⁴⁰¹



International efforts have highlighted the importance of a large-scale transition from animal-derived antibodies to animal-free affinity reagents. In the US, experts and organisations including NICEATM and PETA Science Consortium International are working to increase access to animal-free affinity reagents. In December 2019, both organisations convened a meeting to outline a pathway to improve the quality and reproducibility of research and testing by accelerating their production and use. Steps to overcome hurdles to a comprehensive shift from animal-derived to animal-free, sequence-defined affinity reagents that were identified at the meeting are described in the article “Increasing the use of animal-free recombinant antibodies”.⁴⁰² More information on sources of animal-free affinity reagents, webinars, publications, and the scientific, economic, and ethical advantages of replacing animal-derived antibodies with animal-free options is available at ThePSCI.eu/our-work/antibodies.

In its 2020 Recommendation on Non-Animal-Derived Antibodies, EURL ECVAM stated the following:

EURL ECVAM recommends that animals should no longer be used for the development and production of antibodies for research, regulatory, diagnostic and therapeutic applications. [...] EU countries should no longer authorise the development and production of antibodies through animal immunisation, where robust, legitimate scientific justification is lacking.⁴⁰³

Therefore, the development, production, and import of animal-derived antibodies, especially monoclonal antibodies using the ascites method, should be banned worldwide. In 2022, the Recombinant Antibody Challenge was launched by PETA Science Consortium International, the Physicians Committee for Responsible Medicine, and the Alternatives Research and Development Foundation, offering grants for free catalogue recombinant antibodies for use in research and testing (ThePSCI.eu/funding/recombinant-antibody-challenge). In order to further expedite the replacement of animal-derived antibodies, we recommend the provision of additional grant opportunities for the generation and use of non-animal affinity reagents.

Biologic Drugs

Recommendation: In light of existing non-animal methods and WoE approaches, the use of animals can be dramatically reduced in the production and evaluation of biologic drugs

Many vaccines and other biologic drugs are produced or tested for quality, identity, safety, and efficacy in experiments that require the use of large numbers of animals. These procedures often cause severe suffering before the animals die or are killed. New technology has enabled the production and testing of biologics without animals, but experience has shown that validation and regulatory acceptance of these methods have not guaranteed their use.^{404–408} Activities intended to phase out the use of animals in this context must ensure that regulatory authorities and industry commit to (1) making the transition to non-animal biologic production platforms, (2) ensuring that available non-animal methods are consistently used in place of animal-based tests, and (3) developing non-animal replacements for quality, identity, safety, and efficacy tests for all biologics.

Production platforms are available that replace animal-derived substances with recombinant, cell-based equivalents. Antitoxins, for example, have been produced historically by hyper-immunising horses and other large mammals and isolating the resulting immunoglobulins from their blood. These animal-derived immunoglobulins have disadvantages intrinsic to their animal origin, including the risk of adverse human immune response, high batch-to-batch variability, and the potential to transmit viruses and other sources of disease between species. Animal-derived antitoxins can be replaced with recombinant human antitoxins expressed in cell culture. Several recombinant antibodies have been licensed for marketing,^{409,410} and more are in development,⁴¹¹ including a candidate diphtheria antitoxin based on human recombinant antibodies created with funding from PETA Science Consortium International.⁴¹²



With adequate funding and support from regulators, all biologics of animal origin, including antibodies (described above), can and should be replaced in a similar fashion in order to resolve issues inherent in using antibodies derived from animals.

Non-animal quality tests are available, but no formal mechanism exists to ensure that barriers to their implementation are resolved in a timely manner.⁴⁰⁴ In some instances, manufacturers report difficulty meeting the technical criteria for using validated non-animal methods (as with the *in vitro* *Leptospira* vaccine potency tests).⁴¹³ In other instances, international regulators have yet to agree on technical criteria for using non-animal methods (as with the *in vitro* rabies vaccine potency test).⁴¹⁴ In the absence of formal oversight of the implementation process, these barriers are left to be resolved informally through workshops and decentralised problem-solving by consortia of interested parties, but this approach is prohibitively expensive and slow for companies seeking to use validated non-animal methods. As a consequence, industry adoption of non-animal methods remains limited, despite the documented reduction in animal use when they are implemented successfully.⁴¹⁵ Additional barriers to the implementation of currently available alternative tests have been discussed at length in workshops and the literature for a broad range of human and veterinary therapeutics hormones, vaccines, and other biologics.^{416–418} Accelerating and standardising processes that facilitate the use of these existing replacement methods is crucial.

Regulatory leadership will ensure international regulatory and industrial coordination on best practices to remove these barriers. Regulatory authorities must establish harmonised manufacturing consistency requirements, as tightly controlled manufacturing consistency policies are the foundation of many animal replacement strategies.^{419,420}

Foetal Bovine Serum

Recommendation: Immediately eliminate the use of foetal bovine serum in scientific applications

Foetal bovine serum (FBS) is a supplement for cell culture media that provides an undefined mixture of macromolecules that function to maintain cell viability and facilitate cell metabolism, growth, proliferation, and spreading in culture. When pregnant cows are slaughtered, a large-gauge needle is used to draw the blood from the beating heart of the foetus.^{421,422} Because the unborn calves are not anaesthetised at the time of blood collection, they likely experience pain. It has been estimated that 600,000 litres of FBS are produced globally each year, which translates to the use of up to 1.8 million bovine fetuses for this purpose.⁴²³

Additionally, a number of scientific concerns are associated with the use of FBS, including batch variation leading to reproducibility issues for *in vitro* studies using FBS, the unknown composition of the serum, and the risk of contamination by animal proteins or pathogens, which is especially problematic in the manufacture of biologics for human therapies. Dutch organisations hosted workshops in 2003 and 2009 that called for the transition from FBS to non-animal serum supplements in cell culture.^{424,425} A third workshop on FBS and alternatives was held in 2016, organised by the SET Foundation and the Deutscher Tierschutzbund (German Animal Welfare Federation).⁴²² The workshop report recommends increased funding and continued development of serum-free culture models and the use of serum-free media when establishing new cell lines. Because a universal chemically defined serum-free culture medium is not yet available and there is high demand for different cell types, the report recommends the use of human platelet lysate (hPL) as a replacement for FBS when a serum-free medium is not available.

Animal component-free and chemically defined serum-free media are available for some cell types. For others, researchers still need to optimise the concentration of each supplement to replace FBS. For these cell types, hPL, which is obtained from donated human platelets, contains growth factors essential for cell growth and proliferation and is superior to FBS for culturing cells.



Listings of commercially available products are available on the Science Consortium's website (ThePSCI.eu/fbs) and in the Fetal Calf Serum-Free Database (<https://fcs-free.org>). Expert presentations on replacing FBS in cell culture media while maintaining robust cell growth and cellular functions are also available at ThePSCI.eu/fbs. PETA Science Consortium International has further funded the transition of a commonly used lung cell line to cell culture media without animal-derived products.⁴²⁶

Government and regulatory agencies should move expediently to restrict the production and use of FBS when non-animal media or supplements are available. They should also provide funding for the transition of cells to available non-animal media and for the development and optimisation of non-animal, serum-free media when needed. For cell types in which non-animal supplement concentrations have not yet been optimised and hPL cannot be used, they should require exemptions to be obtained before FBS can be produced or used. To obtain exemptions, measures should be taken to seek non-animal alternatives and a plan to make the transition to non-animal media or supplements should be implemented.



Scientific Advisory Capabilities of PETA Entities

The Netherlands National Committee for the protection of animals used for scientific purposes (NCad) consulted with PETA scientists before publishing its advice report on the transition towards animal-free innovation for the Dutch government. PETA entities stand ready to offer assistance in whatever capacity might be required.

PETA Science Consortium International promotes and funds non-animal research methods and coordinates the scientific and regulatory expertise of its members, PETA entities around the world. With an eye towards championing the best non-animal methods and reducing animal testing, the Science Consortium and its members are actively involved in the development, validation, global implementation, and harmonisation of non-animal test methods. PETA Science Consortium International is an accredited ECHA stakeholder and a member of the EURL ECVAM Stakeholder Forum, the European Food Safety Authority, and the UK Chemicals Stakeholder Forum and regularly comments on OECD test guidelines as a member of the International Council on Animal Protection in OECD Programmes (ICAPO). More information about the work of the Science Consortium can be found at ThePSCI.eu.

The scientists who work for PETA entities have a proven track record of productively assisting many Fortune 100 corporations as well as regulatory and government agencies. This assistance includes providing expert opinions, regulatory advice, and technical support in a broad range of fields. Given the breadth and depth of our expertise, we believe that we can make a valuable contribution to developing and implementing a strategic plan for the future of biomedical research and regulatory testing.



References (Appendices)

- ¹Ahmad FB, Anderson RN. The leading causes of death in the US for 2020. *JAMA*. 2021;325(18):1829-1830.
- ²Wong CH, Siah KW, Lo AW. Estimation of clinical trial success rates and related parameters. *Biostatistics*. 2018;kxx069.
- ³US Centers for Disease Control and Prevention. An update on cancer deaths in the United States. cdc.gov. Updated 23 February 2021. Accessed 24 February 2022. <https://www.cdc.gov/cancer/dccp/research/update-on-cancer-deaths/index.htm>.
- ⁴Anand P, Kunnumakkara AB, Sundaram C, et al. Cancer is a preventable disease that requires major lifestyle changes. *Pharm Res*. 2008;25(9):2097.
- ⁵Errington TM, Mathur M, Soderberg CK, et al. Investigating the replicability of preclinical cancer biology. *Elife*. 2021;10:e71601.
- ⁶Mak IW, Evaniew N, Ghert M. Lost in translation: Animal models and clinical trials in cancer treatment. *Am J Transl Res*. 2014;6(2):114-118.
- ⁷Cekanova M, Rathore K. Animal models and therapeutic molecular targets of cancer: Utility and limitations. *Drug Des Devel Ther*. 2014;8:1911-1922.
- ⁸Ben-David U, Ha G, Tseng YY, et al. Patient-derived xenografts undergo mouse-specific tumor evolution. *Nat Genet*. 2017;49(11):1567-1575.
- ⁹Cheon DJ, Orsulic S. Mouse models of cancer. *Annu Rev Pathol*. 2011;6:95-119.
- ¹⁰Dennis MB. Welfare issues of genetically modified animals. *ILAR J*. 2002;43(2):100-109.
- ¹¹Ormandy EH, Dale J, Griffin G. Genetic engineering of animals: Ethical issues, including welfare concerns. *Can Vet J*. 2011;52(5):544-550.
- ¹²Romania P, Folgiero V, Nic M, et al. *Advanced Non-Animal Models in Biomedical Research: Immuno-Oncology*. Publications Office of the European Union; 2021.
- ¹³Meng F, Meyer CM, Joung D, Vallera DA, McAlpine MC, Panoskaltsis-Mortari A. 3D bioprinted *in vitro* metastatic models via reconstruction of tumor microenvironments. *Adv Mater*. 2019;31(10):1806899.
- ¹⁴Zamprogno P, Wüthrich S, Achenback S, et al. Second-generation lung-on-a-chip with an array of stretchable alveoli made with a biological membrane. *Commun Biol*. 2021;4(1):168.
- ¹⁵Rosenbluth JM, Schackmann RCJ, Gray GK, et al. Organoid cultures from normal and cancer-prone human breast tissues preserve complex epithelial lineages. *Nat Commun*. 2020;11(1):1711.
- ¹⁶Ethier SP, Guest ST, Garrett-Mayer E, et al. Development and implementation of the SUM breast cancer cell line functional genomics knowledge base. *NPJ Breast Cancer*. 2020;6:30.
- ¹⁷ICGC/TCGA Pan-Cancer Analysis of Whole Genomes Consortium. Pan-cancer analysis of whole genomes. *Nature*. 2020;578(7793):82-93.
- ¹⁸Siddiqui SS, Vaill M, Do R, et al. Human-specific polymorphic pseudogenization of SIGLEC12 protects against advanced cancer progression. *FASEB BioAdv*. 2020;3(2):69-82.
- ¹⁹Pantanowitz L, Quiroga-Garza GM, Bien L, et al. An artificial intelligence algorithm for prostate cancer diagnosis in whole slide images of core needle biopsies: A blinded clinical validation and deployment study. *Lancet Digit Health*. 2020;2(8):e407-e416.
- ²⁰Landhuis E. Deep learning takes on tumours. *Nature*. 2020;580(7804):551-553.
- ²¹Cohen A, Ioannidis K, Ehrlich A, et al. Mechanism and reversal of drug-induced nephrotoxicity on a chip. *Sci Transl Med*. 2021;13(582):eabd6299.
- ²²Cimons M, Getlin J, Maugh II TH. Cancer drugs face long road from mice to men. *Los Angeles Times*. Published 6 May 1998. Accessed 11 July 2018. <http://articles.latimes.com/1998/may/06/news/mn-46795>.
- ²³Verma M. Personalized medicine and cancer. *J Pers Med*. 2012;2(1):1-14.
- ²⁴Honig P, Terzic A. Affairs of the heart: Innovation in cardiovascular research and development. *Clin Pharmacol Ther*. 2017;102(2):162-168.
- ²⁵Liao J, Huang W, Liu G. Animal models of coronary heart disease. *J Biomed Res*. 2015;30(1):3-10.
- ²⁶Janssen PML, Elnakish MT. Modeling heart failure in animal models for novel drug discovery and development. *Expert Opin Drug Discov*. 2019;14(4):355-363.
- ²⁷Milani-Nejad N, Janssen PM. Small and large animal models in cardiac contraction research: Advantages and disadvantages. *Pharmacol Ther*. 2014;141(3):235-249.
- ²⁸Barter P, Rye KA. Cholesteryl ester transfer protein inhibition to reduce cardiovascular risk: Where are we now? *Trends Pharmacol Sci*. 2011;32(12):694-699.
- ²⁹Vegter EL, Ovchinnikova ES, Silljé HHW, et al. Rodent heart failure models do not reflect the human circulating microRNA signature in heart failure. *PLoS One*. 2017;12(5):e0177242.
- ³⁰Zaragoza C, Gomez-Guerrero C, Martin-Ventura JL, et al. Animal models of cardiovascular diseases. *J Biomed Biotechnol*. 2011;2011:497841.
- ³¹Chandrasekera PC, Pippin JJ. The human subject: An integrative animal model for 21st century heart failure research. *Am J Transl Res*. 2015;7(9):1636-1647.



