PETA

Advancing Biomedical Research and Regulatory Policies for Human and Animal Health

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Forty percent of all research funding from the National Institutes of Health (NIH) goes toward animal experimentation, even though an increasing number of studies show that animal experiments not only are wasteful but also impede medical progress. Many scientists have come to realize that most of the published findings from animal studies over the past 20 years are either inaccurate or false. A 2015 analysis concluded that between 50 and 89 percent of all preclinical research could not be reproduced, which, at the most conservative U.S. estimate, results in approximately $28 billion per year spent on experimentation that is misleading (Freedman, et al., 2015). Current 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" (Collins and Tabak 2014). Furthermore, an August 2014 poll conducted by the Pew Research Center found that less than half of U.S. adults support the use of animals in scientific research (Figure 1).

We have identified a number of strategic priorities and appended further information regarding areas of both regulatory 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. We would be happy to expand on these suggestions and offer additional insight throughout the administrative transition process.

Limited Predictive Value of Research on Animals

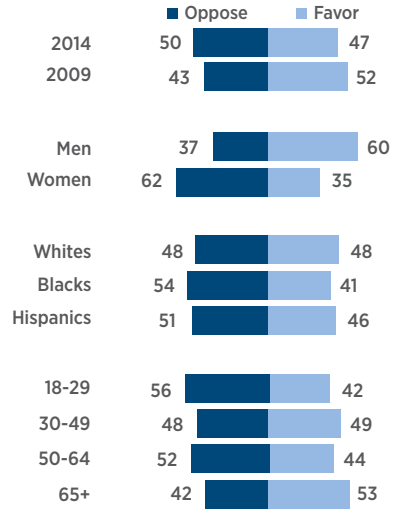
A great deal of scholarly research shows that animal studies are flawed, diverting economic and intellectual resources from methodologies better suited to curing human disease. While there are many factors at play in the failure of animal experimentation to predict human outcomes reliably—including reporting and publication bias, poor study design, and inadequate sample size—intrinsic biological and genetic differences between species contribute significantly to problems in extrapolating results from nonhuman animals to humans.

According to a 2014 review paper in the *British Medical Journal*:

> Several studies have shown that even the most promising findings from animal research often fail in human trials and are rarely adopted into clinical practice. For example, one study found that fewer than 10% of highly promising basic science discoveries enter routine clinical use within 20 years. . . . [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. (Pound and Bracken 2014)

Such limitations are widely recognized, and a number are outlined in the following pages. These difficulties are compounded by the confinement and unnatural conditions of laboratory life that thwart animals' ability

Figure 1
Use of Animals in Scientific Research
% of U.S adults saying they favor/oppose the use of animals in scientific research



Survey of U.S adults Aug. 15-25, 2014. Q24a. "Don't know" responses not shown. Whites and blacks include only non-Hispanics; Hispanics are of any race.
PEW RESEARCH CENTER

http://www.pewinternet.org/2015/07/01/chapter-7-opinion-about-the-use-of-animals-in-research/

to engage in natural behaviors. This deprivation contributes to their stress and alters their physiology and neurobiology causing them to exhibit various psychopathologies. Importantly, the fact that animals in laboratories have altered physiologies and neurobiologies means that they will not be good "models" for their counterparts in the wild. A mouse in a laboratory will not respond to a drug in the same way as a mouse in a field would. One then has to ask, how does this biologically compromised mouse reliably represent the biology of human beings? Furthermore, artificial disease created in the laboratory is not the same as naturally occurring conditions in humans in the real world—further confounding the value of any data gleaned from such approximations of the actual disease.

Becoming World Leaders

The move internationally is clearly away from animal-based research. The Dutch government recently announced its plan to phase out animal experimentation in the Netherlands by 2025 and to focus its efforts on new and rapidly evolving non-animal technologies for biomedical research. PETA scientists were asked by stakeholders in this decision to outline the areas of research in which the use of animals has been an impediment to scientific advancement and where technology has made the use of animals unnecessary.

