PETA

The Research Modernization Deal

People for the Ethical Treatment of Animals

Billions of dollars in research grants awarded by the National Institutes of Health … **are failing to lead to effective treatments** for many of the diseases **that kill and incapacitate humans**.

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Executive Summary

THE RESEARCH MODERNIZATION DEAL

Numerous scientific studies and reviews reveal that experiments on animals fail to lead to effective treatments and cures for human diseases, including the top killers in the U.S. Reliance on animal models is diverting funds away from more promising areas of research and delaying the development of effective drugs and treatments, as well as limiting our ability to protect human and environmental health.

Yet approximately 47% of the budget of the National Institutes of Health (NIH), which is charged with overseeing the health of Americans, funds experiments on animals. NIH has failed to take effective steps to address the following problems:

- 95% of all new drugs that test safe and effective in experiments on animals fail or cause harm in human clinical trials.
- The failure rates of new drugs developed using animals in certain disease research areas exceed 95%. Here are a few examples:
    - Alzheimer's disease.....99.6%
    - Cancer 96.6%
    - HIV/AIDS vaccine.....100.0%
    - Stroke 100.0%
        (1,000 new agents tested in animals and in 100 clinical trials)
    - Sepsis 100.0%
- 90% of basic research fails to lead to any human therapies within 20 years.
- Up to 89% of experiments cannot be reproduced even though reproducibility is a critical component of scientific research.

Promising human-relevant research methods, such as organs-on-chips, sophisticated uses of human stem cells, genomics and proteomics, imaging, and computer modeling, can replace the use of animals.

To improve research results and protect human and environmental health, PETA proposes the following:

- End animal use in research areas in which animals have been demonstrated to be poor "models" of humans and their use has impeded scientific and medical progress.
- Conduct scientific reviews of the efficacy of animal use to identify additional areas in which non-animal methods are available or the use of animals has failed to protect human or environmental health and can, therefore, be ended.
- Redirect funds from animal studies to the use and development of reliable, non-animal methods.
- Implement a harm-benefit analysis system for research involving animals that includes an ethical perspective and consideration of lifelong harm inflicted on animals, such as is used in the U.K.
- Work with other world leaders to harmonize and promote international acceptance of non-animal testing methods for regulatory toxicity testing requirements.
- Educate and train researchers and regulators in the benefits of and how to use non-animal testing approaches.

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Introduction

The observation (right) by best-selling science journalist Richard Harris resonates with each person who is suffering or who knows someone suffering from an incurable disease—and for good reason: Billions of dollars in research grants awarded by the National Institutes of Health (NIH), the world's largest funder of biomedical research, are failing to lead to effective treatments for many of the diseases that kill and incapacitate humans.

The reason for this failure appears to be a misplaced reliance on animal studies. A great deal of scientific research in the last several decades shows that animal studies are flawed and divert both monetary and intellectual resources from more reliable and relevant methodologies. Critically, intrinsic biological and genetic differences among species contribute significantly to inescapable problems in extrapolating results from nonhuman animals to humans, even in the best controlled and best executed study designs.

Along with mounting evidence that experiments on animals do not reliably translate to humans and the increasing development and implementation of technologies that can supplant animal use in laboratories, our society has witnessed growing moral concern regarding experiments on animals. An August 2018 poll conducted by the Pew Research Center found that a majority of U.S. adults oppose the use of animals in scientific research.[2]

"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]

In this report, we offer a strategy for replacing the use of animals in experimentation, identify a number of strategic priorities, and append further information about areas in which there are opportunities for the immediate and near-future replacement of animal use. We have also included information about areas in which further development, validation, and implementation of non-animal methods are needed.

Limited Predictive Value of Research Using Animals

Many in the scientific community are aware of the flaws of studies on animals. NIH reports that novel drugs fail "in about 95 percent of human studies,"[3] even though they appeared safe and effective in preclinical experiments using animals. A 2014 analysis published in *The BMJ* found that studies using animals largely have not furthered knowledge in the field of human health or led to the development of treatments for conditions affecting humans.[4]

Lack of Validity

Problems with internal and external validity contribute to the failure of experiments on animals in the translation of biomedical research from bench to bedside. The internal validity of experiments on animals is undermined by poor study design, including failure to implement processes to prevent bias, such as blinding, in which the individuals conducting the experiments or those analyzing the data do not know whether the animals or samples belong to the treatment or control group.

Following a meta-analysis of systematic reviews of preclinical experiments on animals across a wide variety of disease areas, University of Oxford scientists found that a lack of

measures to reduce bias in experiments on animals likely results in overestimation of the benefits of the treatment studied.[5] The authors concluded, "Biased animal research is less likely to provide trustworthy results, is less likely to provide a rationale for research that will benefit humans, and wastes scarce resources."[5] 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."[5]

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 is unsurprising, therefore, that a 2015 investigation concluded that between 18% and 89% of all preclinical research, a large part of which involves animal testing, could not be reproduced.[6] At the most conservative U.S. estimate, this results in approximately $28 billion per year spent on experimentation that is misleading for human health.[6] Former NIH Director Francis Collins and Acting Director Lawrence Tabak have admitted, "Preclinical research, especially work that uses animal models, seems to be the area that is currently most susceptible to reproducibility issues."[7]

However, the weaknesses of experiments on animals cannot be overcome simply by 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,"[8] can never be achieved. Inherent species differences mean that nonhuman animals cannot serve as analogs for understanding the biological effects of drugs and chemicals on humans. As R.J. Wall and M. Shani write, even the "extrapolated results from studies using tens of millions of animals fail to accurately predict human responses."[9]

Therefore, experiments on animals lack internal and external validity. In other words, they are usually poorly executed, but even if the experimental methods were improved, the results would not translate to humans.

In a 2018 review published 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.[8] Attempts to control for or correct species differences result in what the authors refer to as the "extrapolator's circle". They write, "[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."[8]

Inherent species differences mean that nonhuman animals cannot serve as analogs for understanding the biological effects of drugs and chemicals on humans.

They also discuss the concerning trend among those involved in experiments on animals to minimize the issue of species differences and the effects on external validity, a problem that is acknowledged by a number of researchers.[10,11] 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."[8] There is growing scientific consensus that far more is to be gained from non-animal research methods that are better suited to solving human biomedical research and regulatory assessment questions. As a U.K. industry report emphasized, the time has come to humanize drug discovery and toxicology.[12]

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 behavior.[13,14] This deprivation contributes to their stress and alters their physiology and neurobiology, causing them to exhibit various psychopathologies.[15–20] 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 nature. A mouse in a laboratory will not respond to a drug in the same way a mouse in a field would. One then has to ask, how does this biologically distinct mouse reliably represent the biology of human beings?

Misplaced Resources

Despite the growing evidence that experiments on animals are wasteful and can impede medical progress, approximately 47% of all NIH research funding goes toward them.[21] Federal funds available for biomedical research are a finite resource. In the fiscal year 2020, only 20.6% of grant applications submitted to NIH were awarded funding.[22] Each decision to approve an application carries with it a refusal to fund other projects, leaving a large opportunity cost in terms of human-relevant research that has the potential to help patients.

A 2014 *BMJ* article discussing this issue noted, "[I]f research conducted on animals continues to be unable to reasonably predict what can be expected in humans, the public's continuing endorsement and funding of preclinical animal research seems misplaced."[4]

Funding for biomedical research is allocated into two categories: basic research and applied research. NIH defines

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.[69]**

Oncology drugs, which undergo extensive animal testing, have a success rate of only 3.4%.[70] This theme pervades many human disease areas. There is an abundance of literature documenting the failing of various animal models of neurodegenerative diseases—such as Alzheimer's, for which the clinical failure rate for new drugs is 99.6%.[71] (See the appendices for a comprehensive look at disease areas.)

basic research as that which "supports a broad understanding of human behavior and biology," while applied research is a "systematic study to gain knowledge or understanding" to meet a specific need.[23] A great deal of basic research involves studies on animals.

NIH perceives basic research using animals as important because its intent is to produce foundational knowledge for a better understanding of the causes and determinants of disease. In other words, the results of basic research should point the way toward applied research that should, in turn, benefit humans. However, the evidence shows otherwise. 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 analyzed (63%) described experiments on animals. The researchers' investigation into the application of basic science to clinical applications found that fewer than 10% of these self-proclaimed highly promising basic science discoveries entered routine clinical use within 20 years.[24]

Yet the NIH Office of Budget estimates that the agency consistently spends over half its funding on basic research each year,[23] most of which involves animals.