- ³²Park J, Wu Z, Steiner PR, Zhu B, Zhang JXJ. Heart-on-chip for combined cellular dynamics measurements and computational modeling towards clinical applications. *Ann Biomed Eng.* 2022;50(2):111-137.
- ³³Gitant G, Sager PT, Stockbridge N. Evolution of strategies to improve preclinical cardiac safety testing. *Nat Rev Drug Discov.* 2016;15(7):457-471.
- ³⁴del Álamo JC, Lemons D, Serrano R, et al. High throughput physiological screening of iPSC-derived cardiomyocytes for drug development. *Biochim Biophys Acta.* 2016;1836(7B):1717-1727.
- ³⁵Novoheart Holdings Inc. Novoheart strengthens North American presence opening new R&D location at the world-class Cove Facility, UC Irvine, California. Published 25 October 2017. Accessed 27 October 2022. <https://www.globenewswire.com/en/news-release/2017/10/25/1209725/0/en/Novoheart-Strengthens-North-American-Presence-Opening-New-R-D-Location-at-the-World-class-Cove-Facility-UC-Irvine-California.html>.
- ³⁶Gaudin S. Engineering diseased blood vessels to more accurately test new medications. Worcester Polytechnic Institute. Published 7 June 2018. Accessed 27 October 2022. <https://www.wpi.edu/news/engineering-diseased-blood-vessels-more-accurately-test-new-medications>.
- ³⁷Ren L, Zhou X, Nasiri R, et al. Combined effects of electric stimulation and microgrooves in cardiac tissue—on-a-chip for drug screening. *Small Methods.* 2020;4(10):2000438.
- ³⁸Savchenko A, Cherkas V, Liu C, et al. Graphene biointerfaces for optical stimulation of cells. *Sci Adv.* 2018;4(5):eaat0351.
- ³⁹Gershlak JR, Hernandez S, Fontana G, et al. Crossing kingdoms: Using decellularized plants as perfusable tissue engineering scaffolds. *Biomaterials.* 2017;125:13-22.
- ⁴⁰Hoang P, Wang J, Conklin BR, Healy KE, Ma Z. Generation of spatial-patterned early-developing cardiac organoids using human pluripotent stem cells. *Nat Protoc.* 2018;13(4):723-737.
- ⁴¹Al-Hilal TA, Keshavarz A, Kadry H, et al. Pulmonary-arterial-hypertension (PAH)—on-a-chip: Fabrication, validation and application. *Lab Chip.* 2020;20(18):3334-3345.
- ⁴²Ho L, Hossen N, Nguyen T, Vo A, Ahsan F. Epigenetic mechanisms as emerging therapeutic targets and microfluidic chips application in pulmonary arterial hypertension. *Biomedicines.* 2022;10(1):170.
- ⁴³Richards DJ, Li Y, Kerr CM, et al. Human cardiac organoids for the modelling of myocardial infarction and drug cardiotoxicity. *Nat Biomed Eng.* 2020;4(4):446-462.
- ⁴⁴Pičulin M, Smole T, Žunkovič B, et al. Disease progression of hypertrophic cardiomyopathy: Modeling using machine learning. *JMIR Med Inform.* 2022;10(2):e30483.
- ⁴⁵ScienMag. Cardiovascular treatments could reach patients faster with new Clemson University research. Published 30 April 2018. Accessed 27 October 2022. <https://scienmag.com/cardiovascular-treatments-could-reach-patients-faster-with-new-clemson-university-research>.
- ⁴⁶Passini E, Britton OJ, Lu HR, et al. Human *in silico* drug trials demonstrate higher accuracy than animal models in predicting clinical pro-arrhythmic cardiotoxicity. *Front Physiol.* 2017;8:668.
- ⁴⁷Chandrasekera PC, Pippin JJ. Of rodents and men: Species-specific glucose regulation and type 2 diabetes research. *ALTEX.* 2014;31(2):157-176.
- ⁴⁸Bunner AE, Chandrasekera PC, Barnard ND. Knockout mouse models of insulin signaling: Relevance past and future. *World J Diabetes.* 2014;5(2):146-159.
- ⁴⁹Mir-Coll J, Moede T, Paschen M, et al. Human islet microtissues as an *in vitro* and an *in vivo* model system for diabetes. *Int J Mol Sci.* 2021;22(4):1813.
- ⁵⁰Wang B, Chandrasekera PC, Pippin JJ. Leptin- and leptin receptor-deficient rodent models: Relevance for human type 2 diabetes. *Curr Diabetes Rev.* 2014;10(2):131-145.
- ⁵¹Ali Z, Chandrasekera PC, Pippin JJ. Animal research for type 2 diabetes mellitus, its limited translation for clinical benefit, and the way forward. *Altern Lab Anim.* 2018;46(1):1-10.
- ⁵²Physicians Committee for Responsible Medicine. Using skin cells to model diabetes in humans. Published 20 November 2017. Accessed 28 October 2022. <https://www.pcrm.org/news/ethical-science/using-skin-cells-model-diabetes-humans>.
- ⁵³Kovatchev BP, Breton M, Man CD, Cobelli C. *In silico* preclinical trials: A proof of concept in closed-loop control of type 1 diabetes. *J Diabetes Sci Technol.* 2009;3(1):44-55.
- ⁵⁴Riyaphan J, Pham DC, Leong MK, Weng CF. *In silico* approaches to identify polyphenol compounds as α -glucosidase and α -amylase inhibitors against type-II diabetes. *Biomolecules.* 2021;11(12):1877.
- ⁵⁵Moinul M, Amin SA, Kumar P, et al. Exploring sodium glucose cotransporter (SGLT2) inhibitors with machine learning approach: A novel hope in anti-diabetes drug discovery. *J Mol Graph Model.* 2022;111:108106.
- ⁵⁶Glieberman AL, Pope BD, Zimmerman JF, et al. Synchronized stimulation and continuous insulin sensing in a microfluidic human islet on a chip designed for scalable manufacturing. *Lab Chip.* 2019;19(18):2993-3010.
- ⁵⁷Tao T, Wang Y, Chen W, et al. Engineering human islet organoids from iPSCs using an organ-on-chip platform. *Lab Chip.* 2019;19(6):948-958.
- ⁵⁸Sokolowska P, Zukowski K, Janikiewicz J, Jastrzebska E, Dobrzyn A, Brzozka Z. Islet-on-a-chip: Biomimetic micropillar-based microfluidic system for three-dimensional pancreatic islet cell culture. *Biosens Bioelectron.* 2021;183:113215.



- ⁵⁹Antony JM, MacDonald KS. A critical analysis of the cynomolgus macaque, *Macaca fascicularis*, as a model to test HIV-1/SIV vaccine efficacy. *Vaccine*. 2015;33(27):3073-3083.
- ⁶⁰Centlivre M, Combadière B. New challenges in modern vaccinology. *BMC Immunol*. 2015;16:18.
- ⁶¹Haigwood NL. Update on animal models for HIV research. *Eur J Immunol*. 2009;39(8):1994-1999.
- ⁶²Jülg B, Barouch DH. Novel immunological strategies for HIV-1 eradication. *J Virus Erad*. 2015;1(4):232-236.
- ⁶³Girard M, Habel A, Chaneil C. New prospects for the development of a vaccine against human immunodeficiency virus type 1. An overview. *C R Acad Sci III*. 1999;322(11):959-966.
- ⁶⁴Hu SL. Non-human primate models for AIDS vaccine research. *Curr Drug Targets Infect Disord*. 2005;5(2):193-201.
- ⁶⁵National Institute of Allergy and Infectious Diseases. History of HIV vaccine research. Updated 22 October 2018. Accessed 8 February 2022. <https://www.niaid.nih.gov/diseases-conditions/hiv-vaccine-research-history>.
- ⁶⁶Sekaly RP. The failed HIV Merck vaccine study: A step back or a launching point for future vaccine development? *J Exp Med*. 2008;205(1):7-12.
- ⁶⁷Cohen J. 'It's sobering': A once-exciting HIV cure strategy fails its test in people. Published 25 July 2018. Accessed 7 February 2022. <https://www.science.org/content/article/it-s-sobering-once-exciting-hiv-cure-strategy-fails-its-test-people>.
- ⁶⁸O'Dell R. Sickness and death at Mesa-area monkey farm threaten primate center viability. *azcentral.com*. Published 5 October 2021. Accessed 2 March 2022. <https://www.peta.org/wp-content/uploads/2021/10/2021-10-04-Sickness-and-death-at-Mesa-area-monkey-farm-threaten-primate-center-viability.pdf>.
- ⁶⁹Kumar N, Chahroudi A, Silvestri G. Animal models to achieve an HIV cure. *Curr Opin HIV AIDS*. 2016;11(4):432-441.
- ⁷⁰Matthews H, Hanison J, Nirmalan N. "Omics"-informed drug and biomarker discovery: Opportunities, challenges and future perspectives. *Proteomes*. 2016;4(3):28.
- ⁷¹Rao M, Alving CR. Adjuvants for HIV vaccines. *Curr Opin HIV AIDS*. 2016;11(6):585-592.
- ⁷²Galperin M, Farenc C, Mukhopadhyay M, et al. CD4+ T cell-mediated HLA class II cross-restriction in HIV controllers. *Sci Immunol*. 2018;3(24):eaat0687.
- ⁷³Deeks HM, Walters RK, Hare SR, O'Connor MB, Mulholland AJ, Glowacki DR. Interactive molecular dynamics in virtual reality for accurate flexible protein-ligand docking. *PLoS One*. 2020;15(3):e0228461.
- ⁷⁴Saha I, Saffarian S. Dynamics of the HIV gag lattice detected by localization correlation analysis and time-lapse iPALM. *Biophys J*. 2020;119(3):581-592.
- ⁷⁵Xie G, Luo X, Ma T, et al. Characterization of HIV-induced remodeling reveals differences in infection susceptibility of memory CD4+ T cell subsets *in vivo*. *Cell Rep*. 2021;35(4):109038.
- ⁷⁶Ledford H. Translational research: The full cycle. *Nature*. 2008;453(7197):843-845.
- ⁷⁷Tonks A. Quest for the AIDS vaccine. *The BMJ*. 2007;334:1346-1348.
- ⁷⁸Mestas J, Hughes CCW. Of mice and not men: Differences between mouse and human immunology. *J Immunol*. 2004;172(5):2731-2738.
- ⁷⁹Zschaler J, Schlorke D, Arrhhold J. Difference in innate immune response between man and mouse. *Crit Rev Immunol*. 2014;34(5):433-454.
- ⁸⁰Leist M, Hartung T. Inflammatory findings on species extrapolations: Humans are definitely no 70-kg mice. *Arch Toxicol*. 2013;87(4):563-567.
- ⁸¹Bouvier NM, Lowen AC. Animal models for influenza virus pathogenesis and transmission. *Viruses*. 2010;2(8):1530-1563.
- ⁸²Staeheli P, Grob R, Meier E, Sutcliffe JG, Haller O. Influenza virus-susceptible mice carry Mx genes with a large deletion or a nonsense mutation. *Mol Cell Biol*. 1988;8(10):4518-4523.
- ⁸³Tumpey TM, Szretter KJ, Van Hoeven N, et al. The Mx1 gene protects mice against the pandemic 1918 and highly lethal human H5N1 influenza viruses. *J Virol*. 2007;81(19):10818-10821.
- ⁸⁴Ibricevic A, Pekosz A, Walter MJ, et al. Influenza virus receptor specificity and cell tropism in mouse and human airway epithelial cells. *J Virol*. 2006;80(15):7469-7480.
- ⁸⁵Majde JA, Bohnet SG, Ellis GA, et al. Detection of mouse-adapted human influenza virus in the olfactory bulbs of mice within hours after intranasal infection. *J Neurovirol*. 2007;13(5):399-409.
- ⁸⁶Lowen AC, Mubareka S, Tumpey TM, García-Sastre A, Palese P. The guinea pig as a transmission model for human influenza viruses. *Proc Natl Acad Sci U S A*. 2006;103(26):9988-9992.
- ⁸⁷Wu HJ, Wu E. The role of gut microbiota in immune homeostasis and autoimmunity. *Gut Microbes*. 2012;3(1):4-14.
- ⁸⁸Nguyen TLA, Vieira-Silva S, Liston A, Raes J. How informative is the mouse for human gut microbiota research? *Dis Model Mech*. 2015;8(1):1-16.
- ⁸⁹Cappuccino A, Tieri P, Castiglione F. Multiscale modeling in immunology: A review. *Brief Bioinform*. 2016;17(3):408-418.
- ⁹⁰Brown JA, Codreanu SG, Shi M, et al. Metabolic consequences of inflammatory disruption of the blood-brain barrier in an organ-on-chip model of the human neurovascular unit. *J Neuroinflammation*. 2016;13(1):306.
- ⁹¹Ehling P, Meuth P, Eichinger P, et al. Human T cells *in silico*: Modelling their electrophysiological behaviour in health and disease. *J Theor Biol*. 2016;404:236-250.