We stand ready to offer our assistance in whatever capacity might be required during your transition. PETA scientists are actively involved in the development, validation, global implementation, and harmonization of non-animal test methods, and our scientists have worked behind the scenes with many Fortune 100 corporations and regulatory agencies, providing advice and technical support in a 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 under the Trump administration.

Technology and Job Growth

By mandating a move away from animal experimentation and toward more advanced scientific methods, the U.S. has the opportunity to expand job growth rapidly in science and technology and to reduce healthcare costs for Americans. According to a recent report by Grand View Research, Inc., "[t]he global *in-vitro* [non-animal] toxicology testing market is expected to reach USD 44.7 billion by 2022 growing at an estimated CAGR [Compound Annual Growth Rate] of 10.5% from 2015 to 2022 .... This expected rise in demand can be ascribed to novel and promising technologies in analytical laboratories" (GlobeNewswire 2016). New technologies will streamline drug development, making the process safer, cheaper, and more effective. Developing these technologies allows for the creation of interdisciplinary research teams that will be fundamental in creating the "human disease models of tomorrow" (hDMT Institute 2016).

It is well known that innovation breeds productivity. Under the Trump administration, the U.S. has a unique opportunity to lead the world in technology and medical advancements, while providing safer, more cost-effective healthcare to its citizens.

Strategic Priorities

We have identified five strategic priorities for the replacement of animals in research and for the implementation of more promising methods.

Strategic Priority #1: Immediately eliminate animal use in areas for which animals have been shown to be bad "models" for humans and have impeded progress.

Multiple systematic reviews have documented the overwhelming failure of specific areas of

animal use to benefit human health, 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. Please find appended further elaboration and recommendations on these areas.

Strategic Priority #2: Conduct critical scientific reviews of animal use to determine which areas should be immediately ended.

For those areas of animal use that have not been reviewed, a thorough scientific review should be conducted to determine the efficacy of using animals in those areas of investigation. Such 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 systematic reviews to be conducted before animal studies can receive funding. Scientists at Radboud University Nijmegen Medical Centre published the following 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. A recent article on forbes.com estimates that some major drug companies spend between US$4–$US11 billion per drug, once failure rates are factored in. 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. (Hooijmans and Ritskes-Hoitinga 2013)

There is an instructive and telling example from the Institute of Medicine's recent critical and thorough examination of the scientific necessity of using chimpanzees in behavioral and biomedical research. That scrutiny revealed that harmful research was approved, funded, and conducted for years even though there were alternative methods in virtually every area in which chimpanzees were being used. Institutional oversight bodies and funding agencies had given their stamp of approval to these protocols. However, as we now know, the review processes in place were 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.

Strategic Priority #3: Implement an ethical cost/benefit analysis system, as studies should not be approved when the benefit can never outweigh the suffering.

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 suffered 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; drug, alcohol, and food addiction experiments; and experiments involving the development and testing of tobacco products. Likewise, the use of animal-derived antibodies and fetal bovine serum that are used in scientific applications should be immediately eliminated, given the availability of valid alternative products that do not involve the suffering of animals. Further information regarding this matter follows.

Until all animal studies are ended, a system of analysis for a "risk threshold" or "upper limit,"

similar to that employed in research on humans should be implemented.

Hope Ferdowsian and Agustín Fuentes have commented:

