PETA

The Research Modernization Deal 2021

People for the Ethical Treatment of Animals

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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.

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

- 95% of all new drugs that test safe and effective in animal tests 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.
- 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 animals.

To improve research results and develop more effective human treatments, PETA proposes the following:

- Immediately eliminate animal use in fields of research for which animals have been shown to be bad “models” for humans and have impeded progress.
- Rebalance the public funding of medical research so that the majority goes to sophisticated human-relevant, animal-free research methods.
- Conduct scientific reviews of the efficacy of animal use to identify additional areas in which such use has failed to advance human health, or in which non-animal methods are now available, and can therefore be ended quickly.
- Implement a cost-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 high-tech non-animal testing strategies in regulatory toxicity testing.

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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: The 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 scholarly research in the last 15 years shows that animal studies are flawed and divert both monetary and intellectual resources from methodologies better suited to curing human disease. 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, 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 animal experimentation. 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.[1]

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"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." [483]

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In this report, we offer a roadmap for replacing the use of animals in experimentation, identify a number of strategic priorities, and append further information regarding areas of both regulatory (government-required) and nonregulatory research where there are opportunities for the immediate and near-future replacement of animal use. We have also included information outlining areas in which further development, validation, and implementation of non-animal methods are required.

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,"[2] even though they appeared safe and effective in preclinical experiments using animals. A stunning 2014 analysis published in *The BMJ* found that—contrary to public perception—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.[3]

Lack of Validity

Problems with internal and external validity contribute to the failure of animal experiments in the translation of biomedical research from bench to bedside. The internal validity of animal experiments is undermined by the poor study design, including failure of animal experimenters to implement processes to prevent bias, such as blinding the individuals conducting the experiments or those analyzing the data.

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

to reduce bias in animal experiments likely results in overestimation of the benefits of the treatment studied.[4] 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."[6]

Poor internal validity means that many experiments on animals cannot be reproduced, a critical aspect of the scientific process that speaks to the potential validity of a finding. It can therefore be of little surprise that a 2015 investigation concluded that between 50% and 89% of all preclinical research, a large part of which involves animal testing, could not be reproduced.[7] At the most conservative U.S. estimate, this results in approximately $28 billion per year spent on experimentation that is misleading.[8] NIH Director Francis Collins and Principal Deputy 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."[9]

However, the weaknesses of animal experiments cannot be overcome by simply improving study design, because external validity, or the "extent to which research findings derived in one setting, population or species can be reliably applied to other settings, populations and species,"[10] can never be achieved. Inherent species differences mean that nonhuman animals cannot serve as analogs for understanding the specific biological details necessary to develop safe and effective drugs for 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."[11]

Therefore, animal experiments 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 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.[12] Attempts to control for or correct species differences result in what the authors refer to as the "extrapolator's circle": "[I]f we want to determine whether a mechanism in animals is sufficiently similar to the mechanism in humans to justify extrapolation, we must know how the relevant mechanism in humans operates. But if we already know about the mechanism in humans then the initial animal study is likely to have been redundant."[13] They also discuss the concerning trend among those involved in animal experimentation to minimize the issue of species differences and the effects on external validity, a problem that is acknowledged by a number of researchers.[14,15] 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."[16] There is growing scientific consensus that far more is to be gained from human-relevant research methods and technologies that are better suited to solving human biomedical and regulatory assessment paradigms than from reliance on animal experiments. As a recent U.K. industry report emphasized, the time has come to humanize drug discovery and toxicology.[17]

Inherent species differences mean that nonhuman animals cannot serve as analogs for understanding the specific biological details necessary to develop safe and effective drugs for humans.

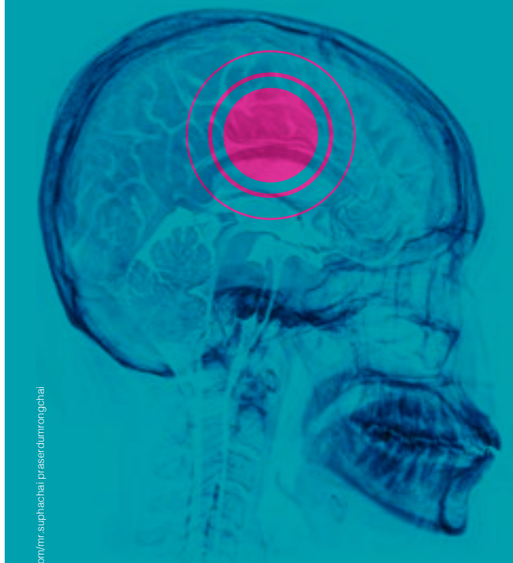
The difficulties in applying data derived from animals to human patients are compounded by the confinement and unnatural conditions of laboratory life, which thwart animals' ability to engage in natural behavior.[18,19] This deprivation contributes to their stress and alters their physiology and neurobiology, causing them to exhibit various psychopathologies.[20,21,22,23,24] Importantly, the fact that animals in laboratories have altered physiology and neurobiology means that they will never be good "models," even for members of their own species who live in the wild. A mouse in a laboratory will not respond to a drug in the same way that a mouse in a field would. One then has to ask, how does this biologically distinct mouse reliably represent the biology of human beings?

Misplaced Resources

Despite the growing evidence that experiments using animals are wasteful and can impede medical progress, approximately 47% of all NIH research funding goes toward animal experimentation.[25] Federal funds available for biomedical research are a finite resource. In fiscal year 2017, only 18.7% of grant applications submitted to NIH were awarded funding.[26] 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.

The 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."[27]

Lack of Clinical Success



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The failure of basic and applied scientific studies involving animals is perhaps most evident in the stark litany of seemingly promising treatments that have simply 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.[471]

Oncology drugs, which also undergo animal testing, have a success rate of only 3.4%.[472] 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%.[473] (See the appendices for a comprehensive look at disease areas.)

Funding for biomedical research is allocated into two categories: basic research and applied research. NIH defines 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.[28] A great deal of basic research involves studies on animals.

NIH perceives basic animal research 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 animal experiments. Their investigation of the application of basic science to clinical applications found that fewer than 10% of highly promising basic science discoveries enter routine clinical use within 20 years.[29]

Yet the NIH Office of Budget estimates that the agency consistently spends over half its funding on basic research each year,[30] most of which involves animals.

The Need for a Paradigm Shift

If our finite public funds are to be used responsibly, they must fund research, whether basic or applied, that leads to effective treatment for humans. 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.

Some within the scientific community have begun to advocate for change. In support of using an evidence-

based approach to accelerating the delivery of useful drugs to the patients who need them, 15 Vanderbilt University researchers published a 2017 article calling for the elimination of experiments using animals where there is clear evidence that animal "models" 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.[31]

The shifting consensus away from the use of animals in experimentation can be be observed in a number of arenas, including in publications documenting the limited predictive value of experiments on animals,[32] in the increased awareness of animal cognition and sentience,[33] and in the fast-eroding public support for animal studies.[34]

For example, *The Turkish Journal of Gastroenterology*—the journal of the Turkish Society of Gastroenterology—officially banned the publication of studies involving experiments on animals from its pages. Journal editor Dr. Hakan Şentürk wrote that the new policy represents "growing concern about the lack of applicability of animal research to humans."[35] 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."[36]

Significantly, a move away from animal-based research will allow for substantial growth in the science and technology sectors and for faster return on investment in drug research and development.[37] An evolution of research funding priorities towards human-relevant methods will get treatments to the patients who need them more safely and likely in less time.[38,39]

Opportunities for Economic Advancement

The High Cost of Drug Development

By mandating a move away from animal experimentation and toward advanced scientific methods, the U.S. has the opportunity not only to advance biomedical research but also to expand job growth rapidly in science and technology and reduce health-care costs for the population. As Meigs and colleagues report in their recent review, "Animal Testing and Its Alternatives—the Most Important Omics Is Economics," "an economy of alternative approaches has developed that is outperforming traditional animal testing."[40]

In the current system, moving a new drug to market may cost up to $2 billion and take as long as 15 years.[41] 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.[42] During a 2017 conference, 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." 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,"[43] 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."[44]

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 clinical trials. Ninety-five percent of drugs that test safe and effective in animals fail in humans[45] because they are either not safe or not effective.[46] Furthermore, it may be that drugs that could be effective in humans are rejected without clinical trials because they were ineffective 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.[47]

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 humans and animals to novel chemical

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, aged 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."[474]

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."[475] Five of the six participants had to remain 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.[476] 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."[477]

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.[478,479] Researchers later remarked on the good fortune that it was not first tested in guinea pigs, for whom the antibiotic is lethal. Had experimenters seen this result, penicillin may have never been tried in humans.[480,481]

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.[48]

Additionally, even in journals that support the "Animal Research: Reporting of In Vivo Experiments" (ARRIVE) guidelines[49]—which aimed to improve the reporting of research using animals—studies continue to demonstrate low reproducibility, poor value for money, and a waste of animals' lives.[50]

With the use of human-relevant technology in place of expensive, time-consuming, and inaccurate animal experiments, the cost of drug discovery has the potential to decrease dramatically. 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-based *in vitro* technology for biomedical research and testing is growing rapidly. For example, the Boston-based startup Emulate, Inc., recently raised an additional $36 million in financing to expand its human organ-on-a-chip technology.[51] It is currently being used by AstraZeneca, Roche, Merck, Johnson & Johnson, and others to predict more accurately the safety and efficacy of drug candidates.[52]

Revisiting Failed Drugs

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An April 2018 study published by Emulate and Janssen Pharmaceuticals 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 animal experiments.[482]

A leading market research company estimates that "[t]he global market for cell-based assays should grow from $20.1 billion in 2018 to $32.7 billion by 2023,"[53] the "global induced pluripotent stem cells (iPSC) market should reach $3.8 billion by 2024,"[54] and the 3D cell culture market "should reach $3.9 billion by 2021."[55] The market researchers also projected that the global regenerative medicine market will reach a volume of $89.5 billion by 2025.[56] 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.