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. But the evidence that basic and applied research involving animals is impeding the development of treatment and cures for human ailments has not prompted sufficient reconsideration of research and funding priorities by NIH and other authorities. Such a paradigm shift is crucial both within and beyond the U.S.

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 the use of animals in experiments in which there is clear evidence that animals are not useful 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.

These complexities inherent in translational research present an important opportunity for exploring novel approaches that successfully and efficiently yield outcomes as proximal as possible to eventual human benefit. 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.[25]

The shift in 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,[4] in the increased awareness of animal cognition and sentience,[2] and in the fast-eroding public support for animal studies.[2]

For example, Dr. Hakan Şentürk, the former editor of *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. Şentürk wrote that the new policy represented "growing concern about the lack of applicability of animal research to humans."[27] He further commented, "When 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."[27] Unfortunately, new editorial leadership does not appear to have maintained this policy.

Significantly, a move away from experiments on animals will allow for substantial growth in the science and technology sectors and for faster return on investment in drug research and development,[28] as seen after the cosmetics testing ban in the E.U., despite initial resistance from some corners of the industry. An evolution of research funding priorities toward human-relevant methods, which recapitulate human physiology and biology without using animals or their tissue, will get treatments to the patients who need them more safely and likely in less time.[29,30]

Opportunities for Economic Advancement

The High Cost of Drug Development

By mandating a move away from experiments on animals and toward advanced scientific methods, the U.S. has the opportunity to advance biomedical research, rapidly expand job growth in science and technology, and reduce healthcare costs. In Meigs and colleagues' review "Animal Testing and Its Alternatives—the Most Important Omics Is Economics," they report that "an economy of alternative approaches has developed that is outperforming traditional animal testing."[28]

In the current system, bringing a new drug to market may cost up to $2 billion and take as long as 15 years.[3] The high costs of research and development (R&D) may be shifted to patients in the form of increasingly unmanageable price tags for prescription drugs.[31] During a 2017 conference, then–U.S. Food and Drug Administration (FDA) Commissioner Scott Gottlieb lamented the high cost of drug development and its effects on patients and the U.S. economy. He discussed the importance of reducing R&D costs "to make sure we're providing an efficient path for the translation of cutting-edge science into practical treatments that are going to benefit patients" and "because the rising cost of drug development is unsustainable."[32] He stated, "Unless we find ways to modernize how we approach our work, and make more efficient use of our resources, then we're going to get fewer medicines, and higher costs," adding, "At a time when people are rightly worried about the rising prices of drugs, and the impact on patient access, we also need to be thinking about these factors that contribute to the high cost of making new medicines."[32]

One factor in the high cost of R&D 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. Ninety-five percent of drugs that test safe and effective in animals fail in humans[3] because they are either not safe or not effective.[29] 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.[33]

Writing in the official journal of the American Society for Clinical Pharmacology & Therapeutics, Tal Burt and his coauthors made the following comments:

> Increasing costs of drug development and ethical concerns about the risks of exposing

The Dangers of Misleading Results

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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, ages 28 to 49, experienced such adverse reactions that they had to be hospitalized. 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."[72]

In his 2010 article "TGN1412: From Discovery to Disaster," Husain Attarwala of Northeastern University 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."[73] Five of the six participants were hospitalized 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.[74] 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."[73]

The opposite is also true: Therapies that have not worked well in animals have sat useless on the shelf while patients have gone without lifesaving 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.[75,76] 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 *never* have been tried in humans.[77,78]

humans and animals to novel chemical entities favor 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.[34]

With the use of human-relevant technology in place of expensive, time-consuming, and inaccurate experiments on animals, the cost of drug discovery has the potential to decrease dramatically. By reducing both the expense and time it takes to get effective therapies to market, manufacturers will be able to pass these savings on to patients.

Job and Economic Growth in the Technology Sector

The market for human cell–based *in vitro* technology for biomedical research and testing is growing rapidly. For example, the Boston-based startup Emulate Inc. raised $36 million in financing to expand its human organ–on-a-chip technology.[35] It is currently being used by AstraZeneca, Roche, Merck, Johnson & Johnson, and others to predict more accurately the safety and efficacy of drug candidates.[35]

A leading market research company has estimated that "[t]he global cell-based assays market should reach $47.3 billion by 2027 from $29.2 billion in 2022"[36] and the "global market for induced pluripotent stem cells should grow from $2.8 billion in 2021 to $4.4 billion by 2026."[37] The firm also projected that the global regenerative medicine market

Revisiting Failed Drugs

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Emulate and Janssen Pharmaceuticals have demonstrated how a blood vessel–on-a-chip was able to predict a human thrombosis caused by an antibody therapy. This therapy had previously been determined to be safe following preclinical animal tests, but clinical trials had to be stopped after humans given the drug developed blood clots, which were not predicted by the experiments on animals.[79]

In a 2021 study, researchers at Johns Hopkins University, the Norwegian Institute of Public Health, and the U.K. patient safety charity Safer Medicines Trust used human-based *in vitro* methods to reevaluate the diabetes drug troglitazone.[80] Troglitazone had been withdrawn from the market due to severe and fatal liver toxicity that killed at least 63 people. The newer *in vitro* tests predicted this potential hazard, while the preclinical animal studies had not. One author of the study commented, "Patients need safer affordable medicines delivered in their lifetime. The pharmaceutical industry is in crisis, with empty pipelines and skyrocketing costs. Focusing on human biology is the route to developing safer medicines faster and with lower total development costs."[81]

will reach a volume of $89.5 billion by 2025.[38] New technology will streamline drug development, making the process safer, cheaper, and more effective. Developing these techniques allows for the creation of interdisciplinary research teams that will be fundamental in creating personalized disease models for precision medicine or developing effective and precise systems for toxicological risk assessment.

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 tests on animals. This is the result of our improved understanding of human 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 tests on animals. 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 tests on animals.[39]

There is growing recognition among regulators and the regulated community that tests on animals do not adequately protect human health. This affects not only drug safety but also the testing of chemicals that humans may be exposed to in their environment. The U.S. National Academies of Sciences, Engineering, and Medicine (NASEM) agrees 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."[40]

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In 2007, NASEM published a landmark report titled "Toxicity Testing in the 21st Century: A Vision and a Strategy." 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 that 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 agency's news release summarizes the approach:

> "The report recommends an approach that would take advantage of rapidly evolving scientific understanding of how 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 health problems. Specifically, the new testing approach would focus on toxicity pathways—cellular pathways that, when sufficiently perturbed, are expected to lead to adverse health effects."[41]

Robust *in vitro* toxicity tests can be designed to evaluate the effects of chemicals on specific events in these toxicity pathways and, therefore, help researchers understand how and at what exposure level an adverse outcome may occur.

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 tests on animals can be prioritized and progress can be monitored.

By eliminating the use of tests on animals for regulatory purposes when replacements exist and by promoting the acceptance and further optimization of methods currently in development, the U.S. 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 to this report. These include tests for eye and skin irritation, skin sensitization, acute systemic effects, genotoxicity, pyrogenicity, endocrine disruption, and carcinogenicity as well as for the safety and efficacy of vaccines and biologics.

Public Opinion and Animal Sentience

Public opposition to the use of animals in experiments has increased steadily, from 8% in 1948[42] to 52% in 2018.[2] As Ormandy and Schuppli report, the public is less approving of animal studies when the experiments are invasive, are viewed as less beneficial or necessary for human health—as in the case of cosmetics testing—and when non-animal methods exist.[43] If members of the public were fully aware of the mountain of evidence that studies on animals may very well be hampering the development of effective treatments, opposition would likely grow substantially.

The minority of the public that continues to support experiments on animals usually predicates its support on the mistaken belief that oversight bodies allow experiments only if they are essential to developing treatments for human disease and when the harm experienced by animals will be outweighed by the benefits to humans. While oversight bodies tasked with approving experimental protocols claim to adhere to government funding policies that require the performance of a harm-benefit analysis,[44,45] a 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."[46] They compared the harm inflicted on animals in preclinical studies for six treatment interventions to the benefits that the studies offered to humans. They concluded that fewer than 7% of studies should have been permitted and that all the studies were of poor quality.