> The roots of the failures in current animal research can be teased out by analysis of current human research standards, where risks of harm to individual subjects are monitored so that risks are minimized and benefits maximized. Within human research, the nature and scope of physical, psychological, social, and other harms and benefits are considered. Even when conflicts occur between commitments to the production of societal benefits and risks of harms to individuals, standards exist to protect human subjects from significant levels of harm. … By contrast, animal research guidelines typically do not reflect the attention to harm avoidance so fundamental to human research. It is basically assumed that nonhuman animals can be exposed to levels of harm without compensating benefits as long as there is a degree of anticipated benefit for humans. If human and nonhuman animals have similar (or shared) capacities for pain, distress, and suffering and similar interests in avoiding these harms, general moral duties to not harm should cover humans and nonhuman animals in relevant similar ways. It is morally suspect to have different sets of ethical norms in similar situations. In the case of invasive and potentially damaging research, it would be prejudicial and ethically problematic to discount serious harms to one species when the same or similar harms are considered to be of serious import in another species. (Ferdowsian and Fuentes 2014)

It has historically been claimed that animal research is the price we pay for medical progress, but we humans do not pay that price, and we generally do not see the animals—none of whom gave their consent—who do. Until we actually look at the price they pay with their suffering and ultimately with their lives, we cannot even begin to decide whether or not we think the results are worth it.

Strategic Priority #4: Work with other world leaders to harmonize and promote international acceptance of non-animal testing methods for regulatory toxicity testing requirements.

The past quarter-century has seen a revolution in the way in which chemicals are tested. This is the result of our better understanding of biological processes, which has allowed for the development of testing methods that can look directly at cellular mechanisms rather than at the crude "black box" results that come from using animals. Mechanistic information about the potential toxicity of a chemical, such as the potential for receptor binding or gene or pathway activation, is more readily obtained *in vitro* than *in vivo*.

Concurrently, there has been a growing recognition among regulators and the regulated community that the animal 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" (National Academies of Sciences 2007a). In 2007, the U.S. National Academies of Sciences published a landmark report titled "Toxicity Testing in the 21st Century: A Vision and a Strategy":

> Toxicity testing is approaching … a scientific pivot point. It is poised to take advantage of the revolutions in biology and biotechnology. Advances in toxicogenomics, bioinformatics, systems biology, epigenetics, and computational toxicology could transform toxicity testing from a system based on whole-animal testing to one

> founded primarily on *in vitro* methods that evaluate changes in biologic processes using cells, cell lines, or cellular components, preferably of human origin. The proposed changes will generate better data on the potential risks humans face from environmental agents, 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 that would take advantage of rapidly evolving scientific understanding of how genes, proteins, and small molecules interact to maintain normal cell function and how some of these interactions can be perturbed in ways that could lead to health problems. Specifically, the new testing approach would focus on toxicity pathways—cellular pathways that, when sufficiently perturbed, are expected to lead to adverse health effects.
>
> 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 and other models to estimate the human exposure necessary to produce responses in these pathways. (National Academies of Sciences 2007b)

The U.S. Environmental Protection Agency has made major advances in implementing these "Tox21" and "ToxCast" programs, and we urge continued momentum in implementing the amended Toxic Substances Control Act, as passed by a bipartisan Congress in 2016. By eliminating the use of tests on animals for regulatory purposes where full replacements exist and promoting the acceptance of methods currently in development, we have the opportunity to shift the regulatory testing paradigm further toward innovative non-animal techniques and thus become world leaders in the application of these methods. Later in this report, we elaborate on opportunities where the use of animals for regulatory testing should be ended immediately or within the next two to five and five to 10 years. These include acute systemic, genotoxicity, and pyrogenicity testing, vaccine and biologics testing, endocrine disruption, and carcinogenicity.

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 recommend that regulatory agencies and industry be mandated to use animal-free testing methods where they are "reasonably and practicably available" (as is required in the European Union). In addition, we recommend the establishment of a public-private center for predictive animal-free toxicology to be coordinated through the National Toxicology Program's Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). 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.

Strategic Priority #5: Divert funds from animal studies to the development of non-animal methods.

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 developed human cell-derived skin models, "organs-on-chips," *in silico* (computer) models, and other methodologies that can replicate

human physiology, diseases, drug responses, and chemical exposure more accurately than experiments on animals.