Regulatory Opportunities for Humane Toxicity Assessment

The past quarter-century has seen a revolution in the way in which chemicals are tested. Non-animal tests are rapidly replacing animal tests. This is the result of our better understanding of biological processes and the emergence of new technology, which has allowed for the development of testing methods that can look directly at cellular mechanisms rather than at the crude, inscrutable results that come from using animals. It is also the result of public pressure and, as explained below, dissatisfaction among scientists with the results from animal tests. Cellular and genetic information about the potential toxicity of a chemical, such as the potential for receptor binding or gene or pathway activation, is obtained more readily with non-animal tests (using human cells *in vitro*) than with animal tests (*in vivo*).[57]

Concurrently, there is growing recognition among regulators and the regulated community that animal-based methods do not adequately protect either human health or the environment and that "the current approach is time-consuming and costly, resulting in an overburdened system that leaves many chemicals untested, despite potential human exposure to them."[58]

In 2007, the U.S. National Academies of Sciences, Engineering, and Medicine 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 and reducing the time, money, and number of animals needed for testing.

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

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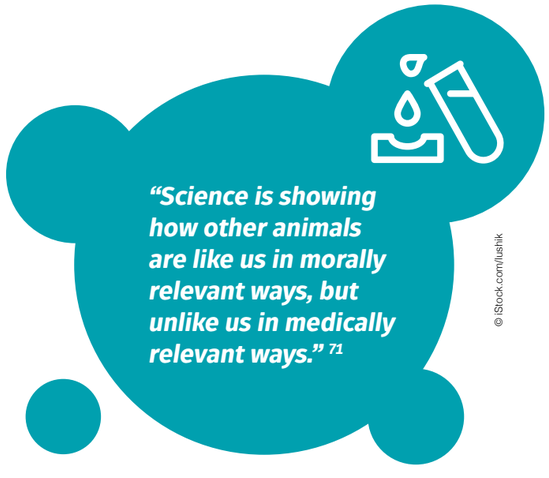
pathways—also known as adverse outcome pathways (AOPs). These are cellular pathways that, when sufficiently perturbed, are expected to lead to adverse health effects.

The committee recommends the use of high-throughput assays—rapid, automated experiments that can test hundreds or thousands of chemicals over a wide range of concentrations—to evaluate chemicals' effects on these toxicity pathways. On the basis of data from these and other experiments, researchers could develop models to describe responses in toxicity pathways as well as models to estimate the human exposure necessary to produce responses in these pathways.[59]

By eliminating the use of tests on animals for regulatory purposes where full replacements exist and by promoting the acceptance of methods currently in development, we have the opportunity to shift the regulatory testing paradigm further towards innovative non-animal techniques and thus become world leaders in the application of these methods. In the appendices to this report, we elaborate on opportunities to end the use of animals for regulatory testing immediately or within the next two to 10 years. The relevant testing areas include acute systemic toxicity, genotoxicity, pyrogenicity, vaccine and biologics, endocrine disruption, and carcinogenicity.

Public Opinion and Animal Sentience

Public opposition to the use of animals in experiments has increased steadily, from 8% in 1948[60] to 52% in 2018.[61] 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.[62] If 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.



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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,[63,64] a recent retrospective analysis by Pandora Pound and Christine J. Nicol concluded that "[t]he regulatory systems in place … failed to safeguard animals from severe suffering or to ensure that only beneficial, scientifically rigorous research was conducted."[65] They compared the harms experienced by animals in preclinical studies for six treatment interventions to 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 animal research. 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.[66]

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."[67] This declaration contrasts with the work of early animal experimenters such as behavioral neuroscientist B.F. Skinner, who asserted that animals' brains were mere automata[68] and who infamously made hungry pigeons peck at each other to receive food[69] and locked animals in electrified boxes[70] to test his hypotheses.

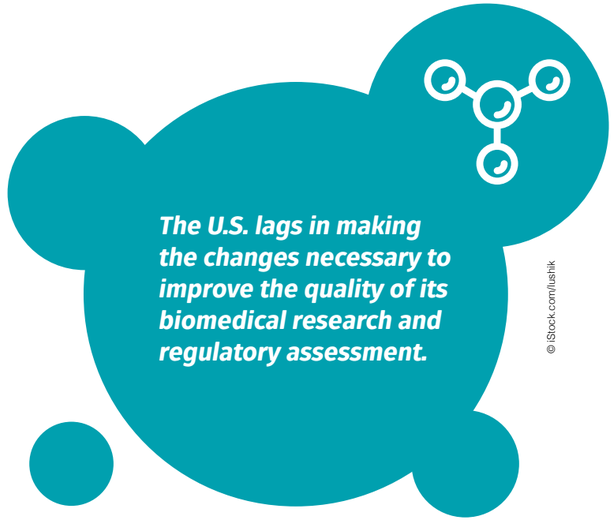
The Cambridge Declaration on Consciousness illustrates that recognition of animal sentience is growing within the scientific community, too. Statistics make clear that animals are not appropriate human surrogates in biomedical research, but when it comes to their ability to suffer, how much like humans need they be before a critical review of animal-based research is considered mandatory? 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."[71]

Over 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.[72] "The deliberate and routine abuse of innocent, sentient animals involving harm, pain, suffering, stressful confinement, manipulation, trade, and death should be unthinkable. Yet animal experimentation is just that: the 'normalisation of the unthinkable,'" write the report's authors. They conclude that experimenting on animals contradicts what we now know about animals' ability to experience not only pain but also shock, fear, foreboding, trauma, anxiety, stress, distress, anticipation, and terror.

World Leadership

There is movement internationally that 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. The European Union, the U.K., Belgium, Austria, Sweden, the Netherlands, New Zealand, Australia, and Japan 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.[73]

In 2016, the Dutch government announced its plan to phase out toxicology tests on animals for chemicals, food ingredients, pesticides, veterinary medicines, and vaccines by 2025. The decision came after the Netherlands National Committee for the Protection of Animals Used for Scientific Purposes (NCad) stressed the need for a paradigm shift away from treating procedures on animals as the gold standard. Its report on the Netherlands' transition to non-animal research included objectives for the country to become an international leader in the field of innovation without animals in applied and translational research.[74] Subsequently, the Transition Programme for Innovation



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Without the Use of Animals 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.[75]

In many parts of the world, cruel and deadly cosmetics tests are now illegal or policies are in development to ban such practices. In addition, Israel and India have ended animal testing for household products and their ingredients and the U.K. Home Office has placed strict limitations on the use of animals for such tests.[76] The U.S. Environmental Protection Agency (EPA) announced in 2019 that it would provide additional funding for the development of non-animal methods and reduce tests on mammals by 30% by 2025, with a view to eliminating these tests completely by 2035.[77] Such changes are necessary to improve the quality of biomedical research and regulatory assessment.

Plan for Action: Recommendations to Modernize U.S. Biomedical Research

1. Immediately eliminate animal use in research areas in which animals have been demonstrated to be poor "models" of humans and their use has impeded scientific progress.

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

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

Poor predictivity of preclinical experiments on animals for toxicity and efficacy in humans has led to high attrition rates in the development of new therapies and is likely the cause of poor investment in the life sciences. 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 skin 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 most recent five-year strategic plan, NIH announced that it would reduce and replace animal experiments:

> 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.[78]

NIH and other federal agencies must now take the next step and end the funding of crude experiments that have failed to provide effective treatments and cures. With greater investment in exciting and innovative non-animal methods and bold policy initiatives, far more promising cures and treatments for humans can be developed. This will also alleviate the almost unimaginable suffering of millions of animals.

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.

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3. Conduct critical scientific reviews of previous animal studies to identify the areas in which the use of animals can be immediately ended.

For those areas of investigation where there is still some question as to whether the use of animals is beneficial, a thorough systematic review should be conducted to determine the efficacy of using animals. Systematic reviews, which critically analyze multiple research studies, are the first step in assessing the effectiveness of animal research. Some countries, such as the Netherlands, require that systematic reviews be conducted before animal studies can receive funding. Scientists at Radboud University Medical Center published the following statement prior to this mandate:

> Making systematic reviews of animal studies a routine is our scientific and societal responsibility, just as with clinical studies in humans. . . . Funding agencies should stimulate and fund systematic reviews. ... Systematic reviews disclose inadequacies in methodology of individual studies. This helps improve future study design, and reduce failure rate of animal studies of new drugs. Specifically, funding agencies can mandate systematic reviews of animal experiments as part of a funding. This will make the choice of animal models more evidence-based and provide better protection for human patients.[79]

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.[80] 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

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bodies and funding agencies had given their stamp of approval to these protocols. However, as we now know, the review processes in place were simply inadequate. Similarly, where thorough and objective scientific reviews of animal use for various areas of inquiry have not been conducted, they should be undertaken.

4. Implement an ethical harm-benefit analysis system.

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 sustained by 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,[81] the report of the Working Group on the Use of Chimpanzees in NIH-Supported Research,[82] and the research of Pandora Pound.[83]

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 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 harmonization and the wider statutory elimination of animal testing methods. Therefore, we advocate that national and international regulatory bodies and standards organizations liaise with industry, research agencies, and relevant NGOs 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[84]). In addition, we recommend that the establishment of a public-private center for predictive animal-free toxicology 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.

Conclusion

The current waste of resources, time, and animal 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.

Information on 29 areas of research and the astonishing failure of animal studies to lead to effective treatments for humans are detailed 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	Environmental Protection Agency
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
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
LAL	Limulus amebocyte lysate test
MAT	monocyte activation test
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NIH	National Institutes of Health
NOS	nitric oxide synthase
NRU	neutral red uptake
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
PD	Parkinson's disease
PDAC	pancreatic ductal adenocarcinoma
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
WoE	weight of evidence

APPENDICES

Please find below further detail 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 the scientists who work for PETA and its international affiliates.

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Basic and Applied Biomedical Research

Cancer

Recommendation: End the use of animals immediately

Oncology drugs have the lowest likelihood of approval among all disease categories. A survey of 4,451 drugs made by 835 companies between 2003 and 2011 found that only 6.7% of cancer drugs were approved after entering the first phase of clinical trials, even though they were all successful in preclinical testing. A 2018 analysis of data collected between 2000 and 2015 shows that the success rate for oncology drugs dropped to 3.4%,[85] suggesting that the problem is getting worse. The authors admit that the "current animal models (e.g., xenograft tumor models in mice) can be poor predictors of clinical outcomes in humans."[86] Even though study design and other logistical issues can be problematic, 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."[87]

Following an analysis of 1,110 mouse xenograft tumor models, which involve the transplantation of human tumor cells into mice, scientists and physicians from Harvard University, Massachusetts Institute of Technology, the Dana-Farber Cancer Institute, and other respected institutions reached a conclusion that challenged the ability of xenograft models to predict 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,[88] invalidating one of the foundational animal models for human cancer research.