- ⁹²Day JD, Metes DM, Vodovotz Y. Mathematical modeling of early cellular innate and adaptive immune responses to ischemia/reperfusion injury and solid organ allotransplantation. *Front Immunol*. 2015;6:484.
- ⁹³Bergers LIJC, Reijnders CMA, van den Broek LJ, et al. Immune-competent human skin disease models. *Drug Discov Today*. 2016;21(9):1479-1488.
- ⁹⁴Rudd KE, Johnson SC, Agesa KM, et al. Global, regional, and national sepsis incidence and mortality, 1990-2017: Analysis for the Global Burden of Disease Study. *Lancet*. 2020;395(10219):200-211.
- ⁹⁵Liu V, Escobar GJ, Greene JD, et al. Hospital deaths in patients with sepsis from 2 independent cohorts. *JAMA*. 2014;312(1):90-92.
- ⁹⁶Torio CM, Moore BJ. National Inpatient Hospital Costs: The Most Expensive Conditions by Payer, 2013. In: *Healthcare Cost and Utilization Project (HCUP) Statistical Briefs*. Rockville (MD): Agency for Healthcare Research and Quality (US); May 2016.
- ⁹⁷Verma S. Laboratory animal models to mimic human sepsis: A review. *JZS*. 2016;4(2):34-39.
- ⁹⁸Seok J, Warren HS, Cuenca AG, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A*. 2013;110(9):3507-3512.
- ⁹⁹Collins F. Of mice, men, and medicine. NIH. Published 19 February 2013. Accessed 31 October 2022. <https://directorsblog.nih.gov/2013/02/19/of-mice-men-and-medicine>.
- ¹⁰⁰Esmon CT. Why do animal models (sometimes) fail to mimic human sepsis? *Crit Care Med*. 2004;32(5):S219-S222.
- ¹⁰¹Rittirsch D, Hoesel LM, Ward PA. The disconnect between animal models of sepsis and human sepsis. *J Leukoc Biol*. 2007;81(1):137-143.
- ¹⁰²Buras JA, Holzmänn B, Sitkovsky M. Animal models of sepsis: Setting the stage. *Nat Rev Drug Discov*. 2005;4(10):854-865.
- ¹⁰³Nemzek JA, Hugunin KM, Opp MR. Modeling sepsis in the laboratory: Merging sound science with animal wellbeing. *Comp Med*. 2008;58(2):120-128.
- ¹⁰⁴Ruiz S, Vardon-Bouines F, Merlet-Dupuy V, et al. Sepsis modeling in mice: Ligation length is a major severity factor in cecal ligation and puncture. *Intensive Care Med Exp*. 2016;4(1):22.
- ¹⁰⁵Redl H, Bahrami S. Large animal models: Baboons for trauma, shock, and sepsis studies. *Shock*. 2005;24(S1):88-93.
- ¹⁰⁶Fink MP. Animal models of sepsis. *Virulence*. 2014;5(1):143-153.
- ¹⁰⁷Hawash MBF, Sanz-Remón J, Grenier JC, et al. Primate innate immune responses to bacterial and viral pathogens reveals an evolutionary trade-off between strength and specificity. *Proc Natl Acad Sci U S A*. 2021;118(13):e2015855118.
- ¹⁰⁸NAGMSC. NAGMSC Working Group on Sepsis final report. Published 17 May 2019. Accessed 9 February 2022. <https://www.nigms.nih.gov/News/reports/Documents/nagmsc-working-group-on-sepsis-final-report.pdf>.
- ¹⁰⁹National Institute of General Medical Sciences. Notice of information: NIGMS priorities for sepsis research. Published 29 July 2019. Accessed 9 February 2022. <https://grants.nih.gov/grants/guide/notice-files/NOT-GM-19-054.html>.
- ¹¹⁰Lilley E, Armstrong R, Clark N, et al. Refinement of animal models of sepsis and septic shock. *Shock*. 2015;43(4):304-316.
- ¹¹¹Jeon H, Lee DH, Jundi B, et al. Fully automated, sample-to-answer leukocyte functional assessment platform for continuous sepsis monitoring via microliters of blood. *ACS Sens*. 2021;6(7):2747-2756.
- ¹¹²Blaurock-Möller N, Gröger M, Siwczak F, et al. CAAP48, a new sepsis biomarker, induces hepatic dysfunction in an *in vitro* liver-on-chip model. *Front Immunol*. 2019;10:273.
- ¹¹³Allen A, Deshmukh H. All on "CHIP": Using microfluidics to study neutrophil ontogeny. *Transl Res*. 2017;190:1-3.
- ¹¹⁴Marik PE, Farkas JD. The changing paradigm of sepsis: Early diagnosis, early antibiotics, early pressors, and early adjuvant treatment. *Crit Care Med*. 2018;46(10):1690-1692.
- ¹¹⁵Goh KH, Wang L, Yeow AYK, et al. Artificial intelligence in sepsis early prediction and diagnosis using unstructured data in healthcare. *Nat Commun*. 2021;12(1):711.
- ¹¹⁶Giacobbe DR, Signori A, Del Puente F, et al. Early detection of sepsis with machine learning techniques: A brief clinical perspective. *Front Med (Lausanne)*. 2021;8:617486.
- ¹¹⁷Rosnati M, Fortuin V. MGP-AttTCN: An interpretable machine learning model for the prediction of sepsis. *PLoS One*. 2021;16(5):e0251248.
- ¹¹⁸Akhtar AZ, Pippin JJ, Sandusky CB. Animal models in spinal cord injury: A review. *Rev Neurosci*. 2008;19(1):47-60.
- ¹¹⁹Angius D, Wang H, Spinner RJ, Gutierrez-Cotto Y, Yaszemski MJ, Windebank AJ. A systematic review of animal models used to study nerve regeneration in tissue-engineered scaffolds. *Biomaterials*. 2012;33(32):8034-8039.
- ¹²⁰Akhtar AZ, Pippin JJ, Sandusky CB. Animal studies in spinal cord injury: A systematic review of methylprednisolone. *Altern Lab Anim*. 2009;37(1):43-62.
- ¹²¹Kaplan HM, Mishra P, Kohn J. The overwhelming use of rat models in nerve regeneration research may compromise designs of nerve guidance conduits for humans. *J Mater Sci Mater Med*. 2015;26(8):226.
- ¹²²Mobini S, Song YH, McCrary MW, Schmidt CE. Advances in *ex vivo* models and lab-on-a-chip devices for neural tissue engineering. *Biomaterials*. 2019;198:146-166.
- ¹²³Zhuang P, Sun AX, An J, Chua CK, Chew SY. 3D neural tissue models: From spheroids to bioprinting. *Biomaterials*. 2018;154:113-133.
- ¹²⁴Shrirao AB, Kung FH, Omelchenko A, et al. Microfluidic platforms for the study of neuronal injury *in vitro*. *Biotechnol Bioeng*. 2018;115(4):815-830.



- ¹²⁵Spijkers XM, Pasteuning-Vuhman S, Dorleijn JC, Vulto P, Wevers NR, Pasterkamp RJ. A directional 3D neurite outgrowth model for studying motor axon biology and disease. *Sci Rep*. 2021;11(1):2080.
- ¹²⁶Ramirez S, Mukherjee A, Sepulveda S, et al. Modeling traumatic brain injury in human cerebral organoids. *Cells*. 2021;10(10):2683.
- ¹²⁷Ramirez S, Mukherjee A, Sepulveda SE, et al. Protocol for controlled cortical impact in human cerebral organoids to model traumatic brain injury. *STAR Protoc*. 2021;2(4):100987.
- ¹²⁸Potashkin JA, Blume SR, Runkle NK. Limitations of animal models of Parkinson's disease. *Parkinsons Dis*. 2010;2011:1-7.
- ¹²⁹Cummings JL, Morstorf T, Zhong K. Alzheimer's disease drug-development pipeline: Few candidates, frequent failures. *Alzheimers Res Ther*. 2014;6(4):37.
- ¹³⁰AstraZeneca. Update on Phase III clinical trials of lanabecestat for Alzheimer's disease. Published 12 June 2018. Accessed 2 November 2022. <https://www.astrazeneca.com/media-centre/press-releases/2018/update-on-phase-iii-clinical-trials-of-lanabecestat-for-alzheimers-disease-12062018.html#>.
- ¹³¹Burns TC, Li MD, Mehta S, Awad AJ, Morgan AA. Mouse models rarely mimic the transcriptome of human neurodegenerative diseases: A systematic bioinformatics-based critique of preclinical models. *Eur J Pharmacol*. 2015;759:101-117.
- ¹³²Lane E, Dunnett S. Animal models of Parkinson's disease and L-dopa induced dyskinesia: How close are we to the clinic? *Psychopharmacology (Berl)*. 2008;199(3):303-312.
- ¹³³Ehrnhoefer DE, Butland SL, Pouladi MA, Hayden MR. Mouse models of Huntington disease: Variations on a theme. *Dis Model Mech*. 2009;2(3-4):123-129.
- ¹³⁴Benatar M. Lost in translation: Treatment trials in the SOD1 mouse and in human ALS. *Neurobiol Dis*. 2007;26(1):1-13.
- ¹³⁵Clerc P, Lipnick S, Willett C. A look into the future of ALS research. *Drug Discov Today*. 2016;21(6):939-949.
- ¹³⁶Menache A, Beuter A. Commentary: Lessons from the analysis of non-human primates for understanding human aging and neurodegenerative diseases. *Front Hum Neurosci*. 2016;10:33.
- ¹³⁷Olsson IA, Hansen AK, Sandøe P. Animal welfare and the refinement of neuroscience research methods – a case study of Huntington's disease models. *Lab Anim*. 2008;42(3):277-283.
- ¹³⁸Pistollato F, Ohayon EL, Lam A, et al. Alzheimer disease research in the 21st century: Past and current failures, new perspectives and funding opportunities. *Oncotarget*. 2016;7(26):38999-39016.
- ¹³⁹Mirbaha H, Chen D, Morazova OA, et al. Inert and seed-competent tau monomers suggest structural origins of aggregation. *Elife*. 2018;7:e36584.
- ¹⁴⁰Gao Y, Liu J, Wang J, et al. Proteomic analysis of human hippocampal subfields provides new insights into the pathogenesis of Alzheimer's disease and the role of glial cells. *Brain Pathol*. 2022;e13047.
- ¹⁴¹Cope TE, Rittman T, Borchert RJ, et al. Tau burden and the functional connectome in Alzheimer's disease and progressive supranuclear palsy. *Brain*. 2018;141(2):550-567.
- ¹⁴²Ochalek A, Mihalik B, Avci HX, et al. Neurons derived from sporadic Alzheimer's disease iPSCs reveal elevated TAU hyperphosphorylation, increased amyloid levels, and GSK3B activation. *Alzheimers Res Ther*. 2017;9(1):90.
- ¹⁴³Bereczki E, Branca RM, Francis PT, et al. Synaptic markers of cognitive decline in neurodegenerative diseases: A proteomic approach. *Brain*. 2018;141(2):582-595.
- ¹⁴⁴Sultzer DL, Lim AC, Gordon HL, Yarns BC, Melrose RJ. Cholinergic receptor binding in unimpaired older adults, mild cognitive impairment, and Alzheimer's disease dementia. *Alzheimers Res Ther*. 2022;14(1):25.
- ¹⁴⁵Santhanam N, Kumanchik L, Guo X, et al. Stem cell derived phenotypic human neuromuscular junction model for dose response evaluation of therapeutics. *Biomaterials*. 2018;166:64-78.
- ¹⁴⁶Dauth S, Maoz BM, Sheehy SP, et al. Neurons derived from different brain regions are inherently different *in vitro*: A novel multiregional brain-on-a-chip. *J Neurophysiol*. 2017;117(3):1320-1341.
- ¹⁴⁷Soscia D, Belle A, Fischer N, et al. Controlled placement of multiple CNS cell populations to create complex neuronal cultures. *PLoS One*. 2017;12(11):e0188146.
- ¹⁴⁸Kim H, Park HJ, Choi H, et al. Modeling G2019S-LRRK2 Sporadic Parkinson's Disease in 3D Midbrain Organoids. *Stem Cell Reports*. 2019;12(3):518-531.
- ¹⁴⁹Nestler EJ, Hyman SE. Animal models of neuropsychiatric disease. *Nat Neurosci*. 2010;13(10):1161-1169.
- ¹⁵⁰Molendijk ML, de Kloet ER. Immobility in the forced swim test is adaptive and does not reflect depression. *Psychoneuroendocrinology*. 2015;62:389-391.
- ¹⁵¹De Pablo JM, Parra A, Segovia S, Guillamón A. Learned immobility explains the behavior of rats in the forced swimming test. *Physiol Behav*. 1989;46(2):229-237.
- ¹⁵²Jefferys D, Funder J. The effect of water temperature on immobility in the forced swimming test in rats. *Eur J Pharmacol*. 1994;253(1-2):91-94.
- ¹⁵³Lucki I, Dalvi A, Mayorga AJ. Sensitivity to the effects of pharmacologically selective antidepressants in different strains of mice. *Psychopharmacology (Berl)*. 2001;155(3):315-322.
- ¹⁵⁴Trunnell ER, Carvalho C. The forced swim test has poor accuracy for identifying novel antidepressants. *Drug Discov Today*. 2021;26(12):2898-2904.
- ¹⁵⁵Carvalho C, Varela SAM, Marques TA, Knight A, Vicente L. Are *in vitro* and *in silico* approaches used appropriately for animal-based major depressive disorder research? *PLoS One*. 2020;15(6):e0233954.