Recognition of animal sentience has also played a role in the public's growing opposition to experiments on animals. This is particularly true for the species with whom humans share

their homes (e.g., dogs and cats) and those perceived as having higher cognitive abilities (e.g., nonhuman primates). However, public concern for other species, such as rats, has also increased.[47]

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 behaviors."[26] *The Cambridge Declaration on Consciousness* illustrates that recognition of animal sentience is growing within the scientific community, too. Statistics make it clear that animals are not appropriate human surrogates in biomedical research, but when it comes to their capacity to suffer, how much like humans do they need to be before a critical review of animal-based research is considered mandatory? Neurologist and public health specialist Aysha Akhtar writes, "Science is showing how other animals are like us in morally relevant ways, but unlike us in medically relevant ways."[48]

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. "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,'"[49] 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.

Changes are necessary for the U.S. to prove itself a world leader in innovative and superior research and testing methods.

COVID-19

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To say that the COVID-19 pandemic has changed life as we know it is an understatement. In a silver lining, it may lead to an entirely new era of biomedical research and vaccine development. In order to speed up the development of COVID-19 vaccines, both the FDA and NIH greenlighted landmark human clinical vaccine trials without first requiring extensive tests on animals. Instead, the two were allowed to proceed in tandem,[82] changes that PETA has encouraged the FDA to extend to all new drugs in development (e-mail communication, May 5, 2020, https://www.peta.org/wp-content/uploads/2020/05/2020.05.05-FDA-Commissioner-COVID-19-letter-FINAL.pdf).

Although time was an obvious factor in this decision, it is important to note that many other species do not respond to SARS-CoV-2 infection in the way humans do. When asked about seemingly promising experimental results in rhesus macaques, Dr. Malcolm Martin, a virologist at NIH "cautioned that monkeys are different from humans in important ways." The interviewer noted that "[t]he unvaccinated monkeys in [the vaccine experiment] didn't develop any of the severe symptoms that some people get following a coronavirus infection" and quoted Martin as saying, "It looks like they got a cold."[83] Mice—who must be genetically engineered to be susceptible to the disease—also show only mild symptoms. Dr. Stanley Perlman, a coronavirologist at the University of Iowa, notes that infecting mice with the novel coronavirus "doesn't really tell you much about how the virus causes disease."[84]

In addition to harming and killing animals—however misguidedly—for COVID-19 vaccine and disease research, university and other business closures caused by the pandemic led to the mass slaughter of countless animals who had been slated to be used in other experiments. Johns Hopkins University, Stanford University, the University of California–Berkeley, the University of Washington, and the University of Michigan were among the institutions calling on faculty to euthanize "extraneous animals" in laboratories.[85]

On the other hand, many scientists are using innovative non-animal methods to study COVID-19 and the novel coronavirus that causes it, including human lung and intestinal organoids, three-dimensional reconstructed human respiratory tissue models, samples of human oral tissue from healthy volunteers, advanced computer simulation and supercomputers, human genetic analyses, human challenge studies, human-derived antibodies, and human organs–on-chips that model human lungs, mouths, eyes, noses, and intestines. An updated list of these examples can be found at https://www.peta.org/blog/coronavirus-covid-19-vaccine-non-animal-tests.

World Leadership

There is an international movement away from using animals in experiments, which reflects the growing consensus in the scientific community that using animals in basic biomedical research or for regulatory assessment requirements is neither ethical nor efficacious. Australia, the European Union, Japan, New Zealand, and the U.K. have all banned or limited the use of great apes (chimpanzees, gorillas, and orangutans) in experimentation, and the U.S. no longer awards federal funding for experiments involving chimpanzees.[50] In many parts of the world, cosmetics tests on animals are now illegal. In addition, India, Israel, and the U.K. have ended animal testing for household products and their ingredients.

In 2016, the Dutch government announced its plan to become a 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.[51] Subsequently, the Transition Programme for Innovation without the use of animals (TPI) was established, aiming to bring together stakeholders and offer a platform for identifying and developing activities to increase the pace of the transition toward animal-free innovation.[52]

The U.S. 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.[53] 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.[53] This is in addition to the Frank R. Lautenberg Chemical Safety for the 21st Century Act (2016), which requires the use of reliable non-animal testing approaches for assessing the safety of industrial chemicals, when they exist.[54]

Also in the U.S., the FDA Modernization Act 2.0 proposes to amend the Federal Food, Drug, and Cosmetic Act to lift the compulsory requirement to test all new drugs on animals in favor of "alternatives to animal testing."[55]

In 2021, members of the European Parliament almost unanimously supported a motion for 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.[56]

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

Plan of Action: Recommendations for Modernizing U.S. Biomedical Research

1. End animal use in research areas in which animals have been demonstrated to be poor "models" of humans and their use has impeded scientific and medical 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, strokes, cancer, diabetes, obesity, inflammation and immune responses, HIV/AIDS research, addiction studies, trauma research, and medical training as well as for regulatory testing. As such, experiments and tests on animals in these research areas should be ended as soon as possible and replaced with more effective and efficient

non-animal methods. Please find further elaboration on and recommendations for these areas in the appendices.

2. Conduct scientific reviews of the efficacy of animal use to identify additional areas in which non-animal methods are available or the use of animals has failed to protect human or environmental health and can, therefore, be ended.

For areas of investigation in which 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 analyze multiple research studies, are the first step in assessing the effectiveness of animal use. Such systematic reviews should include information about the return on investment received by the public from the results of animal-based research funded and conducted by NIH.

Some countries recommend that systematic reviews be conducted before studies are funded. Scientists at Radboud University Medical Center published the following statement: "Making systematic reviews of animal studies a routine is our scientific and societal responsibility, just as with clinical studies in humans."[57]

Several U.S. funding entities, including NIH, the Department of Veterans Affairs, and the Department of Defense, are members of the Ensuring Value in Research Funders' Forum (EViR), a collection of the most prominent international funding bodies formed to address waste in clinical and preclinical research. EViR states as its second guiding principle, "Research should only be funded if set in the context of one or more existing systematic reviews of what is already known or an otherwise robust demonstration of a research gap."[58] It explains, "This is important because new research not set in the context of what is already known leads to unnecessary duplication, studies that cannot change decision making (e.g., will not change the meta analysis), or inappropriate design (e.g., inappropriate outcome measures, incorrect prevalence assumptions, failure to learn from past previous studies)."[58] To apply this principle, EViR says that funders must "[r]outinely assess whether an adequate review has been done and whether the results of that review support the case for further clinical or preclinical research."[59]

The recommendation to conduct scientific reviews of the efficacy of procedures is, therefore, already one that the largest funding bodies in the world agree is a necessary principle for guiding valuable research and reducing waste in research funding.

The National Academy of Medicine, formerly the Institute of Medicine, completed an examination of the scientific necessity of using chimpanzees in behavioral and biomedical research.[60] That effort revealed that harmful studies had been approved, funded, and conducted for years, even though there were alternative methods in virtually every area in which chimpanzees were being used. Institutional oversight bodies and funding agencies had given their stamp of approval to these protocols. However, as we now know, the review processes in place were inadequate. Wherever thorough and objective scientific reviews of animal use for various areas of inquiry have not been conducted, they should be undertaken.

3. Redirect funds from animal studies to the use and development of reliable, non-animal methods.

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. As long as 47% of the NIH funding budget goes to animal studies, the U.S. will be stalled in the development of effective treatments for human disease. Forward-thinking scientists are developing and implementing methods for studying and treating diseases and testing products that do not entail the use of animals and are relevant to human health. Researchers have created human cell–derived models, "organs-on-chips," *in silico* (computer) models, and other methodologies that can replicate human physiology, diseases, and drug responses more accurately than experiments on animals do.

Studies have repeatedly shown that these new methodologies are better at modeling human diseases than crude experiments on animals are. Indeed, in its 2016–2020 strategic plan, NIH announced that it would reduce and replace experiments on animals:

> Petri dish and animal models often fail to provide good ways to mimic disease or predict how drugs will work in humans, resulting in much wasted time and money while patients wait for therapies. To address that challenge, NIH, DARPA, and FDA are collaborating to develop 3D platforms engineered to support living human tissues and cells, called tissue chips or organs-on-chips. An integrated body-on-a-chip is the ultimate goal.[61]

NIH and other federal agencies must now take the next step and end the funding of 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 and help protect human health and the environment.