There are numerous examples of the ways in which rodent models have misled cancer researchers. For brevity, we will present three cases. Scientists now know that endogenous bile acids, if dysregulated, can induce DNA damage and several forms of cancer, such as colon cancer, in humans. However, previous experiments on rats show that bile acids are not carcinogenic on their own. The profiles of bile acids, metabolism of bile acids (by the liver and gut microbiome), and colon epithelial cell accumulated turnover rate (adjusted by age) are all different between rodents and humans, contributing to the discrepancy.[89]

Another example of the disconnect between human cancer and rodent cancer research is the formerly proposed link between soy and breast cancer. It is now recognized that isoflavones in soy may be protective against several types of cancer, such as breast and prostate cancers,[90] particularly if people are exposed to it early in life.[91] However, it was observed that genistein, a major isoflavone in soy, induces estrogen-sensitive tumors in some animals used in studies, including rodents. The inconsistency was later explained to be due to differences in phase II metabolism of genistein in rodents, whose level of unconjugated, and hence active, genistein is about 20 to 150 times higher than that of humans (depending on the strain). Additionally, rodent models had low endogenous estrogen levels and different metabolic profiles compared to humans, and high experimental levels of isoflavones were used in those initial studies.[92]

Rodents are not suitable for radiation-induced carcinogenesis research, including for thyroid cancer. The nuclear architecture and spatial positioning of genes involved in radiation-induced injury are drastically different between rodent and human thyroid cells.[93] Similarly, rodents are not suitable for research into pancreatic ductal adenocarcinoma (PDAC). As some scientists have pointed out, "Although it may seem obvious that there are important differences between men and mice, this is often overlooked by those modeling human disease. … The potential for species differences to be relevant is greatest in models that use nonhuman PDACs, such as genetically engineered mouse models (GEMMs) and syngeneic xenografts."[94]

Given the many shortcomings described above as well as the astonishingly low translational success rate of cancer research, despite the popularity of using rodents in such research, it is clear that they are not good models for any type of human cancer experimentation. Therefore, it is wise to move away from rodent models and focus on human-relevant methods.

The prestigious Institution of Engineering and Technology (IET) global Harvey Engineering Research Prize was recently awarded to Portuguese scientist Rui L. Reis for his work using tissue engineering to create reliable 3-dimensional (3-D) engineered functional cancer disease models. According to IET, his innovative research will "help to predict the efficacy of novel cancer drugs and potential therapies, avoiding a range of unnecessary animal tests, and preclinical and clinical trials of doomed to fail new drugs."[95]

Other recent, human-relevant cancer research includes the development of a human blood vessel-on-a-chip to aid in the advancement of new cancer therapies that may inhibit new blood vessel formation to slow tumor growth,[96] the study of patient-derived human brain organoids to develop personalized therapies for deadly glioblastomas,[97] the use of a tumor microenvironment-on-a-chip to create precision medicine tailored to individual patients and specific cancer types,[98] and the application of 3-D printing to producing precise replicas of tumors using patients' own cells in the bioink.[99] In addition, by sequencing DNA and RNA in human skin cells, researchers at the University of California–San Francisco have analyzed which signaling pathways are disrupted in the evolution of melanoma.[100]

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."[101] Cancer is a highly variable, individualized disease that will require individualized treatment to overcome.[102] 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—instead of rodent—biology.

Cardiovascular Disease

Recommendation: End the use of animals immediately

Cardiotoxicity is a primary reason that drugs fail in clinical trials. Experts point out the "lack of concordance between the effects of compounds in animals (or animal-derived tissues) and those in humans,"[103] that "substantial differences in drug responsiveness between species can limit the effectiveness of predicting clinical outcome from animal toxicity testing,"[104] and the many known species-related differences in cardiac contractile function and calcium handling.[105] In a coauthored review, scientists from Stanford University, the U.S. Food and Drug Administration, and the biopharmaceutical company AbbVie refer to testing cardiotoxicity in animal models as a "black box" approach.[106]

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.[107] This makes the profile of ventricular repolarization and susceptibility of arrhythmia different, leading to varied drug responses. 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.[108] Rodents are also resistant to atherosclerosis, a major cause of many cardiovascular diseases, owing to their lack of cholesteryl ester transfer protein.[109]

For heart failure research, "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]."[110] 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."[111] Scientists in Singapore and New York City are using organ-on-a-chip models of blood vessels and beating heart tissue, respectively, to model human atherosclerosis and test human reactions to various drug compounds.[112,113] 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."[114] 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."[115]

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,[116] the use of a plant-derived cellulose framework as scaffolding to build networks of human veins,[117] and the development of an *in vitro* 3-D model of human early heart development that "could serve as an embryotoxicity screening assay in drug discovery, regulation, and prescription for healthy fetal development."[118] This 3-D "organogenesis-in-a-dish" model could provide a way to determine drug safety in pregnant women.

Computer modeling is also rapidly advancing human cardiovascular research. Recently, 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."[119] His research merges numerical computer models with experimental data to create modern cardiovascular biochemical models.[120] University of Oxford researchers have demonstrated that *in silico* methods are more accurate than animal models at predicting the cardiotoxicity of certain drugs.[121]

Diabetes

Recommendation: End the use of animals immediately

From 1984 to 2014, more than 50 papers were published per month describing experiments on rodent models of type 2 diabetes mellitus (T2DM).[122] 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."[123] 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.[124]

T2DM is a disease of glucose misregulation 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.[125,126] "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."[127] 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.[128] 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.[129] 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."[130]

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 3-D cell culture, the use of human


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organs *ex vivo*, postmortem human tissue, noninvasive human imaging, epidemiological and human genetic studies—including nutrigenomics and nutrigenetics—as well as *in silico* modeling.[131,132] For example, scientists at Glasgow Caledonian University recently 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.[133] Additionally, the U.S. Food and Drug Administration 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."[134]

In their recent 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 [*emphasis added*].[135]

HIV/AIDS

Recommendation: End the use of animals immediately

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

Because macaques are unreceptive to HIV, experimenters who wanted to use them shifted their focus to studying simian immunodeficiency virus (SIV), even though it is known that SIV isn't related to the most widespread HIV virus, HIV-1, but rather is a relative of the rarer and less pathogenic HIV-2.[136] The genetic homology between HIV and SIV is only 55%, and SIV is less genetically diverse than HIV.[137,138] Owing to differences in surface proteins and other molecular markers, antibodies that neutralize SIV have no effect on HIV, and vice versa,[139] making them useless in HIV research. Importantly, the dose of SIV administered to nonhuman primates in experiments is much higher than the typical amount of HIV-1 to which a human is exposed during sexual transmission.[140] AIDS researcher Mark Girard has stressed, "Extrapolating from vaccine protection results in non-human primate [SIV/SHIV] studies to efficacy in man may be misleading."[141]

Immune system and genetic variances between humans and nonhuman primates weaken nonhuman primate HIV/AIDS research. Here are some examples:

- Nonhuman primates have more leukocyte antigen genes and therefore wider variety in antigen recognition than do humans.[142]
- Nonhuman primate T cells contain molecules called siglecs, which act as "brakes" on the immune system, preventing hyper-responsiveness. The absence of siglecs in human T cells dramatically affects how humans respond to infection and treatment.[143]
- The primate TRIM5α gene codes for a restriction factor that affects responsiveness to retroviruses such as SIV, giving some nonhuman primates greater resistance to infection, a function mostly lost in human TRIM5α.[144]
- Even in chimpanzees, humans' closest relatives, transcript expression in the liver differs by 40%,[145] a species difference that becomes more pronounced following the varying translation of these transcripts into proteins.

For these reasons and more, HIV/AIDS vaccine research involving nonhuman primates has been called "one of the most notable failures in animal experimentation translation."[146]

Because of broad failures in nonhuman primate HIV/AIDS research, experimenters have recently shifted some focus to a species even more genetically removed from humans: the mouse. The "humanized" mouse model for HIV/AIDS research is a mouse who has been partially repopulated by human immune cells, allowing the animals to be infected with HIV-1. However, humanized mice are limited in their longevity with the disease and retain murine major histocompatibility complex antigens, "complicating immune response interpretations."[147] Not surprisingly, the use of "humanized" mice has also failed to generate useful results for clinical HIV/AIDS treatment.

Considering the differences between an animal laboratory environment and human society, it is clear that animal experiments will never capture the complexity of this human disease. Animals used in experiments are kept in mostly pathogen-free conditions, 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.[148] Additionally, researchers at Emory University in Atlanta state, "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,"[149] and recognize 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 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."[150]

Scientists admit that even after costly and unreliable animal experiments, 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 state 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."[151] In a comprehensive review of preclinical and clinical data, Bailey reported that of 85 candidate vaccines that were tested in 197 clinical trials, zero were successful; some drugs even increased the risk of HIV infections compared to the placebo.[152] A current search of ClinicalTrials.gov will return more than 700 AIDS vaccine trials, and still, none has been successful.

Recently, 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 virus's spread without any intervening therapy.[153] 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. As Nobel laureate Sydney Brenner declared, "We don't have to look for model organisms anymore because we are the model organism."[154] Similarly, in 2007, the associate editor of *The BMJ* stated, "When it comes to testing HIV vaccines, only humans will do."[155]

Recapitulating neuroAIDS in animal models has not been experimentally possible. To address this issue, research groups at India's National Brain Research Centre are using human neural precursor cells to study HIV-1 neuropathogenesis and neurodegeneration by employing cellular and molecular approaches. Adding a new dimension to the understanding of HIV-1 neuropathogenesis and neurodegeneration, studies have provided novel insights into cellular and molecular mechanisms such as cell cycle disruptions and alterations in the MAPK pathway, the effect of HIV-1B transactivating protein, and co-exposure to drugs that may modulate the cell properties and underlying pathology in HIV/AIDS patients.[156,157,158]

Inflammation and Immunology

Recommendation: End the use of animals immediately

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.[159] 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."[160] Logically, these differences make sense: We humans "do not live with our heads a half-inch off the ground,"[161] and we have considerably longer lifespans and a larger body size than do mice.[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 both the strain of mice and viruses used. The

BALB/c mouse, for example, is an inbred strain and 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 nor 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.[168] This is because human IFV receptors (α2,6-linked sialic acids) are not abundant in the upper airways of mice, who have a different receptor (α2,3-linked sialic acids).[169] Through serial passage, the virus can adapt to the new host and become distinct from the kind that affects humans predominantly.