- ¹⁵⁶Carvalho C, Peste F, Marques TA, Knight A, Vicente LM. The contribution of rat studies to current knowledge of major depressive disorder: Results from citation analysis. *Front Psychol*. 2020;11:1486.
- ¹⁵⁷Carvalho C, Herrmann K, Marques TA, Knight A. Time to abolish the forced swim test in rats for depression research? *J Appl Anim Ethics Res*. 2021;1-9.
- ¹⁵⁸Kato T, Kasahara T, Kubota-Sakashita M, Kato TM, Nakajima K. Animal models of recurrent or bipolar depression. *Neuroscience*. 2016;321:189-196.
- ¹⁵⁹Garner JP. The significance of meaning: Why do over 90% of behavioral neuroscience results fail to translate to humans, and what can we do to fix it? *ILAR J*. 2014;55(3):438-456.
- ¹⁶⁰Jin H, Romano G, Marshall C, Donaldson AE, Suon S, Iacovitti L. Tyrosine hydroxylase gene regulation in human neuronal progenitor cells does not depend on Nurr1 as in the murine and rat systems. *J Cell Physiol*. 2006;207(1):49-57.
- ¹⁶¹Hodge RD, Bakken TE, Miller JA, et al. Conserved cell types with divergent features in human versus mouse cortex. *Nature*. 2019;573(7772):61-68.
- ¹⁶²van der Staay FJ, Arndt SS, Nordquist RE. Evaluation of animal models of neurobehavioral disorders. *Behav Brain Funct*. 2009;5:11.
- ¹⁶³Siekmeier PJ. Computational modeling of psychiatric illnesses via well-defined neurophysiological and neurocognitive biomarkers. *Neurosci Biobehav Rev*. 2015;57:365-380.
- ¹⁶⁴Haggarty SJ, Silva MC, Cross A, Brandon NJ, Perlis RH. Advancing drug discovery for neuropsychiatric disorders using patient-specific stem cell models. *Mol Cell Neurosci*. 2016;73:104-115.
- ¹⁶⁵Adegbola A, Bury LA, Fu C, Zhang M, Wynshaw-Boris A. Concise review: Induced pluripotent stem cell models for neuropsychiatric diseases. *Stem Cells Transl Med*. 2017;6(12):2062-2070.
- ¹⁶⁶Zhong X, Harris G, Smirnova L, et al. Antidepressant paroxetine exerts developmental neurotoxicity in an iPSC-derived 3D human brain model. *Front Cell Neurosci*. 2020;14:25.
- ¹⁶⁷Urresti J, Zhang P, Moran-Losada P, et al. Correction: Cortical organoids model early brain development disrupted by 16p11.2 copy number variants in autism. *Mol Psychiatry*. 2021;26(12):7581.
- ¹⁶⁸Dattaro L. Protein inhibitor normalizes neuronal migration in organoid model of autism. SpectrumNews.org. Published 1 September 2021. Accessed 8 February 2022. <https://www.spectrumnews.org/news/protein-inhibitor-normalizes-neuronal-migration-in-organoid-model-of-autism>.
- ¹⁶⁹Provenza NR, Sheth SA, Dastin-van Rijn EM, et al. Long-term ecological assessment of intracranial electrophysiology synchronized to behavioral markers in obsessive-compulsive disorder. *Nat Med*. 2021;27(12):2154-2164.
- ¹⁷⁰Yassin W, Nakatani H, Zhu Y, et al. Machine-learning classification using neuroimaging data in schizophrenia, autism, ultra-high risk and first-episode psychosis. *Transl Psychiatry*. 2020;10(1):278.
- ¹⁷¹Kalmady SV, Paul AK, Greiner R, et al. Extending schizophrenia diagnostic model to predict schizotypy in first-degree relatives. *NPJ Schizophr*. 2020;6(1):30.
- ¹⁷²Roth S, Liesz A. Stroke research at the crossroads – where are we heading? *Swiss Med Wkly*. 2016;146:w14329.
- ¹⁷³Sutherland BA, Minnerup J, Balami JS, Arba F, Buchan AM, Kleinschnitz C. Neuroprotection for ischemic stroke: Translation from the bench to the bedside. *Int J Stroke*. 2012;7(5):407-418.
- ¹⁷⁴Sommer CJ. Ischemic stroke: Experimental models and reality. *Acta Neuropathol*. 2017;133(2):245-261.
- ¹⁷⁵Krafft PR, Bailey EL, Lekic T, et al. Etiology of stroke and choice of models. *Int J Stroke*. 2012;7(5):398-406.
- ¹⁷⁶Chen Z, Mou R, Feng D, Wang Z, Chen G. The role of nitric oxide in stroke. *Med Gas Res*. 2017;7(3):194-203.
- ¹⁷⁷Lin S, Lin Y, Nery JR, et al. Comparison of the transcriptional landscapes between human and mouse tissues. *Proc Natl Acad Sci U S A*. 2014;111(48):17224-17229.
- ¹⁷⁸Holloway PM, Gavins FN. Modeling ischemic stroke *in vitro*: The status quo and future perspectives. *Stroke*. 2016;47(2):561-569.
- ¹⁷⁹Werth JL, Park TS, Silbergeld DL, Rothman SM. Excitotoxic swelling occurs in oxygen and glucose deprived human cortical slices. *Brain Res*. 1998;782(1-2):248-254.
- ¹⁸⁰Wiesmayer P. “Mini-brains” to replace mouse model in stroke research. InnovationOrigins.com. Published 21 July 2021. Accessed 9 February 2022. <https://innovationorigins.com/en/mini-brains-to-replace-mouse-model-in-stroke-research>.
- ¹⁸¹Nzou G, Wicks RT, VanOstrand NR, et al. Author Correction: Multicellular 3D neurovascular unit model for assessing hypoxia and neuroinflammation induced blood-brain barrier dysfunction. *Sci Rep*. 2020;10(1):20384.
- ¹⁸²Lyu Z, Park J, Kim KM, et al. A neurovascular-unit-on-a-chip for the evaluation of the restorative potential of stem cell therapies for ischaemic stroke. *Nat Biomed Eng*. 2021;5(8):847-863.
- ¹⁸³Wevers NR, Nair AL, Fowke TM, et al. Modeling ischemic stroke in a triculture neurovascular unit on-a-chip. *Fluids Barriers CNS*. 2021;18(1):59.
- ¹⁸⁴Miller C, Padmos RM, van der Kolk M, et al. *In silico* trials for treatment of acute ischemic stroke: Design and implementation. *Comput Biol Med*. 2021;137:104802.
- ¹⁸⁵Guo Y. A new paradigm of “real-time” stroke risk prediction and integrated care management in the digital health era: Innovations using machine learning and artificial intelligence approaches. *Thromb Haemost*. 2022;122(1):5-7.



- ¹⁸⁶Matsoukas S, Morey J, Lock G, et al. AI software detection of large vessel occlusion stroke on CT angiography: A real-world prospective diagnostic test accuracy study. *J Neurointerv Surg*. 2022;neurintsurg-2021-018391.
- ¹⁸⁷Gunda B, Neuhaus A, Sipos I, et al. Improved stroke care in a primary stroke centre using AI-decision support. *Cerebrovasc Dis Extra*. 2022;10.1159/000522423.
- ¹⁸⁸Bosetti F, Koenig JI, Ayata C, et al. Translational stroke research: Visions and opportunities. *Stroke*. 2017;48(9):2632-2637.
- ¹⁸⁹Mozaffarian D, Benjamin EJ, Go AS, et al. Heart disease and stroke statistics – 2016 update: A report from the American Heart Association. *Circulation*. 2016;133(4):e38-e360.
- ¹⁹⁰Tzschentke TM. Where do we stand in the field of anti-abuse drug discovery? *Expert Opin Drug Dis*. 2014;9(11):1255-1258.
- ¹⁹¹Stephens DN, Crombag HS, Duka T. The challenge of studying parallel behaviors in humans and animal models. *Curr Top Behav Neurosci*. 2013;13:611-45.
- ¹⁹²Green AR, King MV, Shortall SE, Fone KC. Lost in translation: Preclinical studies on 3,4-methylenedioxymethamphetamine provide information on mechanisms of action, but do not allow accurate prediction of adverse events in humans. *Br J Pharmacol*. 2012;166(5):1523-1536.
- ¹⁹³Ahmed SH. Validation crisis in animal models of drug addiction: Beyond non-disordered drug use toward drug addiction. *Neurosci Biobehav Rev*. 2010;35(2):172-184.
- ¹⁹⁴Ramsden E. Making animals alcoholic: Shifting laboratory models of addiction. *J Hist Behav Sci*. 2015;51(2):164-194.
- ¹⁹⁵Hyman SE, Malenka RC. Addiction and the brain: The neurobiology of compulsion and its persistence. *Nat Rev Neurosci*. 2001;2(10):695-703.
- ¹⁹⁶Scarnati MS, Halikere A, Pang ZP. Using human stem cells as a model system to understand the neural mechanisms of alcohol use disorders: Current status and outlook. *Alcohol*. 2019;74:83-93.
- ¹⁹⁷Lieberman R, Kranzler HR, Levine ES, Covault J. Examining the effects of alcohol on GABAA receptor mRNA expression and function in neural cultures generated from control and alcohol dependent donor induced pluripotent stem cells. *Alcohol*. 2018;66:45-53.
- ¹⁹⁸De Filippis L, Halikere A, McGowan H, et al. Ethanol-mediated activation of the NLRP3 inflammasome in iPS cells and iPS cells-derived neural progenitor cells. *Mol Brain*. 2016;9(1):51.
- ¹⁹⁹Lee CT, Chen J, Kindberg AA, et al. CYP3A5 mediates effects of cocaine on human neocortigenesis: Studies using an in vitro 3D self-organized hPSC model with a single cortex-like unit. *Neuropsychopharmacology*. 2017;42(3):774-784.
- ²⁰⁰Arzua T, Yan Y, Jiang C, et al. Modeling alcohol-induced neurotoxicity using human induced pluripotent stem cell-derived three-dimensional cerebral organoids. *Transl Psychiatry*. 2020;10(1):347.
- ²⁰¹Tian L, Prasad N, Jang YY. In vitro modeling of alcohol-induced liver injury using human-induced pluripotent stem cells. *Methods Mol Biol*. 2016;1353:271-283.
- ²⁰²Hildebrand F, Andruszkow H, Huber-Lang M, Pape HC, van Griensven M. Combined hemorrhage/trauma models in pigs – current state and future perspectives. *Shock*. 2013;40(4):247-273.
- ²⁰³Staudlbauer KH, Wagner-Berger HG, Raedler C, et al. Vasopressin, but not fluid resuscitation, enhances survival in a liver trauma model with uncontrolled and otherwise lethal hemorrhagic shock in pigs. *Anesthesiology*. 2003;98(3):699-704.
- ²⁰⁴Tsukamoto T, Pape HC. Animal models for trauma research: What are the options? *Shock*. 2009;31(1):3-10.
- ²⁰⁵Xiong Y, Mahmood A, Chopp M. Animal models of traumatic brain injury. *Nat Rev Neurosci*. 2013;14(2):128-142.
- ²⁰⁶Combes RD. A critical review of anaesthetised animal models and alternatives for military research, testing and training, with a focus on blast damage, haemorrhage, and resuscitation. *Altern Lab Anim*. 2013;41(5):385-415.
- ²⁰⁷Brown D, Namas RA, Almahmoud K, et al. Trauma *in silico*: Individual-specific mathematical models and virtual clinical populations. *Sci Transl Med*. 2015;7(285):285ra61.
- ²⁰⁸Zirardo C, Solovyev A, Allegretti A, et al. A computational, tissue-realistic model of pressure ulcer formation in individuals with spinal cord injury. *PLoS Comput Biol*. 2015;11(6):e1004309.
- ²⁰⁹Abboud A, Mi Q, Puccio A, et al. Inflammation following traumatic brain injury in humans: Insights from data-driven and mechanistic models into survival and death. *Front Pharmacol*. 2016;7:342.
- ²¹⁰Schiller AM, Howard JT, Convertino VA. The physiology of blood loss and shock: New insights from a human laboratory model of hemorrhage. *Exp Biol Med (Maywood)*. 2017;242(8):874-883.
- ²¹¹Ehrlich H, McKenney M, Elkbulli A. The niche of artificial intelligence in trauma and emergency medicine. *Am J Emerg Med*. 2021;45:669-670.
- ²¹²Laur O, Wang B. Musculoskeletal trauma and artificial intelligence: Current trends and projections. *Skeletal Radiol*. 2022;51(2):257-269.
- ²¹³Niggli C, Pape HC, Niggli P, Mica L. Validation of a visual-based analytics tool for outcome prediction in polytrauma patients (WATSON Trauma Pathway Explorer) and comparison with the predictive values of TRISS. *J Clin Med*. 2021;10(10):2115.
- ²¹⁴Diebel LN, Marinica AL, Edelman D, Liberati D. The effect of perturbations of the glycocalyx on microvascular perfusion in the obese trauma population: An *in vitro* study. *Trauma Surg Acute Care Open*. 2021;6(1):e000711.
- ²¹⁵Diebel LN, Wheaton M, Liberati DM. The protective role of estrogen on endothelial and glycocalyx barriers after shock conditions: A microfluidic study. *Surgery*. 2021;169(3):678-685.