Currently, the system does not adequately determine the extent to which animals are suffering in these experiments. Until researchers make this critical assessment, they cannot reasonably measure whether or not the results are worth the pain and suffering.

4. Implement a harm-benefit analysis system for research involving animals that includes an ethical perspective and consideration of lifelong harm inflicted on animals.

For the benefit of animal welfare and human health, researchers should focus their considerable talent, time, money, and energy on moving away from archaic animal use—prioritizing areas in which the harm inflicted on the animals involved is so great that no benefit could ever justify the experiment. Examples of such studies would include the following: maternal deprivation experiments; psychology experiments that cause fear, anxiety, or depression; and drug, alcohol, and food addiction experiments. Until all animal studies have ended, a system of analysis for a "risk threshold" or "upper limit," similar to that employed in research on humans, should be implemented. Examples of frameworks by which to conduct harm-benefit analyses of animal experimentation can be found in the reports of the U.K. Animals in Science Committee Harm-Benefit Analysis Sub-Group,[62] the report of the Working Group on the Use of Chimpanzees in NIH-Supported Research,[60] and the research of Pandora Pound.[46]

The harm to animals that is considered should not be restricted to that resulting from specific procedures but should also include the inherent harm caused by life in a laboratory, where animals are denied the opportunity to meet their species-specific needs. Currently, the system does not adequately determine the extent to which animals are suffering in these experiments. Until researchers make this critical assessment, they cannot reasonably measure whether or not the results are worth the pain and suffering.

5. Work with other world leaders to harmonize and promote international acceptance of non-animal testing methods for regulatory toxicity testing requirements.

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

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 and industry be mandated to use a scientifically satisfactory method or testing strategy that does not involve live animals instead of a procedure involving animals wherever possible (as is required in the European Union[63]). In addition, we recommend that the establishment of a public-private center for predictive animal-free research and testing be coordinated, similar to the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM). Such a center would help transform the science of safety assessment with new tools to guide industry, government, consumers, and international trade partners to adopt best practices.

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

Informing the Scientific Community

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 recognize that barriers can exist to adopting new technology and that efforts to build confidence are therefore needed. For example, the U.K.'s innovation agency, Innovate UK, has recognized that overcoming skepticism 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.[64] 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.

Training Opportunities for Early-Career Researchers

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 U.S. can compete with international developments. Because many study programs lack sufficient courses about animal-free methods, supplemental training programs have been developed. For example, in the EU, the European Commission's Joint Research Centre (JRC) hosts a summer school on non-animal approaches.[65] Similar programs could be replicated in the U.S. at a federal level. In Canada, the University of British Columbia has accepted a new undergraduate module offered by the Society for Humane Science called "Non-Animal Methods in Biomedical Science," which focuses on training students in animal-free methods of research and testing.[66] Many online resources by experts in the field also exist, including those offered by PETA Science Consortium International e.V.[67] and the Physicians Committee for Responsible Medicine.[68] 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 center 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 organize Ph.D. and postgraduate programs that use only non-animal methods as well as workshops, seminars, and summer schools on *in vitro* and *in silico* methods.

Training Opportunities for Industry, Funders, and the Regulatory Community

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 adverse outcome pathways. 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 TPI 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 authorizing experiments on animals—and those requiring testing data to meet legislative requirements (e.g., 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.

Conclusion

The current waste of resources, time, and animals' lives has a direct and disastrous effect on human health. Until this plan is implemented, the research funded by U.S. taxpayers will fail to provide the basic and applied research needed to develop effective treatments for human disease.

Detailed information on 29 areas of research and the astonishing failure of animal studies to lead to effective treatments for humans is included in the appendices.

Glossary

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

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.

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Basic and Applied Biomedical Research

Cancer

Recommendation: End the use of animals

Cancer is the second-leading cause of death in the U.S., and officials estimate that close to 600,000 Americans died from cancer in 2020.[86] Even after significant investment in research for cancer therapies, the success rate for oncology drugs is only 3.4%,[70] 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 checkups for screening,[87,88] 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.[89] Even though study design and other logistical issues in research can create problems, cancer physicians at McMaster University in Ontario state, "[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."[90]

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 modeling, 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.[91] Following an analysis of 1,110 mouse xenograft tumor 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.[92] Essentially, when human tumor 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 tumor-suppressing genes.[93] 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 tumor growth seen in natural tumor development. In addition, random integration of the oncogenes can result in unexpected outcomes that would not be present in human patients.[93] These models are also time-consuming and costly to create, and they use large numbers of animals because of the extensive breeding requirements.[94,95]

Given the many shortcomings of cancer modeling 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-tumor immune response, determining molecular features that can represent biomarkers in specific cancer pathogenesis, exploring

adoptive cell therapies and virotherapies, and more.[96]

Some examples of recent human-relevant cancer research include vascular human tumor models—created using three-dimensional bio-printing—that mimic key steps of cancer metastases,[97] patient-specific human lung–on-a-chip models for precision medicine,[98] sophisticated analyses of human mammary tumor organoids[99] and breast cancer cell lines,[100] genomics to improve understanding of uniquely human aspects of cancer,[101,102] artificial intelligence for faster diagnoses[103] and for predicting individual drug responses,[104] and wearable bionic chips to collect real-time data from patients.[105]

Former 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."[106] Cancer is a highly variable, individualized disease that will require individualized treatment to overcome.[107] 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 the U.S. and around the world, yet the development and approval of new drug candidates for treating it have declined over the past two decades.[108]

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.[109,110] 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.[111] 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.[112] This makes the profile of ventricular repolarization 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.[113] Rat and mouse models of heart failure do not exhibit the same miRNA expression profiles as patients with acute heart failure.[113] 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.[114]

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]."[115]

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 that "[t]he 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."[116] Experts point out the "lack of concordance between the effects of compounds in animals (or animal-derived tissues) and those in humans"[117] 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."[118] In a coauthored review, scientists from Stanford University, the FDA, and the biopharmaceutical company AbbVie refer to testing cardiotoxicity in animal models as a "black box" approach.[117] 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."[119] 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."[120] 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."[120] 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 utilized for high-throughput screening for cardiotoxicity.[121]

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,[122] the use of a plant-derived cellulose framework as scaffolding to build networks of human veins,[123] 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."[124] 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.[125] 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."[126] 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.[127]

Computer modeling 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.[128] 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."[129] 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.[130]

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).[131] 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."[131] 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.[132]

T2DM is a disease of glucose misregulation resulting from impaired insulin secretion action and pancreatic ß-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.[131,132] "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."[132] And as Joan Mir-Coll and colleagues point out, "[R]odent ß-cells differ from human ß-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."[133] 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.[134] Mice who have been genetically modified to lack select insulin-signaling 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.[132] 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."[134]

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.[135] [*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*, postmortem human tissue, noninvasive human imaging, epidemiological and human genetic studies—including nutrigenomics and nutrigenetics—and *in silico* modeling.[131,135] 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.[136] Additionally, the FDA has approved a closed-loop insulin pump developed using *in silico* modeling 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."[137] *In silico* models are being used to rapidly assess potential natural and pharmaceutical interventions for T2DM.[138,139] Numerous investigators are using islet-on-a-chip microfluidic systems to study disease mechanisms and test therapeutic agents.[140–142]

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 recognized more than 20 years ago when, in 1995, 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 nonhuman primate 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.[143,144]

Owing to differences in surface proteins and other molecular markers, antibodies that neutralize SIV have no effect on HIV, and vice versa,[145] making them useless in HIV research. Importantly, the dose of SIV administered to nonhuman primates in experiments is often much higher than the typical amount of HIV-1 to which a human is exposed during sexual transmission.[146] 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."[147]

In a peer-reviewed journal, an animal experimenter at the Washington National Primate Research Center admitted that nonhuman primate (NHP) 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."[148] Experimenters have developed dozens of vaccine candidates using monkeys. Only five have reached as far as human trials, and all of them have failed.[149] One of them even increased the likelihood of HIV infection in humans.[150] After one of the human vaccine trials failed in 2018, Anthony Fauci, director of the U.S. National Institute of Allergy and Infectious Diseases, acknowledged that the original positive results of a macaque study "might be a fluke."[151]