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.[170] They do not cough or sneeze.[171] Moreover, the virus does not transmit between mice.[172] Additionally, we now know that gut microbiota are intimately linked to the immune system,[173] 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.[174] 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."[175]

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.[176] 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.[177] Additionally, a University of Tennessee 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.[178]

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, 3-D 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."[179]

Nerve Regeneration

Recommendation: End the use of animals immediately

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: "differences 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."[180] 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."[181] The authors lamented the low quality of described animal experiments, in that 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.[182] 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."[183]

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.[184]

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.[185]

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."[186] 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 humans cells. *Ex vivo* models, such as those that use 3-D engineered scaffolds, bioreactors, neurospheres, and organoids, allow for more controlled studies on specific parameters than do animal experiments.[187] Bioprinting can use bioinks containing human cells and materials to construct heterogeneous tissue models in a single step and with great consistency,[188] an aspect of nerve regeneration research that has been particularly lacking in animal models.[189]

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."[190] Mobini and colleagues note that microfluidics offer advantages in precision, scalability, and cost-effectiveness when compared to traditional cell culture or animal experiments and that these are currently on the market and available for neural regenerative medicine research.[191]

Neurodegenerative Diseases

Recommendation: End the use of animals immediately

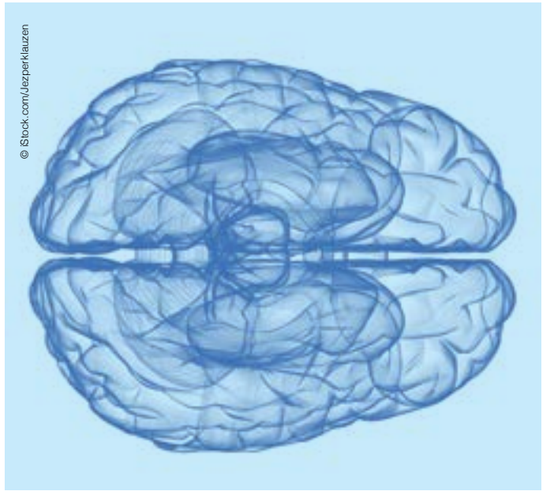
There is sufficient literature documenting the failings of various animal models of neurodegenerative diseases, including Alzheimer's (AD), Parkinson's (PD), Huntington's (HD), and amyotrophic lateral sclerosis (ALS), to write a lengthy appendix for each disease. However, since many of the same limitations 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.[192] For AD research, the clinical failure rate for new drugs is 99.6%.[193] This includes the recent failure of AstraZeneca and Eli Lilly's lanabecestat, which was hailed as extremely promising, due to futility.[194] There have been no new discoveries to treat the symptoms of AD—much less slow its progression—for 17 years.[195]

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.[196]

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,[197] 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.[198] 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.[199] 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,"[200] and that "animal models are not an ideal system for studying ALS or for developing drug therapies."[201] 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."[202] 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";[203] 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 (replacement, reduction, and refinement of animal use) and, in their failure to do so, are compromising not only animal welfare but also the relevance of their studies to HD.[204]

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.[205] 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.[206]
- Thanks to developments in human brain imaging, scientists at the University of Cambridge were able to trace tau protein in human brains.[207] Chemists there also used mathematical modeling to understand the role of cholesterol in the aggregation of amyloid proteins.[208]
- 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.[209]
- 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 subject to proteomic analyses.[210]

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.[211] 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.[212,213]

For many years, animal 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 get these human diseases, 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

Recommendation: End the use of animals immediately

Animal models of neuropsychiatric disorders such as depression, schizophrenia, bipolar disorder, anxiety, and attention deficit spectrum disorders lack two critical aspects of model validity: (1) construct validity, meaning that the mechanistic underpinnings creating the observed symptoms in animals are different than those that lead to the disorder in humans, and (2) face validity, meaning that animals lack the ability to "recapitulate important anatomical, biochemical, neuropathological, or behavioural features of a human disease."[214] 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 depression, for example, is 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 rodent 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 Molendijk and de Kloet discuss, immobility in the forced swim test is simply animals' adaptation to their situation and should not be used to determine their mood.[215] Individual animals who are quicker to float also save their energy and are less likely to sink, meaning that those who pick up on this sooner and spend less time struggling are simply learning this adaptive behavior more readily. Furthermore, the immobility response occurs after treatment with drugs that do not have antidepressant effects at all, such as caffeine and other miscellaneous drugs,[216,217] and is sometimes not observed after treatment with drugs that do.[218] Time spent swimming versus floating is also influenced by an animal's strain as well as experimental variances, such as water depth and temperature.[219,220,221] Nevertheless, thousands of published papers ignore these warnings and use the forced swim test to draw erroneous conclusions about an animal's mood.[222]

Experiments on animals for neuropsychiatric conditions are of poor quality. In a survey of 121 animal studies claiming to investigate attention deficit hyperactivity disorder (ADHD), only five were found to be in any way relevant to the hypotheses of the human medical papers in which they were cited. The authors of the survey concluded that "animal research has contributed very little to contemporary understanding of ADHD."[223] A similar failure of animal studies to translate into a clinical setting has been noted with bipolar depression research,[224] and animal studies have been cited as the primary source of attrition (failure of drugs) in neurobehavioral clinical trials.[225] 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.[226] Misregulation of tyrosine hydroxylase has been implicated in several psychiatric illnesses, such as bipolar disorder and schizophrenia.

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."[227] 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."[228] Funds should be allocated to more relevant, human-based experimental models, such as computational modeling using already well-defined biomarkers[229] 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"[230] 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."[231]

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

- A research group at the University of Michigan used induced pluripotent stem cells from bipolar and nonbipolar individuals to grow patient-specific neurons and glial cells. They found that cells from bipolar people were genetically and behaviorally distinct from those from nonbipolar people and that they responded differently to a commonly used therapeutic. The group is now further characterizing these cells and testing other treatments.[232]
- German neuroscientists are using virtual reality to simulate anxiety-causing events in humans.[233]
- In Australia, researchers performed gene expression studies in postmortem human brains, and their analyses indicated that schizophrenia may be related to the developmental complexity of the human brain.[234]
- Scientists at the Albert Einstein College of Medicine used neurons derived from human induced pluripotent stem cells, along with the gene-editing tool CRISPR-Cas9, to identify misregulated genes following the knock-out of a gene implicated in autism and other disorders.[235]
- A team at the Salk Institute for Biological Studies used a human cellular model of bipolar disorder to pinpoint key features of the disease, such as hyperexcitability of bipolar neurons and differences in responsiveness to lithium.[236]
- At the University of São Paulo, induced pluripotent stem cells were derived from samples collected from three patients with autism spectrum disorder. By generating mixed cell cultures, researchers were able to study the interplay between neurons and astrocytes and pinpoint interleukin-6 as a potential mediator of autism-specific neural defects.[237]

In addition to the lack of applicability of animal neuropsychiatric models to the human condition, animals used in this research 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 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. 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 immediately.

Sepsis

Recommendation: End the use of animals immediately

Sepsis is estimated to affect more than 30 million people worldwide each year.[238] It is a leading cause of death in U.S. hospitals and is one of the most expensive conditions to treat.[239,240] 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.[241] The difficulty in reliably translating results from mice to humans is believed to be the 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.[242]

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."[243] 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![244]

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.[245,246] 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.[247] Unlike humans, mice are rarely given pain relief,[248] 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. 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.[249] In addition, the procedure causes the formation of an abscess, whose effects may disguise or be disguised by the effects of the sepsis itself.[250] 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 reproduces 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.[251] Furthermore, baboons and mice are less sensitive to a species of bacteria commonly used to induce sepsis in experimental settings.[252]

Fortunately, researchers do not have to use animals to study and find treatments for sepsis in humans. In 2015, an expert working group consisting of veterinarians, animal technologists, and scientists issued a report on the implementation of the 3Rs in sepsis research.[253] 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, 3-D 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."[254]

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

- Scientists at Emory University and the Georgia Institute of Technology have engineered a microfluidic vascularized bleeding model that allows them to test the effects of therapies on clot and plug formation in human blood.[255]
- Because the clinical trajectory of sepsis can be drastically different for every individual, University of Chicago researchers propose that human genetic algorithms "can serve as a guide on the path towards true 'precision control' of sepsis."[256]
- 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.[257]
- Researchers from the Harvard T.H. Chan School of Public Health, Brigham and Women's Hospital, and the University of Sheffield compared public datasets of the blood transcriptome profiles of adults and children with sepsis, populations that have different mortality rates from the disease. This led them to identify 10 candidate drugs that had never been linked to sepsis before.[258,259]
- By analyzing blood from patients with sepsis, a German group identified a specific microRNA as an independent risk factor for mortality and a biomarker for discriminating between sepsis and infection.[260]

In fact, there may have already been a breakthrough in sepsis research. Physicians have recently had impressive results by treating sepsis patients with an intravenous vitamin C combination.[261] One patient whose chance of dying from sepsis was nearly 100% was well enough to leave the intensive care unit within seven days of receiving this treatment.[262] An estimated 10% to 20% of intensive care specialists around the world have already started using this therapy, and studies involving 13 hospitals are underway to confirm its efficacy.[263] Importantly, these successes have been achieved using only human patients, not mice or other animals, and many patients were helped tremendously in the process.

Stroke

Recommendation: End the use of animals immediately

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."[264]

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."[265] 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.[266] This illustrates the need to shift away from animal models and focus on human-centered methods.

In a 2017 review,[267] Clemens Sommer, M.D., of the University Medical Center at 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 cell—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,"[268] 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."[269]
- 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.[270]
- 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.[271]

- 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.[272]
- 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.