- ²¹⁶Cattaneo C, Maderna E, Rendinelli A, Gibelli D. Animal experimentation in forensic sciences: How far have we come? *Forensic Sci Int*. 2015;254:e29-e35.
- ²¹⁷Knight B. Forensic science and animal rights. *Forensic Sci Int*. 1992;57(1):1-3.
- ²¹⁸Mole CG, Heyns M. Animal models in forensic science research: Justified use or ethical exploitation? *Sci Eng Ethics*. 2019;25(4):1095-1110.
- ²¹⁹Steadman DW. Multidisciplinary validation study of nonhuman animal models for forensic decomposition research. National Institute of Justice. Published March 2018. Accessed 18 February 2022. <https://nij.ojp.gov/library/publications/multidisciplinary-validation-study-nonhuman-animal-models-forensic>.
- ²²⁰Patronek GJ, Rauch A. Systematic review of comparative studies examining alternatives to the harmful use of animals in biomedical education. *J Am Vet Med Assoc*. 2007;230(1):37-43.
- ²²¹Goodman JR, Borch CA, Cherry E. Mounting opposition to vivisection. *Contexts*. 2012;11(2):68-69.
- ²²²Reznick RK, MacRae H. Teaching surgical skills – changes in the wind. *N Engl J Med*. 2006;355(25):2664-2669.
- ²²³Institute of Medicine. *To Err Is Human: Building a Safer Health System*. Washington, DC: The National Academies Press; 2000.
- ²²⁴Fears D. One last US medical school still killed animals to teach surgery. But no more. *The Washington Post*. Published 30 June 2016. Accessed 16 August 2018. <https://www.washingtonpost.com/news/animalia/wp/2016/06/30/one-last-u-s-medical-school-still-killed-animals-to-teach-surgery-but-no-more>.
- ²²⁵Herrmann K, Pawlowski J, Feinstein D, Crandall M, Gala S. Modernizing biomedical training: Replacing live animal laboratories with human simulation. In: Herrmann K, Jayne K, eds. *Animal Experimentation: Working Towards a Paradigm Change*. Brill; 2019:551-566.
- ²²⁶Hansen LA. Animal laboratories are not needed to train medical students. *J Surg Educ*. 2014;71(4):454.
- ²²⁷Dua A. Letters to the editor. *Mil Med*. 2014;179(7):vii.
- ²²⁸Jin C, Dai L, Wang T. The application of virtual reality in the training of laparoscopic surgery: A systematic review and meta-analysis. *Int J Surg*. 2021;87:105859.
- ²²⁹Schmidt MW, Köppinger KF, Fan C, et al. Virtual reality simulation in robot-assisted surgery: Meta-analysis of skill transfer and predictability of skill. *BJS Open*. 2021;5(2):zraa066.
- ²³⁰Dromey BP, Peebles DM, Stoyanov DV. A systematic review and meta-analysis of the use of high-fidelity simulation in obstetric ultrasound. *Simul Healthc*. 2020;16(1):52-59.
- ²³¹Gaubert S, Blet A, Dib F, et al. Positive effects of lumbar puncture simulation training for medical students in clinical practice. *BMC Med Educ*. 2021;21(1):18.
- ²³²Hanada K, Hoshina K, Tsuyuki S, et al. Ten-hour simulation training improved the suturing performance of medical students. *Ann Vasc Surg*. 2022;S0890-5096(21)01054-2.
- ²³³Sparks D, Kavanagh KR, Vargas JA, Valdez TA. 3D printed myringotomy and tube simulation as an introduction to otolaryngology for medical students. *Int J Pediatr Otorhinolaryngol*. 2020;128:109730.
- ²³⁴Grober ED, Hamstra SJ, Wanzel KR, et al. The educational impact of bench model fidelity on the acquisition of technical skill: The use of clinically relevant outcome measures. *Ann Surg*. 2004;240(2):374-381.
- ²³⁵Ghanem AM, Hachach-Haram N, Leung CC, Myers SR. A systematic review of evidence for education and training interventions in microsurgery. *Arch Plast Surg*. 2013;40(4):312-319.
- ²³⁶Alser O, Youssef G, Myers S, Ghanem A. A novel three-in-one silicone model for basic microsurgery training. *Eur J Plast Surg*. 2020;43(5):621-626.
- ²³⁷SIU Medicine. Microsurgery. SiuMed.org. Accessed 1 March 2022. <https://www.siuMed.org/treatment/microsurgery.html>.
- ²³⁸Abi-Rafeh J, Zammit D, Mojtahed Jaber M, Al-Halabi B, Thibaudeau S. Nonbiological microsurgery simulators in plastic surgery training: A systematic review. *Plast Reconstr Surg*. 2019;144(3):496e-507e.
- ²³⁹Joseph FJ, Weber S, Raabe A, Bervini D. Neurosurgical simulator for training aneurysm microsurgery – a user suitability study involving neurosurgeons and residents. *Acta Neurochir (Wien)*. 2020;162(10):2313-2321.
- ²⁴⁰Steineke TC, Barbary D. Microsurgical clipping of middle cerebral artery aneurysms: Preoperative planning using virtual reality to reduce procedure time. *Neurosurg Focus*. 2021;51(2):E12.
- ²⁴¹Hall A, Riojas R, Sharon D. Comparison of self-efficacy and its improvement after artificial simulator or live animal model emergency procedure training. *Mil Med*. 2014;179(3):320-323.
- ²⁴²Hall A. Letters to the editor. *Mil Med*. 2014;179(7).
- ²⁴³Gala SG, Goodman JR, Murphy MP, Balsam MJ. Use of animals by NATO countries in military medical training exercises: An international survey. *Mil Med*. 2012;177(8):907-910.
- ²⁴⁴Seck H. Coast Guard puts permanent end to wounding animals for training. Military.com. Published 20 March 2018. Accessed 3 November 2022. <https://www.military.com/daily-news/2018/03/20/coast-guard-puts-permanent-end-wounding-animals-training.html>.
- ²⁴⁵The New York Times Editorial Board. Ban animal use in military medical training. *The New York Times*. Published 25 June 2016. Accessed 3 November 2022. <https://www.nytimes.com/2016/06/26/opinion/ban-animal-use-in-military-medical-training.html>.



²⁴⁶Rep. Hank Johnson. Leading medical groups endorse Johnson's military modernization bill. Published 27 June 2016. Accessed 3 November 2022.

<https://web.archive.org/web/20160907033912/https://hankjohnson.house.gov/media-center/press-releases/leading-medical-groups-endorse-johnson-s-military-modernization-bill>.

²⁴⁷Keller J, Hart D, Rule G, Bonnett T, Sweet R. The physiologic stress response of learners during critical care procedures: Live tissue vs. synthetic models. *Chest*. 2018;154(4):229A.

²⁴⁸Hoang TN, LaPorta AJ, Malone JD, et al. Hyper-realistic and immersive surgical simulation training environment will improve team performance. *Trauma Surg Acute Care Open*. 2020;5(1):e000393.

²⁴⁹Mackenzie CF, Tisherman SA, Shackelford S, Sevdalis N, Elster E, Bowyer MW. Efficacy of trauma surgery technical skills training courses. *J Surg Educ*. 2019;76(3):832-843.

²⁵⁰Rubeis G, Steger F. Is live-tissue training ethically justified? An evidence-based ethical analysis. *Altern Lab Anim*. 2018;46(2):65-71.

²⁵¹American College of Surgeons. Alternative models for the ATLS surgical skills practicum. Published 7 November 2001. Accessed 14 February 2022. <https://www.peta.org/wp-content/uploads/2022/02/ACS-ATLS-2001-alternatives-endorsement.pdf>.

²⁵²OECD. Integrated approaches to testing and assessment (IATA). Published 2021. Accessed 15 October 2021. <https://www.oecd.org/chemicalsafety/risk-assessment/iata-integrated-approaches-to-testing-and-assessment.htm>.

²⁵³OECD. Guideline No 497: Defined approaches for skin sensitisation. Published 14 June 2021. Accessed 25 August 2022. <https://www.oecd-ilibrary.org/docserver/b92879a4-en.pdf?expires=1623947885&id=id&accname=guest&checksum=8C4837C649125C05005B006E220193BD>.

²⁵⁴Ball N. Developing the scientific basis for Exposure Based Adaptations (EBA) – technical report no 137. European Centre for Ecotoxicology and Toxicology of Chemicals. Published 2 October 2020. Accessed 28 January 2022. <https://policycommons.net/artifacts/1662601/developing-the-scientific-basis-for-exposure-based-adaptations-eba-technical-report-no/2394251>.

²⁵⁵European Commission. Summary report on the statistics on the number of animals used for experimental and other scientific purposes in the member states of the European Union. Published 14 July 2021. Accessed 25 August 2022. https://ec.europa.eu/environment/chemicals/lab_animals/pdf/SWD_%20part_A_and_B.pdf.

²⁵⁶OECD. Test No 319A: Determination of *in vitro* intrinsic clearance using cryopreserved rainbow trout hepatocytes (RT-HEP). Published 27 June 2018. Accessed 25 August 2022. <https://www.oecd-ilibrary.org/docserver/9789264303218-en.pdf>.

²⁵⁷OECD. Test No 319B: Determination of *in vitro* intrinsic clearance using rainbow trout liver S9 sub-cellular fraction (RT-S9). Published 25 June 2018. Accessed 25 August 2022. <https://www.oecd-ilibrary.org/docserver/9789264303232-en.pdf>.

²⁵⁸OECD. Guidance document on the determination of *in vitro* intrinsic clearance using cryopreserved hepatocytes (RT-HEP) or liver S9 sub-cellular fractions (RT-S9) from rainbow trout and extrapolation to *in vivo* intrinsic clearance. Series on Testing & Assessment No 280. Published 6 July 2018. Accessed 25 August 2022. <https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cot e=ENV/JM/MONO%282018%2912&doclanguage=en>.

²⁵⁹OECD. Test No 305: Bioaccumulation in Fish: Aqueous and Dietary Exposure. Published 2 October 2012. Accessed 25 August 2022. <https://www.oecd-ilibrary.org/docserver/9789264185296-en.pdf>.

²⁶⁰OECD. Test No 236: Fish Embryo Acute Toxicity (FET) Test. Published 26 July 2013. Accessed 25 August 2022. <https://www.oecd-ilibrary.org/docserver/9789264203709-en.pdf>.

²⁶¹ECHA. Joint Report ECHA and UBA. Expert workshop on the potential regulatory application of the Fish Embryo Acute Toxicity (FET) Test under REACH, CLP and the BPR. 3–4 May 2017, Helsinki. Accessed 5 November 2022. https://echa.europa.eu/documents/10162/13630/fet_workshop_proceedings_en.pdf/a987ccab-5d4a-a226-2a73-994be484ca8d.

²⁶²Tanneberger K, Knöbel M, Busser FJM, Sinnige TL, Hermens JLM, Schirmer K. Predicting fish acute toxicity using a fish gill cell line-based toxicity assay. *Environ Sci Technol*. 2013;47(2):1110-1119.

²⁶³OECD. Test No 249: Fish cell line acute toxicity: The RTgill-W1 cell line assay. Published 14 June 2021. Accessed 25 August 2022. <https://www.oecd.org/chemicalsafety/test-no-249-fish-cell-line-acute-toxicity-the-rtgill-w1-cell-line-assay-c66d5190-en.htm>

²⁶⁴OECD. Test No 203: Fish, Acute Toxicity Test. Updated 18 June 2019. Accessed 25 August 2022. <https://www.oecd-ilibrary.org/docserver/9789264069961-en.pdf>.

²⁶⁵HUGIN SWiFT. Published 2020. Accessed 25 August 2022. <https://swift.hugin.com>.

²⁶⁶OECD. Guidance document on aqueous-phase aquatic toxicity testing of difficult test chemicals. Series on Testing & Assessment No 23. 2nd ed. Published 8 February 2019. Accessed 25 August 2022. <https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cot e=ENV/JM/MONO%282000%296/REV1&docLanguage=En>.