Because of broad failures in nonhuman primate HIV/AIDS research, experimenters have shifted some focus to mice—a species even more genetically removed from humans. The "humanized" 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, humanized mice are limited in their longevity with the disease and retain parts of their murine immune systems, "complicating immune response interpretations."[145] Not surprisingly, the use of humanized 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 feces, 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.[143] Nonhuman primates used in HIV research, on the other hand, have been found to be harboring confounding infections like valley fever, which compromises findings when they are used in HIV studies.[152]

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,"[153] and they recognized that human *in vitro* models are needed to replicate this human disease and develop treatment. Thinking progressively about non-animal methods, U.K. 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."[154]

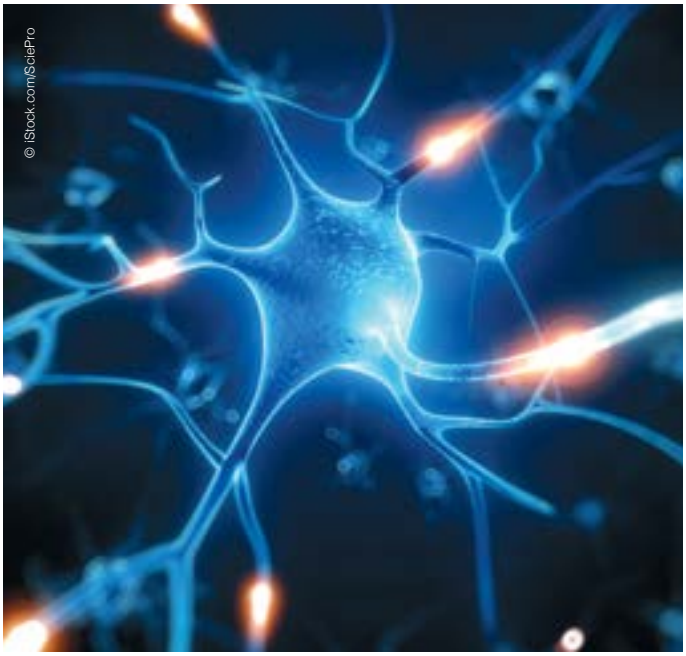
Scientists admit that even after costly and unreliable experiments on animals, human data are still needed to determine whether a drug is fit for the clinical setting. Rao and Alving of the U.S. Military HIV Research Program stated that "human clinical trials still appear to be the only reliable way to determine whether an HIV vaccine candidate will have activity or efficacy in humans."[155] Scientists from Australia, France, Italy, and the U.K. 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.[156] 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,[157] novel imaging techniques to discover previously unknown aspects of HIV structure that open up the potential for new therapies,[158] 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.[159]

Nobel laureate Sydney Brenner declared, "We don't have to look for model organisms anymore because we are the model organism."[160] Similarly, in 2007, the associate editor of *The BMJ* stated, "When it comes to testing HIV vaccines, only humans will do."[161]

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.[162] 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."[163] Logically, these differences make sense: We humans "do not live with our heads a half-inch off the ground,"[164] and we have considerably longer life spans and a larger body size than mice do.[162,163] As concisely stated by Leist and Hartung, "[H]umans are definitely no 70-kg mice."[164] 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 criticized: "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."[165] 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.[166] The lethal dose of a deadly IFV strain (H5N1) is about 100 times lower in BALB/c mice compared to their wild cousins.[167] 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.[165] This is because human IFV receptors (Þ2,6-linked sialic acids) are not abundant in the upper airways of mice, who have a different receptor (Þ2,3-linked sialic acids).[168] 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.[169] They do not cough or sneeze.[165] Moreover, the virus does not transmit between mice.[170] Additionally, we now know that gut microbiota are intimately linked to the immune system,[171] 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.[172] 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."[173]

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.[174] 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.[175] Additionally, a University of Tennessee–Knoxville mathematician, along with surgical and immunological specialists at the University of Pittsburgh, used a mechanistic mathematical model to characterize human immune responses during organ transplantation.[176]

A review summarizing the progress of immune-competent human skin disease models recognizes 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."[177]

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.[178] It is a leading cause of death in U.S. hospitals and is one of the most expensive conditions to treat.[179,180]

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.[181] 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.[182]

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."[183] 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![183]

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 unsterilized, unpredictable environments, who develop sepsis.[184,185] 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.[186] Unlike humans, mice are rarely given pain relief,[187] 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.[188] In addition, the procedure causes the formation of an abscess, whose effects may disguise or be disguised by the effects of the sepsis itself.[186] 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 nonhuman 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.[189] Furthermore, baboons and mice are less sensitive to a species of bacteria commonly used to induce sepsis in experimental settings.[190] A recent study found that rhesus macaques and baboons differ markedly in their innate immune response to pathogens compared to humans.[191]

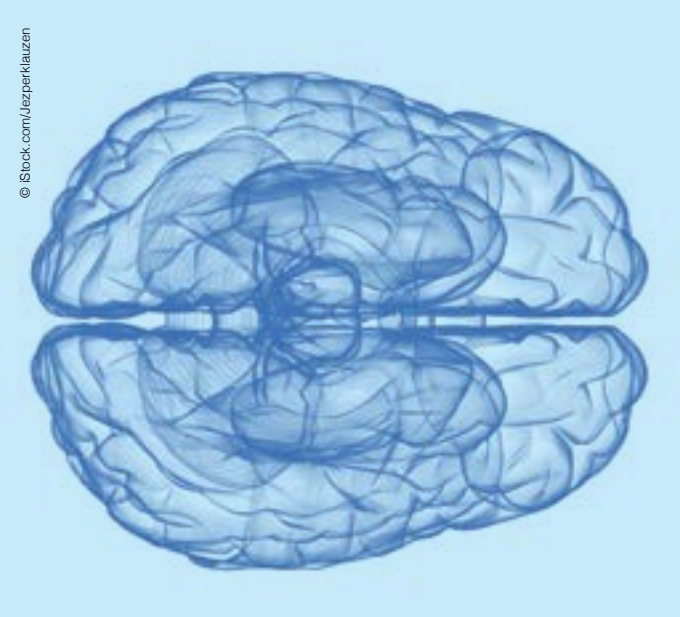
A 2019 report from the National Advisory General Medical Sciences Council (NAGMSC) Working Group on Sepsis states, "Despite decades of intensive study of the underlying mechanisms of this condition, no new drug or significantly new diagnostic technology has emerged. Dozens of prospective trials of agents or strategies targeting the inflammatory basis of sepsis have failed."[192] In its report, the

NAGMSC Working Group on Sepsis recommended that the National Institute of General Medical Sciences (NIGMS), under NIH, "rebalance" its sepsis research–funding portfolio to "include a more clinical focus."[192] 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."[193] In other words, NIGMS intends to prioritize 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.[194] 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 state that genomic information "will complement or even replace the need for mouse models in disease discovery and drug development."[194]

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."[195]
- 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.[196]
- 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.[197]
- Because early detection of sepsis is likely the most important factor in reducing mortality from this condition,[198] researchers around the globe are exploring different artificial intelligence and machine learning tools to aid in sepsis early prediction and diagnosis.[199-201]



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."[202] 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."[203] 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.[204] 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."[204]

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.[205]

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.[205]

University of Florida biomedical engineers Mobini and colleagues add, "We are incapable of truly mimicking human neural injures in animal models because of the extensive anatomical, functional, molecular, immunological, and pathological differences between humans and frequently studied animals."[206] 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 molds, 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.[206] Bioprinting can

use bioinks containing human cells and materials to construct heterogeneous tissue models in a single step and with great consistency,[207] an aspect of nerve regeneration research that has been particularly lacking in animal models.[203]

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."[208] 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.[209] 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.[210,211] 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.[206]

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.[212] For AD research, the clinical failure rate for new drugs is 99.6%.[213] This includes the 2018 failure of AstraZeneca and Eli Lilly's lanabecestat, which was hailed as extremely promising, due to futility.[214]

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.[215]