Kaya and colleagues made the following observation:

> In animal studies, prolonged survival and neurological improvement rates are not documented realistically. Histopathological findings and treatment effects are rarely adequate to reveal the mechanisms in behavioral and functional improvement. There is great difference between animal experiments and clinical practice in terms of outcome evaluation. The cerebral infarct area is used in animal experiments while neurological function and quality of life are more important in humans.[273]

On the other hand, human-based models of stroke do not suffer from these deficiencies. Instead, they allow for high-throughput analyses and are "increasingly important" for "testing novel potentially neuroprotective pharmaceuticals."[274] 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."[275]

Thanks to technological advances, including accurate 3-D 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. As part of a $70 million NIH program, an interdisciplinary team of researchers at Vanderbilt University have engineered a blood-brain barrier-on-a-chip, which they are using to study human brain inflammation induced by various compounds.[276]

Similarly, the Seattle-based biotechnology company Nortis was recently awarded a federal grant to develop its predictive preclinical living model of the blood-brain barrier as an alternative "to traditional pharmaceutical drug development testing on laboratory animals," which will "reduce costs and minimize clinical trial failures."[277] Disruption of the blood-brain barrier following a stroke[278] is a critical factor to consider in attempting to move a potential therapeutic compound from a patient's bloodstream to the brain. Scientists at the University of California, Irvine opine that "[blood-brain barrier]-on-a-chip models offer tremendous potential for recreating microvasculature in the laboratory that will allow controlled study of the mechanics of [blood-brain barrier] permeability and immune infiltration as they relate to the process of stroke,"[279] particularly those that employ human cells, such as human induced pluripotent stem cells, which "can be used to create clinically relevant models for [central nervous system] disease."[280]

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."[281] 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,[282] we cannot afford to spend our limited resources on substandard, animal-based research.

Substance Abuse

Recommendation: End the use of animals immediately

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.[283] 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."[284]

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."[285] 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.[286] Since MDMA is being explored because of not only its illegal use as a recreational drug but also 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.[287] This holds true for primates as well as mice and rats.[288] 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.[289] 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."[290]

The nonhuman animal has been called a "most reluctant collaborator" in studying alcohol addiction and 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."[291] 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**."[292]

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."[293] 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.[294]

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 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."[295]

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.[296] 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.[297] Others are using human induced pluripotent stem cells to study the effects of alcohol on the human liver.[298]

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

Trauma

Recommendation: End the use of animals immediately

After rodents, pigs are the species most commonly used in trauma experimentation. 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.[299] In addition, there are differences in the administration of mechanical ventilation and drugs such as vasopressin and heparin in research.[300,301] 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,[302] if not impossible. In studies of traumatic brain injury, all promising therapeutics identified in animals have failed in human clinical trials.[303] 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.[304]

Fortunately, it has been shown that computer simulation can accurately replicate real-life trauma and predict patient outcomes.[305] 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.[306] 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."[307]

In addition to the already-mentioned human-relevant methods that can be used to study molecular aspects of the side effects of and comorbidities related to trauma, clinical research remains invaluable in this field and informs mathematical and computer modeling. German researchers conducted a study of 35,232 patients over the course of 12 years and revealed a reduction in intubation rates, ventilation, and systemic complications such as sepsis.[308] 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.[309]

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 immediately

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

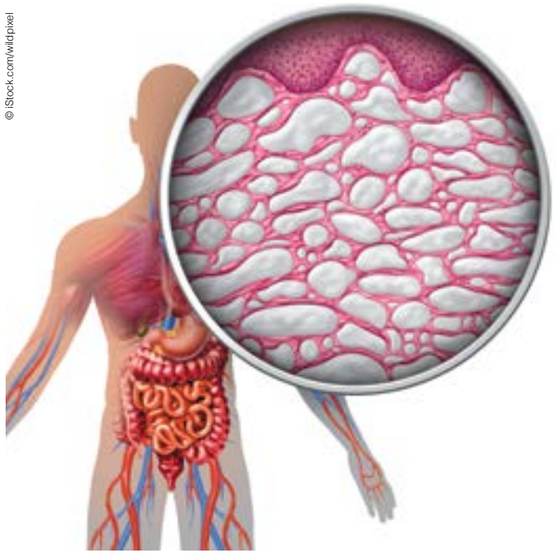
animal species], especially where mankind's actual survival is not at risk."[310]

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."[311] 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."[312]

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."[313] 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.[314] 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.

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,"[315] given the anatomical, physiological, and genetic differences between species. Mole and Heyns 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."[316]

In addition, there are a plethora of alternative methods, such as manikins, simulators, artificial materials, and *in vitro* technology, and "applying alternative methods rather than using animals has provided, in the forensic field, important and reproducible results."[317] Taken together, the ethical problems and scientific and practical issues associated with animal experimentation as well as the abundant and readily available alternative methods signify that forensic research is a prime area for animal use to end immediately.



Medical Training

Recommendation: End the use of animals immediately

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 the following recent developments have contributed to a paradigm shift in this field: improvements in human-patient simulation and computer-assisted learning technology that teaches biomedical education as well as or better than animal dissection and experimentation,[318] rising public opposition to animal use in laboratories,[319] increasing animal laboratory cost burdens,[320] and a renewed focus by the medical community on improving patient safety and reducing clinical errors through simulation-based training.[321]

Human simulation-based teaching has become the gold standard. Now, medical students in the U.S. and Canada learn without using animals throughout the curricula.[322] Medical experts have recommended a transition from an animal-based pedagogy to "a robust curriculum composed of didactics, task trainers, virtual reality, cadavers, computer software, high-fidelity patient simulators, and supervised clinical work."[323] Unlike animal-based laboratories, 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.[324]

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 that have been endorsed as replacements for live-animal use. These include task trainers and 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."[325]

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 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.[326]

Given the non-animal training methods already available, we recommend that the use of animals for military and civilian trauma training and microsurgery training be ended immediately.

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 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."[327] 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."[328]

Non-animal methods are used exclusively instead of animals for military trauma training by nearly 80% of NATO member states,[329] 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.[330] These developments confirm that animal use for trauma training is neither necessary nor justified.

Efforts to replace animals with human simulators in military trauma training have gained many prominent supporters, including, recently, The New York Times Editorial Board[331] 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.[332]

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, and national ATLS programs in numerous countries have made this transition and ended animal use for this purpose.[333]

Toxicity Assessment

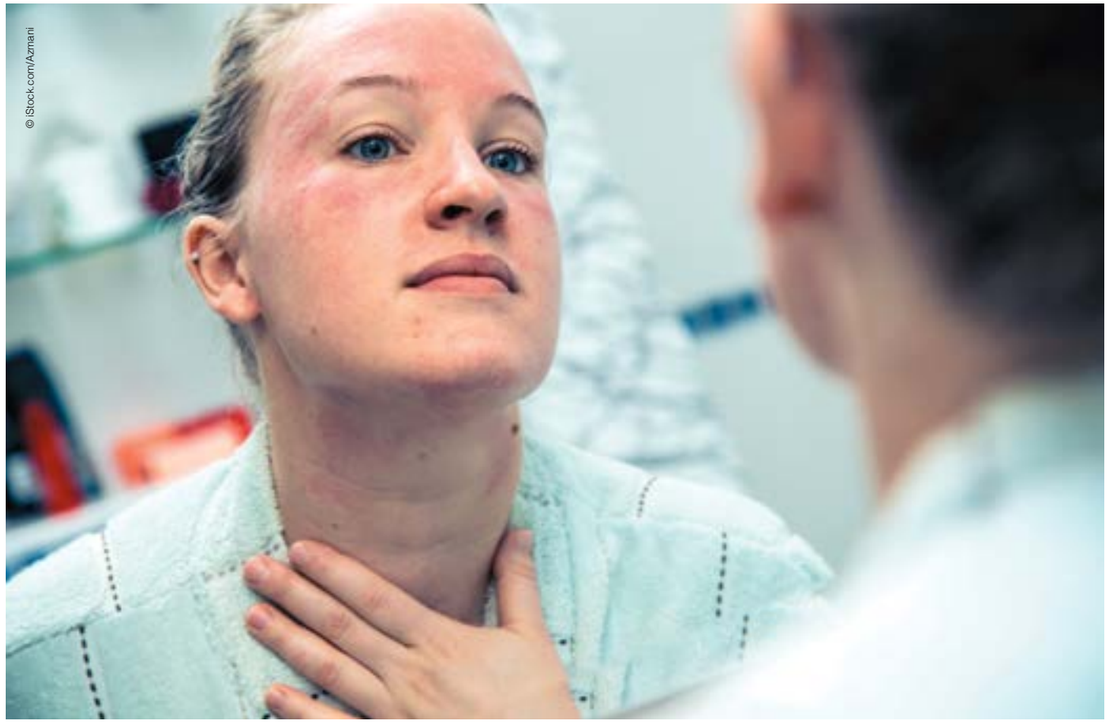
Detailed below are opportunities to end or significantly reduce the use of animals for the toxicity assessment of substances in the context of regulatory toxicity requirements. Also described are areas in which greater support is required to develop innovative methods that are relevant for the assessment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

Please note that where tests are required for regulatory purposes, the Organisation for Economic Co-operation and Development (OECD) website (OECD.org) should be consulted for the most recent versions of test guidelines and guidance documents.

Exposure-Based Assessment

Recommendation: Immediately promote the use of exposure-based waiving as an opportunity to reduce the use of animals dramatically

Exposure-based waiving will reduce animal testing by shifting the focus of regulatory decision-making from a hazard-based to an exposure-based approach. This strategy employs "fit-for-concern" assessments rather than simple "box-ticking" by exploring safety based on real concerns and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avoiding characterizing hazards not relevant to human safety. The pesticide industry is actively seeking ways to promote exposure-based waiving for the assessment of its products.

Further work and collaboration by all stakeholders will be necessary to determine whether exposure-based waiving can be accepted and approved by regulatory authorities and the public.

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 multiple regulatory agencies. In these tests, rabbits are shaved, test substances are applied to their exposed skin, 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. There is no requirement that animals be provided with pain-relieving drugs during this prolonged process.