Studies have repeatedly shown that these new methodologies are better at modeling human diseases than crude experiments on animals. Indeed, NIH in its most recent five-year strategic plan announced that it would reduce and replace animal experiments, stating:

> 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. (National Institutes of Health 2015)

NIH 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 as well as more effective and reliable methods for toxicity assessment can be developed. This will also alleviate the unimaginable suffering of millions of animals.

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APPENDICES

Please find below further detail on opportunities for replacing animals in the following areas of scientific research and testing:

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CANCER

Recommendation: End 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 percent of cancer drugs were approved after entering the first phase of clinical trials, even though they were all successful in preclinical testing. The authors admit that the "current animal models (e.g., xenograft tumour models in mice) can be poor predictors of clinical outcomes in humans" (Hay, David and Craighead). Even though study design and other logistical issues can be problematic, "most futilities in fact originate from molecular mechanisms of the drug(s) tested" and "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" (Mak, Evaniew and Ghert).

Rodent models have misled cancer researchers. For example, we now know that endogenous bile acids, if disregulated, can induce DNA damage and several forms of cancer, such as colon cancer. However, previous research on rats shows 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 the colon epithelial cell accumulated turnover rate (adjusted by age) are all different between rodents and humans, contributing to the discrepancy (Bernstein, Pay and Dvorakova). Another example is the link between soy and breast cancer. It is now recognized that isoflavones, the main phytoestrogen in soy, are protective against several types of cancers, such as breast and prostate cancers (Setchell, Brown and Zhao). However, it was observed that genistein, a major isoflavone in soy, can induce oestrogen-sensitive tumours in some animal studies, including rodents. The inconsistency was later explained to be due to differences in phase II metabolism of genistein in rodents, who have about 20-150 times (depending on the strain) higher level of unconjugated, and hence active, genistein. Additionally, the rodent models also had low endogenous oestrogen levels and different metabolic profiles compared to humans, and high experimental levels of isoflavones were used in those initial studies (Setchell, Brown and Zhao). There are numerous other examples.

It has also been found that rodents are not suitable for research on radiation-induced carcinogenesis such as thyroid cancer and possibly also leukaemia. The nuclear architecture and spatial positioning of genes involved in radiation-induced injury between rodent and human thyroid cells are drastically different (Gandhi and Nikiforov). Similarly, rodents are not suitable for research on pancreatic ductal adenocarcinoma (PDAC). Some scientists have pointed out that "although it may seem obvious that there are important differences between men and mice, this is often overlooked by those modeling human disease" and that "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" (Logsdon, Arumugam and Ramachandran). It is likely true that rodents are not good for any type of cancer research, given their many shortcomings described above, their unparalleled popularity in cancer research, and yet astonishingly low translational success overall. Therefore, it is wise to move away from rodent models and focus on human-relevant methods instead.

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HIV/AIDS

Recommendation: End immediately

Despite being one of the most strongly-contested areas of animal research, animal experiments in the field of HIV and AIDS continue to waste precious time and resources. The failures of animal experiments to translate into useful human application of HIV/AIDS vaccines was recognized more than twenty years ago when in 1995 the National Institutes of Health (NIH) instituted a moratorium on chimpanzee breeding, acknowledging the failure of studies with chimpanzees to produce clinically useful data in this field. At the time, the chimpanzee was the most commonly used animal in HIV/AIDS research, causing much suffering to the animals and leading to a financially burdensome surplus of chimpanzees in laboratories.

Following this acknowledgement that chimpanzees aren’t human-relevant surrogates for HIV and AIDS research, experimenters began to use other nonhuman primates (NHPs), notably macaques. However, macaques were also unreceptive to HIV. Scientists then turned to simian immunodeficiency virus (SIV) for clues, despite the known fact that the SIV isn’t related to the most widespread HIV virus, HIV-1, but is instead simply a relative of the rarer and less pathogenic HIV-2 (Haigwood). The genetic homology between HIV and SIV is only 55 percent, with SIV being the less genetically diverse virus of the two (Antony and MacDonald; Centlivre and Combadiere). Due to differences in surface proteins and other molecular markers, antibodies that neutralize SIV have no effect on HIV and vice versa (Haigwood), making their usefulness in HIV research void. Importantly, the challenge dose of SIV used in NHP experiments is much higher than the typical amount of HIV-1 to which a human is exposed during sexual transmission (Julg and Barouch). 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” (Girard, Habel and Chanel).