²⁶⁷Hilton GM, Odenkirchen E, Panger M, Waleko G, Lowit A, Clippinger AJ. Evaluation of the avian acute oral and sub-acute dietary toxicity test for pesticide registration. *Regul Toxicol Pharmacol*. 2019;105:30-35.



- ²⁶⁸US EPA Office of Pesticide Programs (OPP). Final guidance for waiving sub-acute avian dietary tests for pesticide registration and supporting retrospective analysis. Published February 2020. Accessed 25 August 2022. <https://www.epa.gov/sites/default/files/2020-02/documents/final-waiver-guidance-avian-sub-acute-dietary.pdf>.
- ²⁶⁹LaLone CA, Villeneuve DL, Lyons D, et al. Editor's highlight: Sequence alignment to predict across species susceptibility (SeqAPASS): A web-based tool for addressing the challenges of cross-species extrapolation of chemical toxicity. *Toxicol Sci*. 2016;153(2):228-245.
- ²⁷⁰Gore AC, Chappell VA, Fenton SE, et al. EDC-2: The Endocrine Society's second scientific statement on endocrine-disrupting chemicals. *Endocr Rev*. 2015;36(6):1-150.
- ²⁷¹La Merrill MA, Vandenberg LN, Smith MT, et al. Consensus on the key characteristics of endocrine-disrupting chemicals as a basis for hazard identification. *Nat Rev Endocrinol*. 2020;16(1):45-57.
- ²⁷²Kahn LG, Philippat C, Nakayama SF, Slama R, Trasande L. Endocrine-disrupting chemicals: Implications for human health. *Lancet Diabetes Endocrinol*. 2020;8(8):703-718.
- ²⁷³Iwanowicz LR, Blazer VS, Pinkney AE, et al. Evidence of estrogenic endocrine disruption in smallmouth and largemouth bass inhabiting Northeast US national wildlife refuge waters: A reconnaissance study. *Ecotoxicol Environ Saf*. 2016;124:50-59.
- ²⁷⁴Bókony V, Üveges B, Ujhegyi N, et al. Endocrine disruptors in breeding ponds and reproductive health of toads in agricultural, urban and natural landscapes. *Sci Total Environ*. 2018;634:1335-1345.
- ²⁷⁵AOP Wiki. Published 2021. Accessed 15 October 2021. <https://aopwiki.org>.
- ²⁷⁶OECD. Integrated Approaches to Testing and Assessment (IATA). 2021.
- ²⁷⁷Vandenberg LN, Welshons WV, Vom Saal FS, Toutain PL, Myers JP. Should oral gavage be abandoned in toxicity testing of endocrine disruptors? *Environ Health*. 2014;13(1):46.
- ²⁷⁸Moroni L, Barbaro F, Caiment F, et al. SCREENED: A multistage model of thyroid gland function for screening endocrine-disrupting chemicals in a biologically sex-specific manner. *Int J Mol Sci*. 2020;21(10):1-23.
- ²⁷⁹Browne P, Judson RS, Casey WM, Kleinstreuer NC, Thomas RS. Screening chemicals for estrogen receptor bioactivity using a computational model. *Environ Sci Technol*. 2015;49(14):8804-8814.
- ²⁸⁰Kleinstreuer NC, Ceger PC, Allen DG, et al. A curated database of rodent uterotrophic bioactivity. *Environ Health Perspect*. 2016;124(5):556-562.
- ²⁸¹Judson RS, Magpantay FM, Chickarmane V, et al. Integrated model of chemical perturbations of a biological pathway using 18 *in vitro* high-throughput screening assays for the estrogen receptor. *Toxicol Sci*. 2015;148(1):137-154.
- ²⁸²EPA. Use of high throughput assays and computational tools in the Endocrine Disruptor Screening Program. Updated 7 March 2022. Accessed 25 August 2022. <https://www.epa.gov/endocrine-disruption/use-high-throughput-assays-and-computational-tools-endocrine-disruptor>.
- ²⁸³Noyes PD, Friedman KP, Browne P, et al. Evaluating chemicals for thyroid disruption: Opportunities and challenges with *in vitro* testing and adverse outcome pathway approaches. *Environ Health Perspect*. 2019;127(9).
- ²⁸⁴Luechtefeld T, Maertens A, Russo DP, Rovida C, Zhu H, Hartung T. Analysis of Draize eye irritation testing and its prediction by mining publicly available 2008–2014 REACH data. *ALTEX*. 2016;33(2):123-134.
- ²⁸⁵OECD. Guidance document on considerations for waiving or bridging of mammalian acute toxicity tests. Series on Testing & Assessment No 237. Published 8 February 2019. Accessed 25 August 2022. <https://www.oecd.org/env/guidance-document-on-considerations-for-waiving-or-bridging-of-mammalian-acute-toxicity-tests-9789264274754-en.htm>.
- ²⁸⁶OECD. Guidance document on an integrated approach on testing and assessment (IATA) for serious eye damage and eye irritation. Series on Testing & Assessment No 263. 2nd ed. Published 25 July 2019. Accessed 25 August 2022. https://www.oecd-ilibrary.org/environment/second-edition-guidance-document-on-integrated-approaches-to-testing-and-assessment-iata-for-serious-eye-damage-and-eye-irritation_84b83321-en.
- ²⁸⁷EPA. Alternate testing framework for classification of eye irritation potential of EPA-regulated pesticide products. Updated 1 June 2022. Accessed 25 August 2022. <https://www.epa.gov/pesticide-registration/alternate-testing-framework-classification-eye-irritationpotential-epa>.
- ²⁸⁸Clippinger AJ, Raabe HA, Allen DG, et al. Human-relevant approaches to assess eye corrosion/irritation potential of agrochemical formulations. *Cutan Ocul Toxicol*. 2021;40(2):145-167.
- ²⁸⁹ECHA. Guidance on Information Requirements and Chemical Safety Assessment. Chapter R.7a: Endpoint specific guidance. Version 6. Published July 2017. doi:10.2823/337352.
- ²⁹⁰ECHA. Guidance on the Biocidal Products Regulation. Volume III: Human health, Part A: Information requirements. Version 1.2. Published May 2018. doi:10.2823/443383.



- ²⁹¹Regulation (EC) no 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. Published 24 November 2009. Accessed 25 August 2022. <https://eur-lex.europa.eu/eli/reg/2009/1107/oj>.
- ²⁹²ICH. ICH guideline S2 (R1) on genotoxicity testing and data interpretation for pharmaceuticals intended for human use. Updated February 2013. Accessed 25 August 2022. <https://www.ema.europa.eu/en/ich-s2-r1-genotoxicity-testing-data-interpretation-pharmaceuticals-intended-human-use>.
- ²⁹³Corvi R, Madia F. EURL ECVAM Genotoxicity and Carcinogenicity Consolidated Database of Ames Positive Chemicals. European Commission Joint Research Centre. Updated 20 December 2018. Accessed 25 August 2022. <http://data.europa.eu/89h/jrc-eurl-ecvam-genotoxicity-carcinogenicity-ames>.
- ²⁹⁴Buick JK, Williams A, Gagné R, et al. Flow cytometric micronucleus assay and TGx-DDI transcriptomic biomarker analysis of ten genotoxic and non-genotoxic chemicals in human HepaRGTM cells. *Genes Environ*. 2020;42(1):5.
- ²⁹⁵Li H-H, Yauk CL, Chen R, et al. TGx-DDI, a Transcriptomic Biomarker for Genotoxicity Hazard Assessment of Pharmaceuticals and Environmental Chemicals. *Front Big Data*. 2019;2:36.
- ²⁹⁶Baltazar MT, Cable S, Carmichael PL, et al. A next-generation risk assessment case study for coumarin in cosmetic products. *Toxicol Sci*. 2020;176(1).
- ²⁹⁷Hendriks G, Derr RS, Misovic B, Morolli B, Calléja FMGR, Vrieling H. The extended ToxTracker assay discriminates between induction of DNA damage, oxidative stress, and protein misfolding. *Toxicol Sci*. 2016;150(1):190-203.
- ²⁹⁸Hendriks G, Atallah M, Morolli B, et al. The ToxTracker assay: Novel GFP reporter systems that provide mechanistic insight into the genotoxic properties of chemicals. *Toxicol Sci*. 2012;125(1):285-298.
- ²⁹⁹US Food and Drug Administration (FDA) Center for Drug Evaluation and Research. Letter to HESI Committee on Genomics. Subject: Biomarker Letter of Support. 24 October 2017. Accessed 4 March 2022. <https://www.fda.gov/media/112682/download>.
- ³⁰⁰OECD. Work plan for the Test Guidelines Programme (TGP). July 2021. Accessed 25 August 2022. <https://www.oecd.org/env/ehs/testing/work-plan-test-guidelines-programme-july-2021.pdf>.
- ³⁰¹ECHA. N,N,4-trimethylpiperazine-1-ethylamine. Registration Dossier. Accessed 26 January 2022. <https://echa.europa.eu/nl/registration-dossier/-/registered-dossier/27533/7/7/1>.
- ³⁰²Pfuhler S, Pirow R, Downs TR, et al. Validation of the 3D reconstructed human skin Comet assay, an animal-free alternative for following-up positive results from standard *in vitro* genotoxicity assays. *Mutagenesis*. 2021;36(1):19-35.
- ³⁰³Pfuhler S, Downs TR, Hewitt NJ, et al. Validation of the 3D reconstructed human skin micronucleus (RSMN) assay: An animal-free alternative for following-up positive results from standard *in vitro* genotoxicity assays. *Mutagenesis*. 2021;36(1):1-17.
- ³⁰⁴Bernauer U, Bodin L, Chaudhry Q, et al. The SCCS Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation, 11th revision, 30–31 March 2021, SCCS/1628/21. *Regul Toxicol Pharmacol*. 2021;127:105052.
- ³⁰⁵Pfuhler S, van Benthem J, Curren R, et al. Use of *in vitro* 3D tissue models in genotoxicity testing: Strategic fit, validation status and way forward. Report of the working group from the 7th International Workshop on Genotoxicity Testing (IWGT). *Mutat Res Genet Toxicol Environ Mutagen*. 2020;850-851:503135.
- ³⁰⁶Moxon TE, Li H, Lee MY, et al. Application of physiologically based kinetic (PBK) modelling in the next generation risk assessment of dermally applied consumer products. *Toxicol Vitro*. 2020;63:104746.
- ³⁰⁷OECD. Case study on grouping and read-across for nanomaterials – genotoxicity of nano-TiO₂. Series on Testing & Assessment No 292. Published 21 September 2018. Accessed 25 August 2022. [https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cot e=ENV/JM/MONO\(2018\)28&docLanguage=En](https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cot e=ENV/JM/MONO(2018)28&docLanguage=En).
- ³⁰⁸OECD. Case study on the use of integrated approaches for testing and assessment for *in vitro* mutagenicity of 3,3'-dimethoxybenzidine (DMOB) based direct dyes. Series on Testing & Assessment No 251. Published 12 September 2016. Accessed 25 August 2022. [https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cot e=env/jm/mono\(2016\)49&doclanguage=en](https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cot e=env/jm/mono(2016)49&doclanguage=en).
- ³⁰⁹OECD. Report on considerations from case study on integrated approaches for testing and assessment (IATA). Series on Testing & Assessment No 350. Published 27 October 2021. Accessed 25 August 2022. [https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cot e=env/cbc/mono\(2021\)36&doclanguage=en](https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cot e=env/cbc/mono(2021)36&doclanguage=en).
- ³¹⁰Gottmann E, Kramer S, Pfahringer B, Helma C. Data quality in predictive toxicology: Reproducibility of rodent carcinogenicity experiments. *Environ Health Perspect*. 2001;109(5):509-514.
- ³¹¹Boobis AR, Cohen SM, Dellarco VL, et al. Classification schemes for carcinogenicity based on hazard-identification have become outmoded and serve neither science nor society. *Regul Toxicol Pharmacol*. 2016;82:158-166.
- ³¹²Cohen SM, Klaunig J, Meek ME, et al. Evaluating the human relevance of chemically induced animal tumors. *Toxicol Sci*. 2004;78(2):181-186.