Despite years of use, animal-based skin irritation studies have never been properly validated. Evidence exists that they are highly variable, of limited reliability, and generally poor predictors of human skin reactions. 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.[334]

The OECD has developed an integrated approach to testing and assessment (IATA) for skin irritation using *in vitro* skin irritation and corrosion methods that avoid or minimize animal use.[335]

- **OECD Test No. 439: *In Vitro* Skin Irritation: Reconstructed Human Epidermis (RHE) Test Method.** 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 (GHS), as category 2, category 3, or nonclassified chemicals. May be used as a stand-alone test or in a tiered testing strategy.
- **OECD Test No. 430: *In Vitro* Skin Corrosion: Transcutaneous Electrical Resistance (TER) Test Method.** May be used for the identification of noncorrosive and corrosive test chemicals in accordance with the GHS.
- **OECD Test No. 431: *In Vitro* Skin Corrosion: RHE Test Method.** May be used for the identification of corrosive chemical substances and mixtures. May also distinguish between severe and less severe skin corrosives.
- **OECD Test No. 435: *In Vitro* Membrane Barrier Test Method for Skin Corrosion.** Allows for the subcategorization of corrosive chemicals into the three GHS subcategories of corrosivity.

Recently, OECD Test No. 439 was validated for use in assessing the ability of medical device extracts to cause skin irritation, and the ISO 10993 guidance is currently being updated to include this test.[336,337] A number of the above methods are currently undergoing evaluation in a joint effort by the U.S. Environmental Protection Agency (EPA), industry, and the U.S. NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) for use with pesticide products. This evaluation consists of side-by-side comparison and analysis of existing *in vitro* and *in vivo* data generated by pesticide companies for their products. Depending on the outcome of these efforts, additional work may be needed to validate the use of these methods with certain classes of chemicals that were not covered during OECD validation efforts.

Additionally, there are opportunities available to waive these tests based on criteria described in the OECD guidance document on considerations for the waiving or bridging of mammalian acute toxicity tests.[338]

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 75 years ago, and advanced replacements have since been developed and validated. Furthermore, 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.[339] 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. The majority of category 2A and 2B chemicals were classified differently in repeat testing: 59.4% of category 2A chemicals and 80.2% of category 2B chemicals were classified as no category in a second test.

While no single *in vitro* test can predict the full range of serious eye damage/irritation categories, it is possible to categorize a test substance using only one method. A top-down approach is used when chemicals are expected, based on existing information, to have a high irritancy potential or induce serious eye damage. Conversely, a bottom-up approach may be used when chemicals are expected, based on existing information, not to cause sufficient eye irritation to require a classification. An OECD guidance document on an IATA of serious eye damage and irritation was published in 2017.[340]

- **OECD Test No. 491: Short Time Exposure (STE) *In Vitro* Test Method.** May be used to identify chemicals causing serious eye damage (GHS category 1) or not requiring classification (GHS no category). May also allow the classification of irritants as minimal, moderate, or severe.
- **OECD Test No. 492: Reconstructed human Cornea-like Epithelium (RhCE) Test Method (EpiOcular™, MatTek).** May be used to identify chemicals not classified for eye irritation or causing serious eye damage (GHS no category).
- **OECD Test No. 460: Fluorescein Leakage Test Method.** May be used to identify chemicals causing serious eye damage (GHS category 1) or not requiring classification (GHS no category). 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.** May be used to identify chemicals causing serious eye damage (GHS category 1) or not requiring classification. Validated by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), and the Japanese Center for the Validation of Alternative Methods (JaCVAM).
- **OECD Test No. 438: Isolated Chicken Eye Test Method.** May be used to identify chemicals causing serious eye damage (GHS category 1) or not requiring classification. Validated by ICCVAM, EURL ECVAM, and JaCVAM. Recommended as the first step within a top-down or bottom-up testing strategy.

These methods are generally validated for use with cosmetics and industrial chemicals that fall under the European Union (EU) Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation, and there may be limitations for some methods with certain types of chemicals (e.g., surfactants, solids, etc.). None of the current OECD-approved assays is recommended for directly determining category 2 eye irritants in a regulatory setting, but category 2 can be inferred if a substance is demonstrated not to be category 1 (severe eye damage) or no category. There is a vital need for validation of a non-animal method that can directly predict category 2 (irritant) substances for use in a regulatory setting.

The EPA currently accepts the use of *in vitro* methods for the determination of eye irritation and corrosion when classifying antimicrobial cleaning products and other pesticide products on a case-by-case basis, and it has published a guidance document describing the testing framework that industry can use for this endpoint.[341] Also, the agency, in collaboration with the PETA International Science Consortium Ltd., NICEATM, and industry members, is currently engaged in evaluating these methods for use with agrochemical formulations through a side-by-side comparison of *in vitro* and *in vivo* data.

India, as per the modifications in the Drugs and Cosmetics

(Amendment) Act, 2017, accepts the OECD-validated *in vitro* methods for eye irritation for all the products under its mandate.

Additionally, there are opportunities available to waive these tests based on criteria described in the OECD guidance document on considerations for waiving or bridging of mammalian acute toxicity tests.[342]

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 or to the ears of mice, who are later killed. Fortunately, for industrial chemicals and drugs, the regulatory requirement to test for skin sensitization can be fully replaced with a combination of *in vitro* and *in chemico* assays that each address a different key event in the adverse outcome pathway (AOP) for this endpoint.[343] The methods distinguish between sensitizers and nonsensitizers and are recommended to be used in an IATA.

- **OECD Test No. 442C: *In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA).** The DPRA 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 the Key Event on Activation of Dendritic Cells on the Adverse Outcome Pathway for Skin Sensitisation.** This method addresses the third key event of the skin sensitization AOP.

A recent study showed that non-animal approaches to predicting skin sensitization are as good as or better than the mouse test when compared to human data.[344,345] While none of the methods is endorsed for potency determination, several approaches—for instance, the human cell line activation test (h-CLAT)—show promise in this regard.[346] Further efforts are underway to explore this potential.

The OECD has published a guidance document on the reporting of defined approaches to be used within an IATA for skin sensitization.[347] In general, the methods can be used to test cosmetics and industrial chemicals. The EPA accepts the use of non-animal approaches to testing single chemicals and is conducting a validation study with a goal of expanding this policy to formulations in the near-term future.[348] Likewise, the U.K. accepts *in vitro* methods for addressing the potential of pesticides to cause skin sensitization for plant-protection products.[349] Additionally, there is an effort underway to validate non-animal skin sensitization methods to replace the ISO 10993–required guinea pig skin sensitization test for assessing medical device biocompatibility.[350] There are opportunities to waive these tests based on criteria described in the OECD guidance document on considerations for waiving or bridging of mammalian acute toxicity tests.[351]

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.

The rabbit pyrogen test (RPT) requires that rabbits be injected with a test substance and subsequently restrained for three hours, during which changes in their body temperature are monitored rectally. In Europe alone, more than 100,000 rabbits are used each year in the RPT,[352] 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.[353]

The Limulus amebocyte lysate test (LAL), also called the bacterial endotoxins test, detects only bacterial endotoxins and no other pyrogens. It requires the use of hemolymph from captured horseshoe crabs. After the biomedical bleeding process, up to 30% of the crabs die. Those who live have a reduced likelihood of surviving in their natural habitat.[354] A synthetic version of the LAL, in which the hemolymph is replaced by a recombinant reagent (the recombinant factor C assay), is available, but sensitivity is still limited to bacterial endotoxins.

Since 2010, the monocyte activation test (MAT) has been validated and included in the *European Pharmacopoeia* (*Ph. Eur.*) as a test for assessing pyrogen contamination.[355] It mimics the innate human fever response *in vitro*, exposing human whole blood or isolated human monocytes to test articles followed by tests to detect pro-inflammatory cytokines released during exposure, and it is compatible with drugs and medical devices.[356] It avoids the aforementioned problems with the RPT and the LAL, and case studies document instances in which the MAT detected pyrogen contamination in products that had passed the RPT and the LAL but caused fever in human patients.[357]

Regulators in the EU, India, 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.[358] To eliminate the use of animals in pyrogen tests, regulatory authorities and standards organizations must make increased effort to integrate and harmonize a preference for the MAT in international testing requirements and to encourage drug and device manufacturers to use and submit data from the MAT in their product dossiers. In September 2018, participants at a workshop organized by the PETA International Science Consortium Ltd. and NICEATM discussed non-animal approaches to medical device pyrogen testing. Publication of the resulting report is forthcoming.[359]

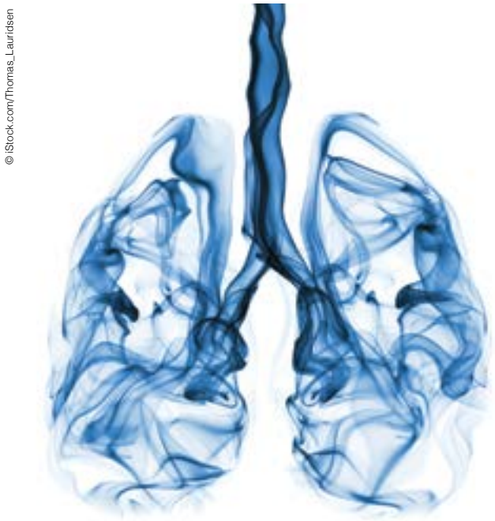
Following a survey of pyrogen test users, the European Directorate for the Quality of Medicines & HealthCare (EDQM) revised the *Ph. Eur.* general chapter on the MAT to improve the method's usability and to emphasize that it is considered a replacement for animal-based pyrogen tests.[360,361] This endorsement is repeated in statements from the European Medicines Agency.[362] 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.[363] In the eighth edition of *Indian Pharmacopoeia*, the Indian Pharmacopoeia Commission revised the pyrogen testing general chapter, introduced the monograph on MAT, and replaced the RPT with the LAL.[364] Drug and device manufacturers report discomfort with regulatory ambiguity about the applicability of the MAT as a stand-alone pyrogen test, and the RPT and LAL will continue to be used until this is resolved.