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,[216] 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 behavioral phenotypes, in part because of the transgenes used, inconsistencies in transgene insertion and expression, and mouse background strains.[217] 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.[217] 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,"[218] and that "animal models are not an ideal system for studying ALS or for developing drug therapies."[219] In PD, even nonhuman primate studies do not "constitute a valid scientific modality for the complete understanding of PD and for the development of future neuromodulation therapeutic strategies."[220]

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."[221] 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.[221]

As animal studies fall short, scientists and policymakers are realizing 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 toward more promising techniques involving patient-derived induced pluripotent stem cell models, "omic" technology (genomics, proteomics, etc.), *in silico* models, neuroimaging, and epidemiological studies.[222] 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.[223]
- Collaborators from numerous medical schools in China, using resources from the Chinese National Human Brain Bank for Development and Function, recently analyzed 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.[224]
- Thanks to developments in human brain imaging, scientists at the University of Cambridge were able to trace tau protein in human brains.[225]
- 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 modeling both forms of the disease.[226]
- 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.[227]
- 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) azetidinylmethoxy) 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.[228]

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.[229] When the researchers tested three known drugs on this model, the results matched live human data. Scientists at Harvard University and Lawrence Livermore National Library 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.[230,231]

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.[232]

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"[233] 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 behavior representing hallmarks of these disorders cannot be produced or properly assessed in animals.

Human depressive disorders, for example, are characterized, in part, by a generalized feeling of sadness, hopelessness, and despair. In an effort to measure "despair" in rodents, the most commonly used behavioral 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.[234] 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 behavior 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.[235-237]

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.[238]

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.[239-241] A similar failure of animal studies to contribute to clinical knowledge has been noted with bipolar depression research,[242] and animal studies have been cited as the primary source of attrition (failure of drugs) in neurobehavioral clinical trials.[243] Nevertheless, thousands of published papers ignore these warnings and use the forced swim test to draw erroneous conclusions about an animal's mood[234] 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.[244] 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 analyzed 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."[245]

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 behaviorists 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."[246] 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."[246]

Funds should be allocated to more relevant, human-based experimental models, such as computational modeling using already well-defined biomarkers[247] and the use of patient-specific stem cells for personalized medicine, which "affords the ability to generate neuronal cell-based models that recapitulate key aspects of human disease"[248] 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."[249]

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.[250]
- 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.[251] The authors noted that mouse models of this genetic mutation have phenotypes that are the opposite of what is observed in humans and that a "patient-derived model will be ideal and more beneficial than looking at the mouse."[252]
- 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.[253] Along with behavioral biomarkers, the team used machine learning to examine correlations between real-life behavioral 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.[254]
- 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.[255]

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."[256]

Many factors contribute to this failure, such as flaws in experimental designs, 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."[257] 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.[257] These realities illustrate the need to shift away from animal models and focus on human-centered methods.

In a 2017 review,[258] Clemens Sommer, M.D. 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 signaling 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,"[259] 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."[258]
- 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-signaling molecule during stroke.[260]
- 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.[258]

- 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.[261]
- 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."[262]

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.[263] 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.[264] 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.[265] 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 discovery[266] 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.[267] Clinical researchers are now utilizing artificial intelligence to improve stroke prevention, detection, and care.[268-270]

A report authored by 42 scientists following a National Institute of Neurological Disorders and Stroke workshop 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."[271] 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 U.S. suffers a stroke and that every four minutes, someone dies of one,[272] we cannot afford to spend our limited resources on substandard, animal-based research.

Substance Abuse

Recommendation: End the use of animals

Fundamental aspects of nonhuman 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.[273] It has been argued that attempts to model human disorders such as addiction in nonhuman 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."[274]

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."[275] 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.[275]

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.[276] 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.[276] 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."[276]

The nonhuman 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."[277] National Institute of Mental Health researchers 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."[278]

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."[273] 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.

Non-invasive human research methods can provide us with answers to the questions that nonhuman 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."[279]

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.[280] 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.[281] Researchers at the National Institute on Drug Abuse are using three-dimensional neocortical organoids to study the effects of prenatal cocaine exposure on the developing human brain.[282] 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 fetal alcohol spectrum disorders.[283] Other investigators are using human iPSCs to study the effects of alcohol on the human liver.[284]

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 programs.

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.[285] In addition, there are differences in the administration of mechanical ventilation and drugs such as vasopressin and heparin in research.[285,286] 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,[287] if not impossible. In studies of traumatic brain injury, all promising therapeutics identified in animals have failed in human clinical trials.[288]

There is a significant amount of discussion regarding the limitations of animal models of trauma and hemorrhagic shock, which is summarized 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 traumatisation, 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.[289]

Fortunately, it has been shown that computer simulation can accurately replicate real-life trauma and predict patient outcomes.[290] 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.[291] This Pittsburgh group also used data-driven and mechanistic modeling 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."[292]

In addition, clinical research remains invaluable in this field and both informs and benefits from mathematical and computer modeling. A study conducted at the U.S. 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 hemorrhage. 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.[293]

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.[294–296] 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.[297,298] 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

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 anesthesia. 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."[299]

The use of animals in forensic research was heavily criticized 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."[300] 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."[300]

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 anesthetized."[299] 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.[301] 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."[301]

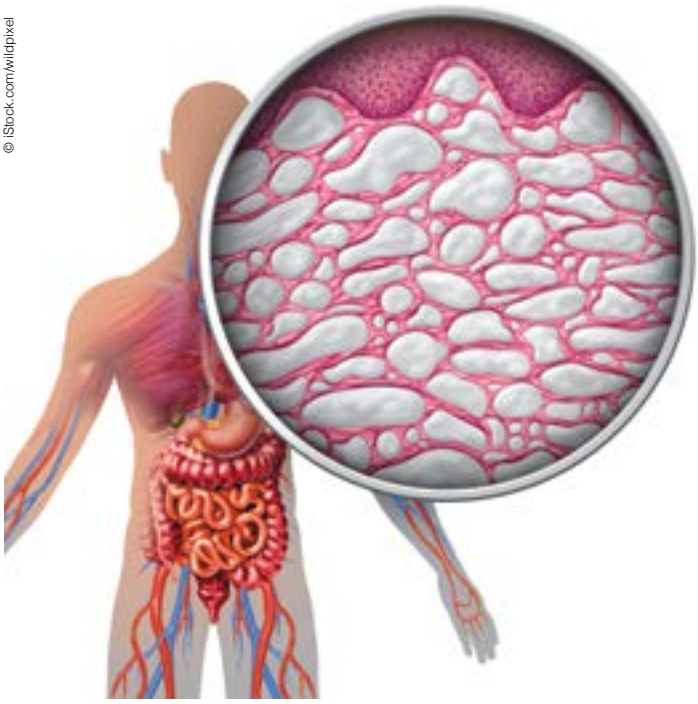
Cruelty aside, Cattaneo and colleagues stress, "[T]he history of forensic sciences has provided us with much evidence of the inapplicability of data obtained from studies performed on animal models,"[299] given the anatomical, physiological, and genetic differences between species. For example, recent research funded by the National Institute of Justice and conducted at the Forensic Anthropology Center at the University of Tennessee indicates that decomposition data from nonhuman animals varies considerably from humans and is not recommended for use in forensic casework.[302]

In addition, there is a plethora of alternative methods, such as manikins, simulators, artificial materials, and *in vitro* technology, and it has been recognized that "applying alternative methods rather than using animals has provided, in the forensic field, important and reproducible results."[299] 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,[303] rising public opposition to animal use in



laboratories,[304] increasing animal laboratory cost burdens,[305] and a renewed focus by the medical community on improving patient safety and reducing clinical errors through simulation-based training.[306]

Human simulation–based teaching has become the gold standard. Now, medical students in Canada, India, and the U.S. learn without using animals throughout the undergraduate curricula.[307,308] Medical experts have recommended a transition away from an animal-based pedagogy and toward "a robust curriculum composed of didactics, task trainers, virtual reality, cadavers, computer software, high-fidelity patient simulators, and supervised clinical work."[309] 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.[310]

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.[311] 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.[312] 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."[313] Other evidence supports using simulations to improve skills and/or clinical performance in lumbar punctures,[314] suturing,[315] myringotomy,[316] 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 tumors, 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."[317]