- ³¹³Gori GB. Regulatory forum opinion piece: Long-term animal bioassays: Is the end near? *Toxicol Pathol.* 2013;41(5):805-807.
- ³¹⁴Osimitz TG, Droege W, Boobis AR, Lake BG. Evaluation of the utility of the lifetime mouse bioassay in the identification of cancer hazards for humans. *Food Chem Toxicol.* 2013;60:550-562.
- ³¹⁵Bourcier T, McGovern T, Stavitskaya L, Kruhlak N, Jacobson-Kram D. Improving prediction of carcinogenicity to reduce, refine, and replace the use of experimental animals. *J Am Assoc Lab Anim Sci.* 2015;54(2):163-169.
- ³¹⁶Cohen SM. The relevance of experimental carcinogenicity studies to human safety. *Curr Opin Toxicol.* 2017;3:6-11.
- ³¹⁷Billington R, Lewis RW, Mehta JM, Dewhurst I. The mouse carcinogenicity study is no longer a scientifically justifiable core data requirement for the safety assessment of pesticides. *Crit Rev Toxicol.* 2010;40(1):35-49.
- ³¹⁸Sistare FD, Morton D, Alden C, et al. An analysis of pharmaceutical experience with decades of rat carcinogenicity testing: Support for a proposal to modify current regulatory guidelines. *Toxicol Pathol.* 2011;39(4):716-744.
- ³¹⁹ICH. The ICHS1 regulatory testing paradigm of carcinogenicity in rats: Status report. Published 2 March 2016. Accessed 25 August 2022. https://database.ich.org/sites/default/files/S1%28R1%29EWG_StatusReport_Mar2016.pdf.
- ³²⁰ICH. The ICHS1 regulatory testing paradigm of carcinogenicity in rats: Status report 2019. Accessed 25 August 2022. https://database.ich.org/sites/default/files/S1_StatusReport_2019_0802.pdf.
- ³²¹ICH. Addendum to the guideline on testing for carcinogenicity of pharmaceuticals S1B(R1). Draft version. Endorsed on 10 May 2021. Accessed 25 August 2022. https://database.ich.org/sites/default/files/ICH_S1BR1_Step2_DraftGuideline_2021_0510.pdf.
- ³²²Hilton GM, Adcock C, Akerman G, et al. Rethinking chronic toxicity and carcinogenicity assessment for agrochemicals project (ReCAAP): A reporting framework to support a weight of evidence safety assessment without long-term rodent bioassays. *Regul Toxicol Pharmacol.* 2022;131:105160.
- ³²³JRC, Institute for Health and Consumer Protection. EURL ECVAM recommendation on the cell transformation assay based on the Bhas 42 cell line. Publications Office of the European Union; 2013. Accessed 25 August 2022. <http://dx.doi.org/10.2788/42908>.
- ³²⁴Stokes W, Jacobs A. Bhas 42 cell transformation assay validation study report. OECD. Published 30 July 2012. Accessed 25 August 2022. http://www.oecd.org/env/ehs/testing/Text_Bhas_Validation_Study_Report.pdf.
- ³²⁵Sakai A, Sasaki K, Hayashi K, et al. An international validation study of a Bhas 42 cell transformation assay for the prediction of chemical carcinogenicity. *Mutat Res.* 2011;725(1-2):57-77.
- ³²⁶Benigni R, Bossa C. Alternative strategies for carcinogenicity assessment: An efficient and simplified approach based on *in vitro* mutagenicity and cell transformation assays. *Mutagenesis.* 2011;26(3):455-460.
- ³²⁷OECD. Guidance document on the *in vitro* Bhas 42 cell transformation assay. Series on Testing & Assessment No 231. Published 8 January 2016. Accessed 25 August 2022. [http://www.oecd.org/env/ehs/testing/ENV_JM_MONO\(2016\)1.pdf](http://www.oecd.org/env/ehs/testing/ENV_JM_MONO(2016)1.pdf).
- ³²⁸OECD. The OECD QSAR toolbox. 2020. Accessed 25 August 2022. <https://www.oecd.org/chemicalsafety/oecd-qsar-toolbox.htm>.
- ³²⁹EPA. OncoLogic™ – an expert system to evaluate the carcinogenic potential of chemicals. Updated 16 February 2022. Accessed 25 August 2022. <https://www.epa.gov/tsca-screening-tools/oncologicm-computer-system-evaluate-carcinogenic-potential-chemicals>.
- ³³⁰Jacobs MN, Colacci A, Corvi R, et al. Chemical carcinogen safety testing: OECD expert group international consensus on the development of an integrated approach for the testing and assessment of chemical non-genotoxic carcinogens. *Arch Toxicol.* 2020;94:2899-2923.
- ³³¹Felter SP, Bhat VS, Botham PA, et al. Assessing chemical carcinogenicity: Hazard identification, classification, and risk assessment. Insight from a Toxicology Forum state-of-the-science workshop. *Crit Rev Toxicol.* 2022;51(8):653-694.
- ³³²Luijten M, Corvi R, Mehta J, et al. A comprehensive view on mechanistic approaches for cancer risk assessment of non-genotoxic agrochemicals. *Regul Toxicol Pharmacol.* 2020;118:104789.
- ³³³Stalford SA, Cayley AN, de Oliveira AAF. Employing an adverse outcome pathway framework for weight-of-evidence assessment with application to the ICH S1B guidance addendum. *Regul Toxicol Pharmacol.* 2021;127:105071.
- ³³⁴Pharmacy Times. Drug-induced photosensitivity: Focus on antibiotics. *Pharmacy Times.* Published 24 August 2016. Accessed 25 August 2022. <https://www.pharmacytimes.com/view/drug-induced-photosensitivity-focus-on-antibiotics>.
- ³³⁵European Medicines Agency (EMA). ICH Guidance S10 on photosafety evaluation of pharmaceuticals. Published 25 August 2015. Accessed 25 August 2022. https://www.ema.europa.eu/en/documents/regulatory-procedural-guideline/ich-guideline-s10-photosafety-evaluation-pharmaceuticals-step-5_en.pdf.
- ³³⁶OECD. Test No 498: *In vitro* phototoxicity: Reconstructed human epidermis phototoxicity test method. Published 14 June 2021. Accessed 25 August 2022. <https://doi.org/10.1787/7b2f9ea0-en>.
- ³³⁷European Commission. Summary Report on the statistics on the use of animals for scientific purposes in the member states of the European Union and Norway in 2018.



- ³³⁸Daneshian M, Akbarsha MA, Blaauboer B, et al. A framework program for the teaching of alternative methods (replacement, reduction, refinement) to animal experimentation. *ALTEX*. 2011;28(4):341-352.
- ³³⁹Hartung T, Borel A, Schmitz G. Detecting the broad spectrum of pyrogens with the human whole-blood monocyte activation test. *Bioprocess Int*. 2016;14(3):38-56.
- ³⁴⁰Anderson RL, Watson WH, Chabot CC. Sublethal behavioral and physiological effects of the biomedical bleeding process on the American horseshoe crab, *Limulus polyphemus*. *Biol Bull*. 2013;225(3):137-151.
- ³⁴¹Piehler M, Roeder R, Blessing S, Reich J. Comparison of LAL and rFC assays – participation in a proficiency test program between 2014 and 2019. *Microorganisms*. 2020;8(3):418.
- ³⁴²EDQM. Monocyte-activation test. *European Pharmacopoeia* 6.7, Chapter 2.6.30. Strasbourg, France: Council of Europe; 2010.
- ³⁴³Fennrich S, Hennig U, Toliashvili L, Schlensak C, Wendel HP, Stoppelkamp S. More than 70 years of pyrogen detection: Current state and future perspectives. *Altern Lab Anim*. 2016;44(3):239-253.
- ³⁴⁴Hasiwa N, Daneshian M, Bruegger P, et al. Evidence for the detection of non-endotoxin pyrogens by the whole blood monocyte activation test. *ALTEX*. 2013;30(2):169-208.
- ³⁴⁵FDA. Guidance for industry. Pyrogen and endotoxins testing: Questions and answers. Published June 2012. Accessed 25 August 2022. <http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM310098.pdf>.
- ³⁴⁶Brown J, Clippinger AJ, Fritz Briglia, et al. Using the monocyte activation test as a stand-alone release test for medical devices. *ALTEX*. 2021;38(1):151-156.
- ³⁴⁷EDQM. Monocyte-activation test. *Pharmeuropa*. 2016;27(4):15-26.
- ³⁴⁸EDQM. European Pharmacopoeia Commission adopts revised general chapter on Monocyte-activation test to facilitate reduction in testing on laboratory animals. 23 June 2016.
- ³⁴⁹EMA Committee for Medicinal Products for Veterinary Use. Reflection paper providing an overview of the current regulatory testing requirements for veterinary medicinal products and opportunities for implementation of the 3Rs. 2016. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2016/04/WC500205609.pdf.
- ³⁵⁰EDQM. *European Pharmacopoeia* to put an end to the rabbit pyrogen test. Published 28 June 2021. Accessed 25 August 2022. <https://www.edqm.eu/en/news/european-pharmacopoeia-put-end-rabbit-pyrogen-test>.
- ³⁵¹Indian Pharmacopoeia Commission. Monocyte activation test. Indian Pharmacopoeia. 8th ed. General Chapter Monograph 2.2.25.
- ³⁵²Rovida C, Hartung T. Re-evaluation of animal numbers and costs for *in vivo* tests to accomplish REACH legislation requirements for chemicals – a report by the transatlantic think tank for toxicology (t4). *ALTEX*. 2009;26(3):187-208.
- ³⁵³Rovida C, Longo F, Rabbit RR. How are reproductive toxicity and developmental toxicity addressed in REACH dossiers? *ALTEX*. 2011;28(4):273-294.
- ³⁵⁴Rolaki A, Nepelska M, Bremer S, Graepel R, Price A, Worth A. Reproductive toxicity – effects on fertility and developmental toxicity. In: Worth A, Barroso J, Bremer S, et al, eds. *JRC Science and Policy Reports: Alternative Methods for Regulatory Toxicology: A State-of-the-Art Review*. 2014. <https://publications.jrc.ec.europa.eu/repository/handle/JRC91361>.
- ³⁵⁵AOP Wiki. Aromatase (Cyp19a1) reduction leading to impaired fertility in adult female. Updated 4 June 2021. Accessed 25 August 2022. <https://aopwiki.org/aops/7>.
- ³⁵⁶ReProTest. Development of a novel approach in hazard and risk assessment or reproductive toxicity by a combination and application of *in vitro*, tissue and sensor technologies. Cordis. Updated 15 December 2010. Accessed 25 August 2022. https://cordis.europa.eu/project/rcn/75291_en.html.
- ³⁵⁷van der Burg B, Wedebye EB, Dietrich DR, et al. The ChemScreen project to design a pragmatic alternative approach to predict reproductive toxicity of chemicals. *Reprod Toxicol*. 2015;55:114-123.
- ³⁵⁸EPA. Virtual tissue modeling: Types of virtual tissue models. Updated 3 August 2022. Accessed 25 August 2022. <https://www.epa.gov/chemical-research/virtual-tissue-modeling-0>.
- ³⁵⁹Sachana M, Shafer TJ, Terron A. Toward a better testing paradigm for developmental neurotoxicity: OECD efforts and regulatory considerations. *Biology (Basel)*. 2021;10(2):86.
- ³⁶⁰Health Canada. Science Approach Document – Bioactivity Exposure Ratio: Application in Priority Setting and Risk Assessment. Published March 2021. Accessed 28 January 2022. <https://www.canada.ca/en/environment-climate-change/services/evaluating-existing-substances/science-approach-document-bioactivity-exposure-ratio-application-priority-setting-risk-assessment.html>.
- ³⁶¹Rooney JP, Choksi NY, Ceger P, et al. Analysis of variability in the rabbit skin irritation assay. *Regul Toxicol Pharmacol*. 2021;122:104920.
- ³⁶²Robinson MK, Cohen C, de Fraissinette AB, Ponec M, Whittle E, Fentem JH. Non-animal testing strategies for assessment of the skin corrosion and skin irritation potential of ingredients and finished products. *Food Chem Toxicol*. 2002;40(5):573-592.