Tobacco and E-Cigarette Testing

Recommendation: Immediately eliminate the use of animals for developing and testing 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 e-cigarettes. In such tests, rats may be squeezed into narrow tubes, immobilized, 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) appropriately states that, in light of the EU policy banning animal studies for chemicals to be used in voluntary products such as



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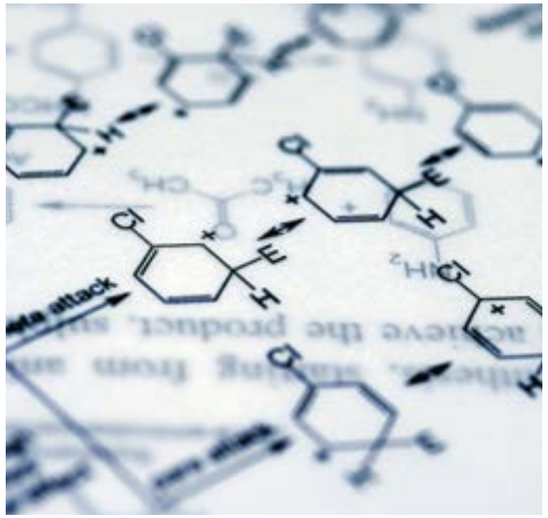
cosmetics, animal studies are not endorsed to assess the safety of tobacco additives.[365] In addition, Belgium, Estonia, Germany, Slovakia, and the U.K. already prohibit animal tests for tobacco products because of ethical concerns.[366,367,368,369,370]

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, cell transformation assays (CTAs), and genomic analyses.[371,372,373,374] These techniques have been used to investigate cytotoxicity, genotoxicity, inflammation, and gene expression. They are more relevant to actual human exposure than are animal tests that have historically under-predicted the hazards of tobacco.

Genotoxicity

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

Currently, the assessment of genotoxicity typically follows a step-wise approach, beginning with a core battery of *in vitro* tests that may be followed up by *in vivo* studies if the *in vitro* results are positive. The major endpoints that must be evaluated are gene mutation, structural chromosomal aberrations, and numerical chromosomal aberrations. In its "Strategy to Avoid and Reduce Animal Use in Genotoxicity Testing," EURL ECVAM recommends the Ames test to identify gene mutations, combined with the *in vitro* micronucleus test to identify both structural and numerical chromosomal aberrations.[375] If a substance produces negative results in both tests, it can be categorized as having no genotoxic



potential and no further testing is indicated. If a substance produces positive results in either test, certain regulatory applications currently specify *in vivo* tests as the next step. This is because while *in vitro* tests are highly sensitive, producing false negative results at a low rate, they are less specific, producing false positive results at a higher rate. The number of false positive results can be reduced by using p53-competent human cells, evaluating cytotoxicity based on cell proliferation, and testing at reduced maximum concentrations.[376] These considerations have been incorporated into recent revisions of OECD test guidelines.

- **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. 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.
- **OECD Test No. 471: Bacterial Reverse Mutation Test.** This test uses amino acid–requiring *Salmonella typhimurium* and *Escherichia coli* to detect point mutations by base substitutions or frameshifts.
- **OECD Test No. 473: *In Vitro* Mammalian Chromosomal Aberration Test.** This test identifies chemical substances that cause structural chromosomal aberrations in cultured mammalian somatic cells.
- **OECD Test No. 476: *In Vitro* Mammalian Cell Gene Mutation Test Using Hrpt and xrpt Genes.** These tests can detect gene mutations induced by chemicals.

To undertake a better assessment of the genotoxic potential of substances that produce positive results in the core battery, additional *in vitro* tests can be used in place of *in vivo* tests. In its "Notes of Guidance for the Testing of Cosmetic Ingredients and Their Safety Evaluation," the European Commission's Scientific Committee on Consumer Safety (SCCS) recommends using a micronucleus test on 3-D reconstructed human skin or a comet assay either in mammalian cells or on 3-D reconstructed human skin.[377] However, negative results produced in these alternative tests do not necessarily rule out genotoxic potential. In such cases, expert judgment as well as mechanistic investigations may be helpful in evaluating the WoE. For example, *in vitro* toxicogenomics-based tests can provide information on the mode of action of potential genotoxicants by identifying global gene expression changes.

Validation studies of the micronucleus test and comet assay on 3-D reconstructed human skin are currently being conducted and thus providing further opportunities for phasing out the use of animals for genotoxicity testing.[378]

Acute Systemic Toxicity

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

To determine the danger of acute exposure to a product or chemical, a substance is administered to animals in extremely high doses through force-feeding (oral), skin contact (dermal), and/or forced inhalation. In this test, 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 calculated. Animals may endure severe abdominal pain, diarrhea, convulsions, seizures, paralysis, or bleeding from the nose, mouth, or genitals before they ultimately die or are killed. The LD_{50} and its adaptations have never been scientifically validated, and their accuracy in predicting chemical effects in humans remains questioned. One 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.[379]

Regulatory authorities may issue waivers for acute toxicity testing in animals if certain criteria are met. The OECD has published guidance for waiving or bridging acute toxicity testing,[380] and the EPA has published similar guidance for pesticides and pesticide products.[381] This includes the use of existing data for read-across and the consideration of the physicochemical properties of the test substance.

Acute Oral Toxicity

NICEATM and ICCVAM organized a project to develop predictive models for acute oral systemic toxicity.[382] The outcome was consensus quantitative structure-activity relationship (QSAR) models for the prediction of acute oral toxicity to meet various regulatory needs, which were presented at an April 2018 workshop.[383] The models are being optimized and will be posted on the NICEATM and EPA websites.

EURL ECVAM's strategy to replace, reduce, and refine the use of animals in the assessment of acute mammalian systemic toxicity focuses on the *in vitro* 3T3 neutral red uptake (NRU) cytotoxicity assay, which can be used in a WoE approach to support the identification of nonclassified substances.[384] *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.[385,386,387] In addition, it has proposed an approach to identifying nonclassified substances using information from 28-day repeated dose toxicity studies, thereby avoiding acute systemic toxicity testing.[388]

In its "Guidance on Information Requirements and Chemical Safety Assessment," the European Chemicals Agency (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.[389] 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 unnecessary animal testing pursuant to REACH Articles 13(1) and 25(1).[390]

Acute Dermal Toxicity

Testing by the dermal route of exposure can be waived if data on oral toxicity are available. 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 waiver requests.[391] In addition, dermal studies can be waived for substances that are nonclassified by the oral route and not absorbed dermally. The European Commission recently amended REACH Annex VIII so that substances that are nonclassified by the oral route do not require dermal data.

Acute Inhalation Toxicity

Testing by the inhalation route of exposure can be waived if substances demonstrate low volatility and are not aerosolized or otherwise made respirable under conditions of use. In addition, promising research efforts are underway to develop non-animal methods for acute inhalation toxicity.[392,393] A recent series of webinars (www.PISCLtd.org.uk/inhalation-webinars) and a workshop hosted by the PETA International Science Consortium Ltd. and NICEATM presented several approaches that could eventually replace animal testing for this endpoint.[394,395]

Carcinogenicity

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

The carcinogenicity study currently requires that testing be conducted on rats (or other species when justified) for the majority of their life (up to two years for rodents). The test requires the use of 50 animals of each sex per dose and a minimum of three doses and control for each study, which equates to a minimum total of 400 rats or mice per chemical. Some chemical regulation requires carcinogenicity tests on both rats and mice (OECD Test No. 451 and No. 453), meaning that approximately 1,000 animals are used to meet toxicity testing requirements for one chemical.

While carcinogenicity studies are still routinely conducted, the test has been under scientific scrutiny since the early 1970s for its lack of reproducibility[396] and its inability to predict human outcomes.[397] Several reviews have been conducted over the past three decades to highlight the overall lack of reliability in the rodent cancer bioassays.[398,399,400,401,402,403,404,405,406,407,408,409,410,411,412,413,414] There are two assumptions that underlay these bioassays: (1) rodent carcinogens are human carcinogens, and (2) high-dose chemical exposure in rodents is indicative of an environmentally relevant dose.[415] Both have been proved incorrect by 50 years' worth of carcinogenicity data.

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.[416] 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.[417] This study highlights the opportunity to use a WoE approach to determine whether the carcinogenicity study can be waived for chemicals that meet certain criteria.

Additionally, *in vitro* CTAs recapitulate a multistage process that closely models *in vivo* carcinogenesis, and they have the potential to detect both genotoxic and nongenotoxic

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.[418] In a validation study, the Bhas 42 CTA was tested with 98 substances, including carcinogens and noncarcinogens; for predicting carcinogenicity, its performance was equivalent or superior to conventional genotoxicity assays. As the protocols were transferable and reproducible between laboratories, they are recommended for routine use. In addition, because the Bhas 42 CTA is based on a cell line rather than primary cells, no animals are required.

In its guidance document on the Bhas 42 CTA, the OECD recommends that it be used as part of a testing strategy rather than as a stand-alone assay. 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 the use of *in vivo* testing.[419]

The structural alerts (SAs) rulebase has recently been expanded with a large number of new SAs for nongenotoxic carcinogenicity and has been incorporated into the OECD QSAR Toolbox version 4.4.1 Additionally, the EPA has published a computer model, OncoLogic™, to evaluate chemicals for carcinogenic potential,[420] and commercial options are also available, such as the Lhasa Carcinogenicity Database, MultiCASE, UL Cheminformatics, and Leadscope. Ultimately, the identification of DNA-reactive chemicals with the Ames test or genotoxic SAs can potentially be combined with the identification of nongenotoxic carcinogens using nongenotoxic SAs, leaving CTAs to model most of what is left unexplained in a WoE approach. There is an expert group at the OECD working to generate an IATA for nongenotoxic carcinogens.[421]

Endocrine Disruption

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

In the 1990s, the EPA's Endocrine Disruptor Screening Program (EDSP) was established to screen approximately 10,000 chemicals for their effects on the human body's hormone systems and on wildlife. The program has the potential to use millions of animals in testing. In order to reduce the number of animals used and rapidly and effectively screen such a high volume of chemicals, the agency has turned to several non-animal methods.

Its Toxicity Forecaster (ToxCast) ranks and prioritizes chemicals using more than 700 high-throughput screening assays, which cover a variety of high-level cell responses and approximately 300 signaling pathways, as well as computational toxicology approaches. Data have already been generated on thousands of chemicals of interest to the EPA.

ToxCast is being used successfully for these purposes. After a comparative study of ToxCast estrogen pathway assay results and uterotrophic assay results,[422] the EPA announced that it will accept ToxCast data as an alternative to at least one animal test—the uterotrophic assay—that screens for effects on the estrogen pathway.[423] The agency is working to finalize the use of ToxCast data as an alternative to the rat Hershberger assay, which screens for effects on the androgen pathway.