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.[318]

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 favorably for acquiring basic microsurgical skills. The authors state that the Surgitate model "could be particularly useful in enhancing suturing skills as a replacement or reduction in the use of chicken models."[319] Given that plastic surgery is a subspecialty that often uses microsurgical techniques,[320] 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."[321]

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.[322]

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.[323]

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 U.S. 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."[324] 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."[325]

Non-animal methods are used exclusively instead of animals for military medical education by more than 70% of NATO member states,[326] and the U.S. Coast Guard has become the first branch of the U.S. Armed Forces to end the use of animals for this practice.[327] 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 Board[328] as well as numerous medical and veterans organizations representing more than 255,000 physicians and doctors-in-training, which have former U.S. surgeons general among their leadership.[329]

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."[330]

One study examined the training of U.S. Navy and U.S. 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."[331]

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)."[332]

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."[333]

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,[334] and national ATLS programs in numerous countries have made the transition to ending animal use for this purpose.[335]

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 for 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)[336] 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.[337] 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 "check box" approach to a risk-centric approach that allows for the minimization of tests on animals.[338]

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.[339] 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 hepatocytes[340] and rainbow trout liver S9 subcellular fraction[341] and an associated guidance document[342] 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.[343]

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 test[344] in a WoE approach[345] 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,[346] and the respective OECD test guideline was adopted in 2021.[347] This *in vitro* assay has the potential to reduce or even replace the use of fish in the acute fish toxicity test.[348]

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 centered around a probabilistic Bayesian network approach.[349] 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.[350] 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 U.S. 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 fulfill 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 U.S. EPA to retrospectively assess the use of avian oral and dietary tests in risk management decision-making.[351] 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.[352]

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 analyze 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 modernize ecological testing using predictive computational methods that have the potential to reduce testing on terrestrial animals while improving ecological protection.[353]

Global harmonization 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) of India require the use of a single test species for the avian reproduction test, while the U.S. 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,[354] 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 estrogen, androgen, thyroid, and steroidogenesis (EATS) systems and, to a lesser degree, the retinoid pathway.[355]

Much is understood about the complex mechanisms through which chemicals can interfere with endocrine pathways in humans[356] and wildlife.[357,358] Numerous AOPs related to endocrine disruption are included in the AOP-Wiki,[359] and the OECD has published several case studies on IATAs.[360] 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).[361] 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 project[362] aims to develop three-dimensional *in vitro* tools to screen for the influence of endocrine disruptors on the thyroid gland.

The U.S. 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 prioritizes 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 U.S. and the EU. Following a comparative study of ToxCast estrogen pathway assay results and uterotrophic assay results,[363] the EPA announced that it will accept the data from the ToxCast ER Bioactivity Model as an alternative to at least one animal test[360,364,365]—the uterotrophic assay—that screens for effects on the estrogen pathway.[366] 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 estrogen or the androgen pathways. In collaboration with other organizations, 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.[367]

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, hemorrhaging, 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.[368] 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.[369] An OECD guidance document on an IATA of serious eye damage and irritation was published in 2017,[370] 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.[371] Also, the EPA, in collaboration with PETA Science Consortium International, the U.S. 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.[372]

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.[373,374] However, if a substance produces negative results in the *in vitro* tests, it can be categorized 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).[375,376]

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.[377]

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,[378,379] and the ToxTracker assay[380–382] can provide information on the mode of action of potential genotoxicants and have been submitted to formal regulatory "qualification" programs.[383,384] 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.[385]

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.[386,387] The information requirements for genotoxicity assessment on cosmetics[388] 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.[389]

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.[380,390]

The genotoxicity[391] and mutagenicity[392] case studies on IATA, under the OECD IATA case studies project,[393] 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 reproducibility[394] and its inability to predict human outcomes.[395] 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.[395–400]

For example, in an assessment of 202 pesticide evaluations from the EU review program, 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 labeling purposes.[401] 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.[402] This study was used to support an international collaboration that developed a WoE approach to fulfill some of the carcinogenicity test requirements without the two-year test on rats.[403,404] 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.[405] 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.[406]

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.[407] 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.[408,409] 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.[410,411]

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.[412] Additionally, the EPA has published a computer model, OncoLogic™, to evaluate chemicals for carcinogenic potential,[413] 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.[414]

Given the complexity of carcinogenesis, experts recognize 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.[415] 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.[406,414,416,417]

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.[418]

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.[419]

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.[420] 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 emphasizes the relevance of OECD Test No 432.[421]

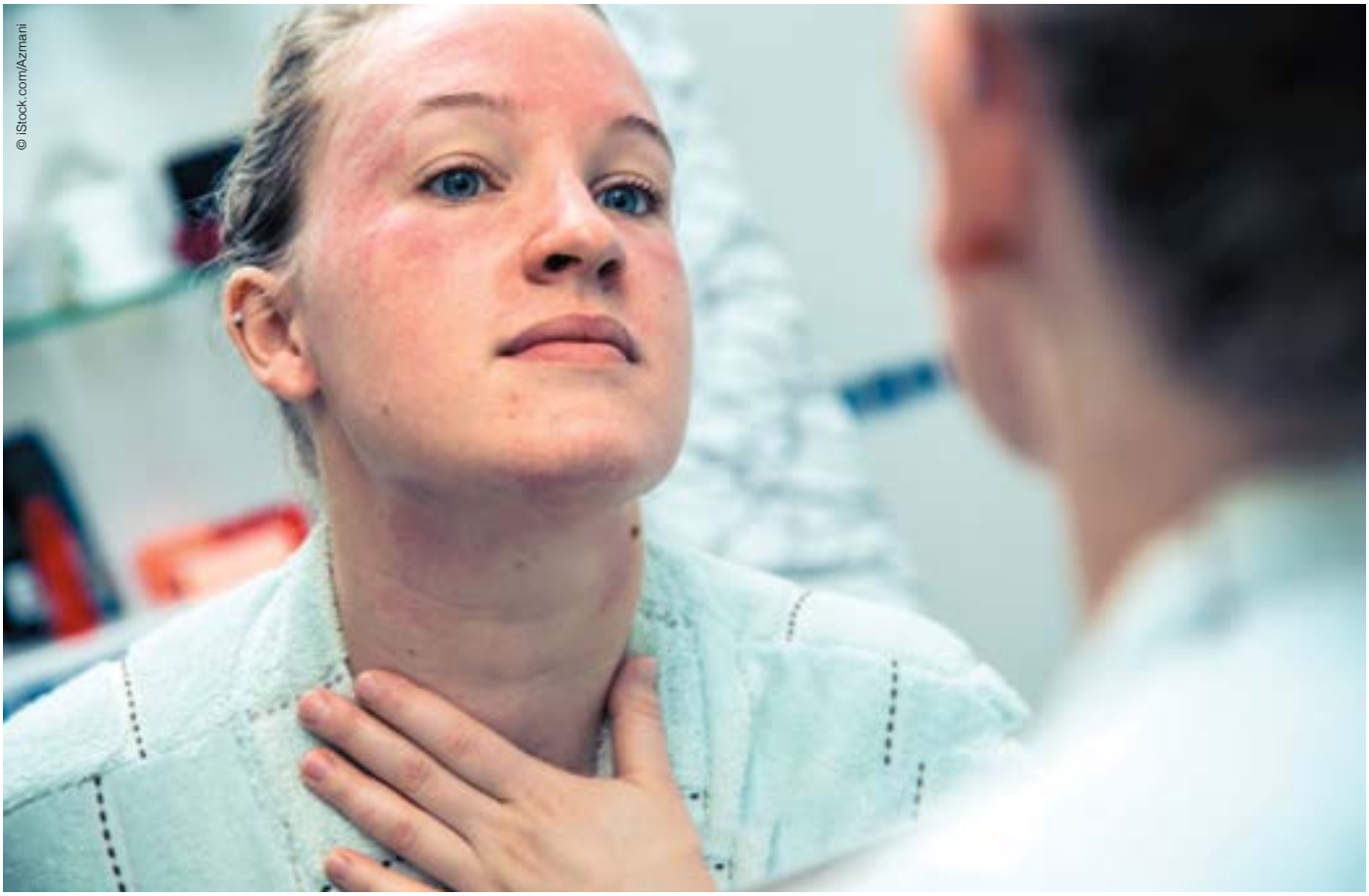
Pyrogenicity

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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temperature are monitored rectally. In Europe alone, more than 200,000 rabbits were used between 2015 and 2019 in the RPT,[422] 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.[423]