- ³⁶³OECD. New guidance document on an integrated approach on testing and assessment (IATA) for skin corrosion and irritation. Series on Testing & Assessment No 203. Published 11 July 2014. Accessed 25 January 2022. [http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono\(2014\)19&doclanguage=en](http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2014)19&doclanguage=en).
- ³⁶⁴ISO. ISO 10993-23:2021 Biological Evaluation of Medical Devices – Part 23: Tests for Irritation. 2021. <https://www.iso.org/standard/74151.html>.
- ³⁶⁵Hoffmann S, Kleinstreuer N, Alépée N, et al. Non-animal methods to predict skin sensitization (I): The Cosmetics Europe database. *Crit Rev Toxicol*. 2018;48(5):344-358.
- ³⁶⁶Kleinstreuer NC, Karmaus AL, Mansouri K, Allen DG, Fitzpatrick JM, Patlewicz G. Predictive models for acute oral systemic toxicity: A workshop to bridge the gap from research to regulation. *Comput Toxicol*. 2018;8:21-24.
- ³⁶⁷Karmaus AL, Mansouri K, To KT, et al. Evaluation of variability across rat acute oral systemic toxicity studies. *Toxicol Sci*. 2022;188(1):34-47.
- ³⁶⁸EPA OPP. Guidance for waiving or bridging of mammalian acute toxicity tests for pesticides and pesticide products (acute oral, acute dermal, acute inhalation, primary eye, primary dermal, and dermal sensitization). Published 1 March 2012. Accessed 25 August 2022. <https://www.epa.gov/sites/default/files/documents/acute-data-waiver-guidance.pdf>.
- ³⁶⁹Pham LL, Watford SM, Pradeep P, et al. Variability in *in vivo* studies: Defining the upper limit of performance for predictions of systemic effect levels. *Comput Toxicol*. 2020;15:100126.
- ³⁷⁰Guth S, Roth A, Engeli B, et al. Comparison of points of departure between subchronic and chronic toxicity studies on food additives, food contaminants and natural food constituents. *Food Chem Toxicol*. 2020;146:111784.
- ³⁷¹EFSA Scientific Committee. Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data. *EFSA J*. 2012;10(3):1-32.
- ³⁷²NICEATM. Predictive Models for Acute Oral Systemic Toxicity. 2018. https://ntp.niehs.nih.gov/whatwestudy/niceatm/test-method-evaluations/acute-systemic-tox/models/index.html?utm_source=direct&utm_medium=prod&utm_campaign=ntpgolinks&utm_term=tox-models.
- ³⁷³Mansouri K, Grulke CM, Judson RS, Williams AJ. OPERA models for predicting physicochemical properties and environmental fate endpoints. *J Cheminform*. 2018;10(1):10.
- ³⁷⁴National Toxicology Program. Integrated Chemical Environment (ICE). Accessed 7 February 2022. <https://ice.ntp.niehs.nih.gov>.
- ³⁷⁵Prieto P, Burton J, Graepel R, Price A, Whelan M, Worth A. EURL ECVAM Strategy to Replace, Reduce and Refine the Use of Animals in the Assessment of Acute Mammalian Systemic Toxicity. Publications Office of the European Union; 2014.
- ³⁷⁶Hamm J, Sullivan K, Clippinger AJ, et al. Alternative approaches for identifying acute systemic toxicity: Moving from research to regulatory testing. *Toxicol In Vitro*. 2017;41:245-259.
- ³⁷⁷Prieto P, Kinsner-Ovaskainen A, Stanzel S, et al. The value of selected *in vitro* and *in silico* methods to predict acute oral toxicity in a regulatory context: Results from the European Project ACuteTox. *Toxicol In Vitro*. 2013;27(4):1357-1376.
- ³⁷⁸Prieto P, Graepel R, Gerloff K, et al. Investigating cell type specific mechanisms contributing to acute oral toxicity. *ALTEX*. 2019;36(1):39-64.
- ³⁷⁹Commission Regulation (EU) 2016/863 of 31 May 2016 amending Annexes VII and VIII to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as regards skin corrosion/irritation, serious eye damage/eye irritation and acute toxicity. <http://eur-lex.europa.eu/eli/reg/2016/863/oj>.
- ³⁸⁰EPA OPP. Guidance for waiving acute dermal toxicity tests for pesticide formulations and supporting retrospective analysis. Published 9 November 2016. Accessed 25 August 2022. https://www.epa.gov/sites/products/files/2016-11/documents/acute-dermal-toxicity-pesticide-formulations_0.pdf.
- ³⁸¹EPA OPP. Guidance for waiving acute dermal toxicity tests for pesticide technical chemicals and supporting retrospective analysis. Published 31 December 2020. Accessed 25 August 2022. <https://www.epa.gov/sites/default/files/2021-01/documents/guidance-for-waiving-acute-dermal-toxicity.pdf>.
- ³⁸²EPA. Revocation of Significant New Use Rule for a Certain Chemical Substance (P-16-581), 85 FR 52274. 25 August 2020 (to be codified at 40 CFR 721).
- ³⁸³EPA. Chlorothalonil: Revised Human Health Draft Risk Assessment for Registration Review. Published 21 May 2021. Accessed 25 August 2022. <https://www.regulations.gov/document/EPA-HQ-OPP-2011-0840-0080>.
- ³⁸⁴Clippinger AJ, Allen D, Behrsing H, et al. Nonanimal approaches to assessing the toxicity of inhaled substances: Current progress and future promise. *Appl In Vitro Toxicol*. 2018;4(2):82-88.
- ³⁸⁵Clippinger AJ, Allen D, Jarabek AM, et al. Alternative approaches for acute inhalation toxicity testing to address global regulatory and non-regulatory data requirements: An international workshop report. *Toxicol In Vitro*. 2018;48:53-70.



- ³⁸⁶Clippinger AJ, Allen D, Behrsing H, et al. Pathway-based predictive approaches for non-animal assessment of acute inhalation toxicity. *Toxicol In Vitro*. 2018;52:131-145.
- ³⁸⁷Barosova H, Maione AG, Septiadi D, et al. Use of EpiAlveolar lung model to predict fibrotic potential of multiwalled carbon nanotubes. *ACS Nano*. 2020;14(4):3941-3956.
- ³⁸⁸SCHEER. Opinion on additives used in tobacco products (Opinion 2). Tobacco additives II. European Union. https://ec.europa.eu/health/sites/health/files/scientific_committees/scheer/docs/scheer_o_001.pdf.
- ³⁸⁹Service public de Wallonie. Le bien-être animal en Wallonie. Article D.66. 7. Accessed 22 December 2021. <http://bienetreanimal.wallonie.be/home/legislation/legislationlist/liste-de-legislations-bea/bienetre067-W.html>.
- ³⁹⁰Parve V. *National Regulations on Ethics and Research in Estonia*. European Commission; 2004. <https://op.europa.eu/en/publication-detail/-/publication/4510a49f-3151-4e1b-8dad-91c0672620b5>.
- ³⁹¹German Federal Ministry of Justice. German Animal Welfare Act. § 7 | 4 TierSchG. Accessed 7 February 2022. https://www.gesetze-im-internet.de/tierschg/BJNR01277097_2.html.
- ³⁹²Glasa J. *Slovak Republic – Regulations on Ethics and Research*. European Commission; 2004. <https://op.europa.eu/en/publication-detail/-/publication/bccce559-870b-4591-9c7b-c14b62d8bda7>.
- ³⁹³UK Home Office. *Guidance on the operation of the Animals (Scientific Procedures) Act 1986*. Section 5.23.
- ³⁹⁴Moore MM, Clements J, Desai P, et al. Workshop series to identify, discuss, and develop recommendations for the optimal generation and use of *in vitro* assay data for tobacco product evaluation: Phase 1 genotoxicity assays. *Appl In Vitro Toxicol*. 2020;6(2):49-63.
- ³⁹⁵Manuppello JR, Sullivan KM. Toxicity assessment of tobacco products *in vitro*. *Altern Lab Anim*. 2015;43(1):39-67.
- ³⁹⁶Groff K, Brown J, Clippinger AJ. Modern affinity reagents: Recombinant antibodies and aptamers. *Biotechnol Adv*. 2015;33(8):1787-1798.
- ³⁹⁷Research Councils UK, National Centre for Replacement, Refinement & Reduction of Animals in Research. *Animal welfare standards expected of suppliers of antibodies to Research Council establishments*. NC3Rs. Accessed 25 January 2022. <https://www.nc3rs.org.uk/sites/default/files/documents/Funding/Antibody%20supplier%20policy%20-%20will%20be%20updated.pdf>.
- ³⁹⁸Bradbury A, Plückthun A. Reproducibility: Standardize antibodies used in research. *Nature*. 2015;518(7537):27-29.
- ³⁹⁹Baker M. Reproducibility crisis: Blame it on the antibodies. *Nature*. 2015;521(7552):274-276.
- ⁴⁰⁰Bradbury ARM, Trinklein ND, Thie H, et al. When monoclonal antibodies are not monospecific: Hybridomas frequently express additional functional variable regions. *MABs*. 2018;10(4):539-546.
- ⁴⁰¹Gray AC, Sidhu SS, Chandrasekera PC, Hendriksen CFM, Borrebaeck CAK. Animal-friendly affinity reagents: Replacing the needless in the haystack. *Trends Biotechnol*. 2016;34(12):960-969.
- ⁴⁰²Groff K, Allen D, Casey W, Clippinger A. Increasing the use of animal-free recombinant antibodies. *ALTEX*. 2020;37(2):309-311.
- ⁴⁰³Viegas Barroso JF, Halder ME, Whelan M. *EURL ECVAM Recommendation on Non-Animal-Derived Antibodies*. EUR 30185 EN. Publications Office of the European Union; 2020. <https://op.europa.eu/en/publication-detail/-/publication/3f74f5ea-94c1-11ea-aac4-01aa75ed71a1/language-en>.
- ⁴⁰⁴Dozier S, Brown J, Currie A. Bridging the gap between validation and implementation of non-animal veterinary vaccine potency testing methods. *Animals*. 2011;1(4):414-432.
- ⁴⁰⁵Draayer H. Overview of currently approved veterinary vaccine potency testing methods and methods in development that do not require animal use. *Procedia Vaccinol*. 2011;5:171-174.
- ⁴⁰⁶Bristow A, Schulster D, Jeffcoate S. Report of an international workshop on assays, standardization and labelling requirements of somatotropin. *Pharmeuropa*. 1994;6:60-67.
- ⁴⁰⁷EDQM. Harmonisation with VICH Guidelines 41 and 44 and deletion of the TABST, adopted at the 142nd session of the European Pharmacopoeia Commission. *Pharmeuropa*. 2012;S7.7:1-5.
- ⁴⁰⁸Winsnes R, Sesardic D, Daas A, Terao E, Behr-Gross ME. Collaborative study on a guinea pig serological method for the assay of acellular pertussis vaccines. *Pharmeuropa Bio Sci Notes*. 2009;1:27-40.
- ⁴⁰⁹FDA. FDA authorizes REGEN-COV monoclonal antibody therapy for post-exposure prophylaxis (prevention) for COVID-19. Published 10 August 2021. Accessed 2 February 2022. <https://www.fda.gov/drugs/drug-safety-and-availability/fda-authorizes-regen-cov-monoclonal-antibody-therapy-post-exposure-prophylaxis-prevention-covid-19>.
- ⁴¹⁰FDA. FDA authorizes bamlanivimab and etesevimab monoclonal antibody therapy for post-exposure prophylaxis (prevention) for COVID-19. Published 16 September 2021. Accessed 2 February 2022. <https://www.fda.gov/drugs/drug-safety-and-availability/fda-authorizes-bamlanivimab-and-etesevimab-monoclonal-antibody-therapy-post-exposure-prophylaxis>.
- ⁴¹¹Alfaleh MA, Alsaab HO, Mahmoud AB, et al. Phage display derived monoclonal antibodies: From bench to bedside. *Front Immunol*. 2020;11(1986):1-37.
- ⁴¹²Wenzel EV, Bosnak M, Tierney R, et al. Human antibodies neutralizing diphtheria toxin *in vitro* and *in vivo*. *Sci Rep*. 2020;10(571):1-21.



⁴¹³Stokes W, Srinivas G, McFarland R, et al. Report on the international workshop on alternative methods for *Leptospira* vaccine potency testing: State of the science and the way forward. *Biologicals*. 2013;41(5):279-294.

⁴¹⁴Stokes W, McFarland R, Kulpa-Eddy J, et al. Report on the international workshop on alternative methods for human and veterinary rabies vaccine testing: State of the science and planning the way forward. *Biologicals*. 2012;40(5):369-381.

⁴¹⁵Veterinary Medicines Directorate. Animal usage in quality control tests for the batch release of Immunological Veterinary Medicinal Products (IVMPs) via the UK from 2007 to 2012. London: VMD; 2016. Published 2016. Accessed 25 August 2022. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/438916/_518852-v8-Animal_Usage_for_QC_Batch_Release_of_IVMPs_2007-2012.pdf.

⁴¹⁶Jungbäck C, ed. *Potency Testing for Veterinary Vaccines for Animals: The Way From In Vivo to In Vitro*. Langen, Germany: International Alliance for Biological Standardization; 2012. <http://www.epsv.fiocruz.br/upload/d/silviovalle/VaccineforAnimals.pdf>.

⁴¹⁷van den Biggelaar RHGA, Hoefnagel MHN, Vandebriel RJ, et al. Overcoming scientific barriers in the transition from *in vivo* to non-animal batch testing of human and veterinary vaccines. *Expert Rev Vaccines*. 2021;20(10):1221-1233.

⁴¹⁸Akkermans A, Chapsal JM, Coccia EM, et al. Animal testing for vaccines. Implementing replacement, reduction and refinement: Challenges and priorities. *Biologicals*. 2020;68:92-107.

⁴¹⁹De Mattia F, Chapsal JM, Descamps J, et al. The consistency approach for quality control of vaccines – a strategy to improve quality control and implement 3Rs. *Biologicals*. 2011;39(1):59-65.

⁴²⁰De Mattia F, Hendriksen C, Buchheit KH, et al. The vaccines consistency approach project: An EPAA initiative. *Pharmeur Bio Sci Notes*. 2015;2015:30-56.

⁴²¹Jochems CEA, Van der Valk JBF, Stafleu FR, et al. The use of fetal bovine serum: Ethical or scientific problem? *ATLA*. 2002;30(2):219-227.

⁴²²van der Valk J, Bieback K, Buta C, et al. Fetal bovine serum (FBS): Past – present – future. *ALTEX*. 2018;35(1):99-118.

⁴²³Brindley DA, Davie NL, Culme-Seymour EJ, Mason C, Smith DW, Rowley JA. Peak serum: Implications of serum supply for cell therapy manufacturing. *Regen Med*. 2012;7(1):7-13.

⁴²⁴van der Valk J, Mellor D, Brands R, et al. The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture. *Toxicol In Vitro*. 2004;18(1):1-12.

⁴²⁵van der Valk J, Brunner D, De Smet K, et al. Optimization of chemically defined cell culture media – replacing fetal bovine serum in mammalian *in vitro* methods. *Toxicol In Vitro*. 2010;24(4):1053-1063.

⁴²⁶Chary A, Groff K, Stucki AO, et al. Maximizing the relevance and reproducibility of A549 cell culture using FBS-free media. *Toxicol In Vitro*. 2022;83:105423.





Postbus 2570
1000 CN Amsterdam
020 369 9733
Info@peta.nl
PETA.nl