The thyroid pathway has more complexity than either the estrogen or the androgen pathways. Although ToxCast is showing promising results, more research is required in this area, and use of this system to replace tests on animals is still several years away. There are complementary efforts at the international level. An OECD scoping document for *in vitro* approaches to the thyroid signaling pathway was published in 2014.[424] The OECD Molecular Screening Group's *in vitro* Thyroid Subgroup is working to bring relevant *in vitro* thyroid assays to the attention of OECD member countries and provide recommendations for their development and use. More research and development is needed to obtain non-animal approaches to screening for thyroid disruption potential in humans and wildlife populations.

Repeat Dose, Reproductive, and Developmental Toxicity

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

In repeat dose toxicity studies, animals are exposed repeatedly to substances for one to three months in order to measure the effects of multiple chemical exposures. Chemicals are usually administered to animals using an oral gavage.

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

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. The European Commission's Detection of Endpoints and Biomarkers of Repeated Dose Toxicity Using *In Vitro* Systems (DETECTIVE) project was one of the six research projects

funded 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 "-omics" 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, -omics 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.

None of the *in vivo* methods used for testing reproductive and developmental toxicity have been validated for its relevance to humans.[425] There are considerable limitations surrounding the *in vivo* methods, with a predictivity of only around 60% and large interspecies variations.[426,427]

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.[428,429] The EU FP6 project ReProTect has also investigated possible strategies to cover the entire mammalian reproductive cycle, resulting in a series of published works.[430] Furthermore, the ChemScreen FP7 project has been designed to generate a rapid screening system that is relatively simple and cost-effective.[431]

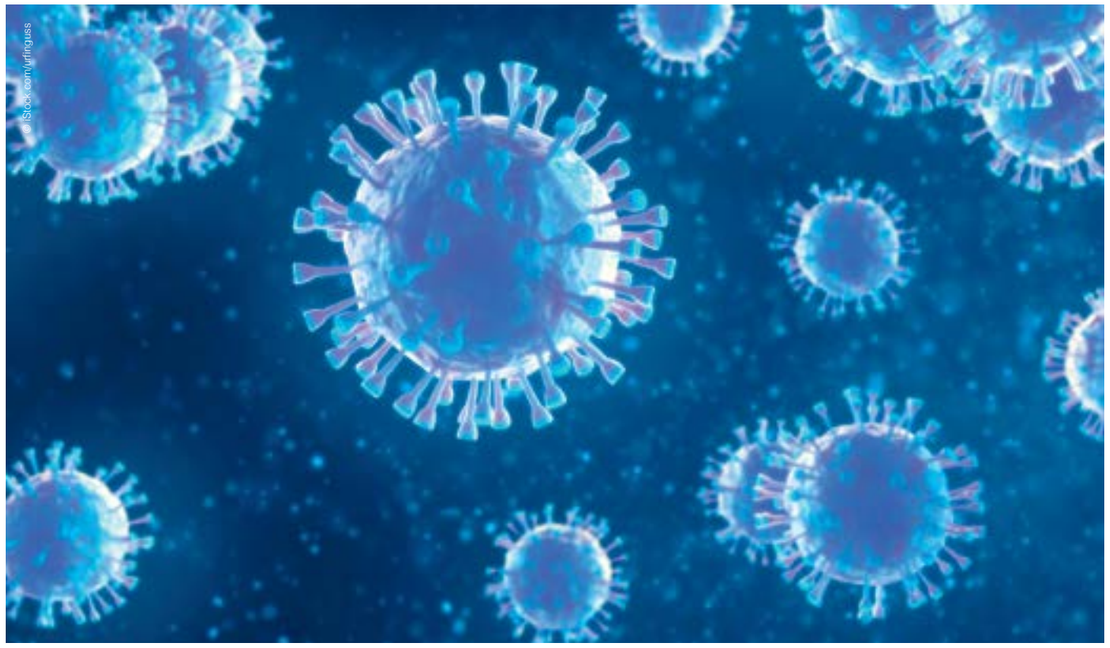
The EPA's National Center for Computational Toxicology is also exploring the potential for chemicals to disrupt prenatal development through the use of its virtual embryo model, v-Embryo™, which integrates *in vitro* and *in silico* modeling approaches.[432] While the field is gradually moving toward IATA strategies in order to cover the majority of possible mechanisms, much more research is required.

Aquatic Toxicity Testing

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

Aquatic toxicity tests are conducted to measure the effects of chemicals on the environment and wildlife. In 2011, nearly 180,000 fish were used for toxicological and other safety assessments in the EU.[433] As assessment of aquatic toxicity is required in various regulatory frameworks, strategies to replace testing using aquatic animals are urgently needed.

Several non-animal alternatives to the use of live animals are available now. In 2018, two OECD test guidelines for *in vitro* intrinsic clearance using cryopreserved rainbow



trout hepatocytes[434] and rainbow trout liver S9 subcellular fraction[435] and an associated guidance document[436] were adopted. 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 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 fish in OECD Test No. 305 on bioaccumulation in fish.[437]

To reduce the number of juvenile and adult fish used in acute aquatic toxicity testing, ECHA will accept data from the fish embryo acute toxicity test[438] in a WoE approach[439] 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.[440] This *in vitro* assay has the potential to reduce or even replace the use of fish in the acute fish toxicity test.[441] A ring trial on transferability and both intra- and inter-laboratory reproducibility of the assay organized by the Swiss Federal Institute of Aquatic Science and Technology has been completed,[442] and a Standard Operating Procedure has been submitted to the ISO. A project to develop an OECD test guideline on the fish cell line acute toxicity test using the RTgill-W1 cell line assay was included in the work plan of the OECD Test Guideline Programme in 2019. Adoption of the test guideline is planned for April 2021.

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.

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.[443,444,445,446] 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 hyperimmunizing horses and other large mammals and isolating the resulting immunoglobulins from animals' blood. These animal-derived immunoglobulins can be replaced with recombinant human antitoxin expressed in cell culture. Several recombinant antitoxins have been licensed for marketing, and more are in development.[447] With adequate funding and support from regulators, all biologics of animal origin, including antibodies (described below), 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.[448] 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).[449] 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).[450] 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. For companies seeking to use validated non-animal methods, this approach is prohibitively expensive and slow. As a consequence, industry adoption of non-animal methods remains limited, despite the documented reduction in animal use when they are implemented successfully.[451] Additional barriers to the implementation of currently available alternative tests have been discussed at length in the literature for erysipelas, clostridial, and tetanus vaccines and for recombinant therapeutic hormones.[452] 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.[453,454]

Antibody Production

Recommendation: Immediately eliminate the use of animal-derived antibodies in 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, such as Australia, Canada, Germany, the Netherlands, Switzerland, and the U.K., have restricted or banned the production of antibodies via the ascites method because of animal welfare concerns.[455]

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 February 2015 *Nature* commentary, 109 academic and industry scientists joined Andrew Bradbury of the Los Alamos National Laboratory in the U.S. and Andreas Plückthun, head of the Department of Biochemistry at the University of Zurich, to call for an international shift to the use of recombinant antibodies for reasons that include increased reliability and reduced lot-to-lot variability in affinity reagents.[456] Bradbury and Plückthun note that they believe that poorly characterized antibodies were in large part to blame in a study in which the scientific results of only six out of 53 landmark preclinical studies could be replicated. In addition, a May 2015 *Nature* news feature reports that antibodies may be the laboratory tool most commonly contributing to the "reproducibility crisis."[457] 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.[458] This issue is not limited to monoclonal antibodies. Because only 0.5% to 5% of the antibodies in a polyclonal reagent bind to their intended target and polyclonal reagents have significant batch-to-batch variation, in 2015, 111 academic and industry scientists called for polyclonal antibodies to be phased out of research completely.[459]

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.[460] 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.[461,462] 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.[463]

International efforts have highlighted the importance of a large-scale transition from animal-derived antibodies to animal-free affinity reagents. In December 2018, a working group of the Scientific Advisory Committee of EURL ECVAM reviewed the scientific validity and benefits of using animal-free technology to produce affinity reagents, concluding that the use of animal-free affinity reagents would improve scientific reproducibility and that scientists should work toward the replacement of animal-derived antibodies.[464] In the U.S., experts and organizations, including NICEATM and the PETA International Science Consortium Ltd., are working to increase access to animal-free affinity reagents. In December 2019, NICEATM and the Science Consortium 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."[465] 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 www.PISCLtd.org.uk/our-work/antibodies/.

A federal ban on the *in vivo* production of monoclonal antibodies using the ascites method should be introduced, in line with the one that has been in place in the Netherlands for more than 20 years, and the U.S. should further move to eliminate the import of animal-derived monoclonal antibodies and the use of animals in the hybridoma method.[466] In order to expedite such a ban, we recommend that government agencies and research funding bodies provide grant opportunities for the generation and implementation of non-animal affinity reagents.

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. Because the unborn calves are not anaesthetized 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.[467]

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.[468,469] A third workshop on FBS and alternatives was held in 2016, organized by the SET Foundation and the Deutscher Tierschutzbund (German Animal Welfare Federation).[470] 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 a superior alternative to FBS for culturing cells.

Listings of commercially available products and FBS-free media recipes published in the scientific literature are available on the PETA International Science Consortium Ltd.'s website (www.PISCLtd.org.uk/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 www.PISCLtd.org.uk/fbs.

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 development and optimization of non-animal, serum-free medium. 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 and Its Affiliates

The Dutch government consulted with PETA scientists before making its decision to phase out certain experiments using animals. PETA and its international affiliates stand ready to offer our assistance in whatever capacity might be required.

The PETA International Science Consortium Ltd. promotes and funds non-animal research methods and coordinates the scientific and regulatory expertise of its members, the international PETA affiliates. With an eye toward championing the best non-animal methods and reducing animal testing, the Science Consortium and its members are actively involved in the development, validation, global implementation, and harmonisation of non-animal test methods. Briefly, the Science Consortium is an accredited ECHA stakeholder and a member of the EURL ECVAM stakeholder forum and regularly comments on OECD test guidelines as a member of the International Council on Animal Protection in OECD Programmes (ICAPO).

The scientists who work for PETA and its international affiliates 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.[3]

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