The Limulus amoebocyte lysate test (LAL), also called the bacterial endotoxins test, requires the use of hemolymph 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.[424] A synthetic version of the LAL, in which the hemolymph 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.[425]

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.[426] 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.[427] 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.[428]

Regulators in the EU, India, the U.K., and the U.S accept the MAT, and the pharmacopeias used in these regions all allow its use following product-specific validation. Nevertheless, animal tests are still used despite their well-documented limitations.[429] To eliminate the use of animals in pyrogen tests, regulatory authorities and standards organizations must make an increased effort to integrate and harmonize 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 organized 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.[430]

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 emphasize that it is considered a replacement for animal-based pyrogen tests.[431,432] This endorsement is repeated in statements from the European Medicines Agency[433,434] 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.[427] In the 8th edition of *Indian Pharmacopoeia*, the Indian Pharmacopeia Commission revised the pyrogen testing general chapter, introduced the monograph on the MAT, and replaced the RPT with LAL.[435] 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.[436]

None of the *in vivo* methods used for testing reproductive and developmental toxicity have been formally validated for their relevance to humans.[437] 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. PPARγ activation leading to impaired fertility.[438,439] The EU FP6 project ReProTect has also investigated possible strategies to cover the entire mammalian reproductive cycle, resulting in a series of published works.[440] Furthermore, the ChemScreen FP7 project has been designed to generate a rapid screening system that is relatively simple and cost-effective.[441]

In addition, the EU-ToxRisk project integrates advancements in cell biology, "omic" technology, systems biology, and computational modeling 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* modeling approaches.[442] 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, with the partner agencies working on case studies that apply to different chemical classes.[443] In 2021, Health Canada[444] 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 $POD_{Traditional}$.[444]

While the field is gradually moving toward 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.[445] 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.[446]

There are opportunities to avoid the animal test based on criteria described in OECD guidance document no. 237.[369] Furthermore, the OECD has developed an IATA for skin

irritation using *in vitro* skin irritation and corrosion methods that avoids or minimizes animal use.[447]

- **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. This 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 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.[448]

Skin Sensitization

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

The assessment of skin sensitization 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 maximization test or to the ears of mice in the local lymph node assay.

The regulatory requirement to test for skin sensitization 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.[337] 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 sensitization 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 sensitization 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 sensitization AOP.

The non-animal approaches to predicting skin sensitization are as good as or better than the local lymph node assay when compared to human data.[449]

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 (LD_{50}) or lethal concentration 50 (LC_{50}) for inhalation testing—is determined. The LD_{50} 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,[450] while another analysis of existing acute oral LD_{50} data demonstrated that replicate studies result in the same hazard categorization on average 60% of the time.[451] 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,[369] and the EPA has published similar guidance for pesticides and pesticide products.[452] 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.[453,454]

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 modeling 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.[454] 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.[455]

Oral Route

NICEATM and ICCVAM organized a project to develop predictive models for acute oral systemic toxicity.[450] 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.[456] CATMoS is implemented through Open Structure-Activity/Property Relationship App (OPERA), a freely available and open-source QSAR tool.[457] This model is routinely optimized, and updates are available on the NICEATM Integrated Chemical Environment (ICE) and EPA websites.[458] PETA Science Consortium International, Physicians Committee for Responsible Medicine, and 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 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.[459] *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.[460–462]

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.[373] 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).[463] 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 analyzed the relative contributions of data from acute oral and dermal toxicity tests to pesticide hazard classification and labeling. 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.[464,465] In addition, dermal studies are not required for substances that are non-classified by the oral route and not absorbed dermally.[369] 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 aerosolized or otherwise made respirable under conditions of use). However, in instances in which testing is required, non-animal methods can be applied to fulfill the informational requirements. For example, to fulfill 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.[466] In another example, the EPA is considering data from *in silico* computational fluid dynamic modeling and *in vitro* testing using three-dimensional reconstructed human lung tissues to fulfill the re-registration requirements for a pesticide.[467] Several other promising research efforts are underway to develop non-animal methods for inhalation toxicity.[468]

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.[469,470] Additionally, the Science Consortium has funded method development and organized several awards to provide researchers with equipment and *in vitro* respiratory tissues to conduct inhalation toxicity studies.[471] 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) states 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.[472] In addition, Belgium, Estonia, Germany, Slovakia, and the U.K. already prohibit the use of animals for the development and testing of tobacco products because of ethical concerns.[473–477]

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 vapor at the air–liquid interface, CTAs, and genomic analyses.[470,478,479] 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 U.K., restricted or banned the production of antibodies obtained via the ascites method because of animal welfare concerns.[480,481]

Growing concern about the lack of quality and reproducibility of animal-derived antibodies, which often show poor specificity or fail to recognize 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.[482] In addition, a 2015 *Nature* news feature reported that antibodies may be the laboratory tool most commonly contributing to the "reproducibility crisis."[483] In fact, poorly characterized 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.[484] 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.[482]

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.[482] 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.[480,485] 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.[485]

International efforts have highlighted the importance of a large-scale transition from animal-derived antibodies to animal-free affinity reagents. In the U.S., experts and organizations including NICEATM and PETA Science Consortium International are working to increase access to animal-free affinity reagents. In December 2019, both organizations 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."[486] 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.[487]

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 catalog 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.[488-492] 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-immunizing 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,[493,494] and more are in development,[495] including a candidate diphtheria antitoxin based on human recombinant antibodies created with funding from PETA Science Consortium International.[496]

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.[488] 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).[497] 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).[498] In the absence of formal oversight of the implementation process, these barriers are left to be resolved informally through workshops and decentralized 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.[499] 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.[500-502] Accelerating and standardizing 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 harmonized manufacturing consistency requirements, as tightly controlled manufacturing consistency policies are the foundation of many animal replacement strategies.[503,504]

Fetal Bovine Serum

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

Fetal bovine serum (FBS) is a supplement for cell culture

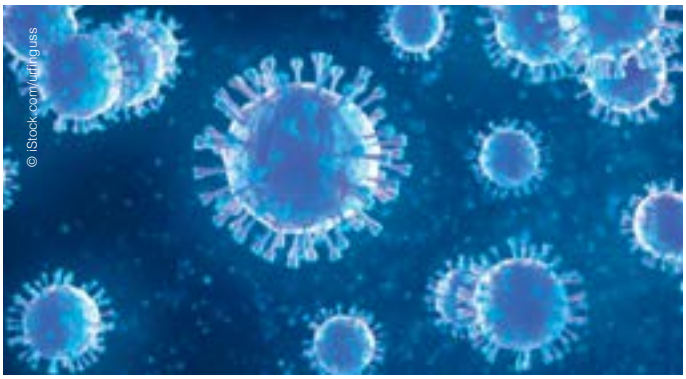
media that provides an undefined mixture of macromolecules that function to maintain cell viability and facilitate cell metabolism, growth, proliferation, and spreading in culture. When pregnant cows are slaughtered, a large-gauge needle is used to draw the blood from the beating heart of the fetus.[505,506] Because the unborn calves are not anesthetized at the time of blood collection, they likely experience pain. It has been estimated that 600,000 liters of FBS are produced globally each year, which translates to the use of up to 1.8 million bovine fetuses for this purpose.[507]

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 organizations hosted workshops in 2003 and 2009 that called for the transition from FBS to non-animal serum supplements in cell culture.[508,509] A third workshop on FBS and alternatives was held in 2016, organized by the SET Foundation and the Deutscher Tierschutzbund (German Animal Welfare Federation).[506] 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 optimize 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.[510]

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 optimization of non-animal, serum-free media when needed. For cell types in which non-animal supplement concentrations have not yet been optimized 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 toward 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 harmonization 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 U.K. Chemicals Stakeholder Forum and regularly comments on OECD test guidelines as a member of the International Council on Animal Protection in OECD Programs (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.

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> **“[I]f research conducted on animals continues to be unable to reasonably predict what can be expected in humans, the public’s continuing endorsement and funding of preclinical animal research seems misplaced.”[4]**

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