Immune systems as well as genetic variances between NHPs and humans are also aspects of the species-specific differences that weaken NHP HIV/AIDS research. Here are some examples: NHPs have more leukocyte antigen genes and therefore a wider variety in antigen recognition than do humans (Kumar, Chahroudi and Silvestri). NHP T-cells contain molecules called siglecs which act as “brakes” on the NHP’s immune system, preventing hyper-responsiveness. The absence of siglecs in human T cells dramatically changes how humans respond to infection and to treatment (Nguyen, Hurtado-Ziola and Gagneux). The primate TRIM5α gene codes for a restriction factor that impacts responsiveness to retroviruses such as SIV, conferring some NHPs with greater resistance to infection, a function mostly lost in human TRIM5α (Song, Javanbakht and Perron). Differences in gene transcription play a huge part in why diseases and treatments affect one species differently or more dramatically than others. For example, even in chimpanzees, our closest nonhuman relatives, transcript expression in the liver differs by 40 percent (Gilad, Oshlack and Smith), a gap that only widens following translation into protein. For these reasons and more, HIV/AIDS vaccine research involving NHPs has been called “one of the most notable failures in animal experimentation translation” (Akhtar).

Due to broad failures in NHP HIV/AIDS research, experimenters have recently shifted some focus to a species even farther genetically removed from humans -- the mouse. The

"humanized" mouse model for HIV/AIDS research has been partially repopulated by human immune cells, allowing the animals to be infected with HIV-1. But humanized mice are limited in their duration and longevity with the disease and also retain murine major histocompatibility complex (MHC) antigens, "complicating immune response interpretations" (Haigwood). In 2014, Boska and colleagues reported that the "obstacles in generating a small animal model require that viral tropism, neuroimmune activation, cognitive impairments, and CD4+ T cell losses are operative" and that "This has remained an unmet goal" (Boska, Dash and Knibbe). Not surprisingly, mice have also failed to generate useful results for clinical HIV/AIDS treatment.

Considering the differences of the laboratory environment from human society, it is clear that animal experiments will never capture the complexity of this human disease. Experimental animals are kept in 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 (Antony and MacDonald). As noted by Kumar and colleagues, "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" (Kumar, Chahroudi and Silvestri). Thinking ahead to alternative methods, UK scientists have stated that "Existing animal models predicting clinical translations are simplistic, highly reductionist, and therefore, not fit for purpose" and that clinical attrition data "focuses 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" (Matthews, Hanison and Nirmalan). Others have recognized that human *in vitro* models are needed to truly replicate disease and treatment mechanisms (Kumar, Chahroudi and Silvestri).

As recent as November 2016, scientists admitted that even after costly animal experiments, human data are still needed to determine if a drug is fit for the clinical setting. Rao and Alving of the US Military HIV Research Program stated that "human clinical trials still appear to be the only reliable way to determine whether an HIV vaccine candidate will have activity or efficacy in humans" (Rao and Alving). In a comprehensive 2008 review of preclinical and clinical data, Jarrod 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 (Bailey). As the associate editor of the *British Medical Journa*l declared in 2007, "When it comes to testing HIV vaccines, only humans will do" (Tonks).

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CARDIOVASCULAR DISEASES AND STROKE

Recommendation: End immediately

Cardiotoxicity is a main reason that drugs fail in clinical trials. Experts point out that there is "a lack of concordance between the effects of compounds in animals (or animal-derived tissues) and those in humans" (Gintant, Sager and Stockbridge), "substantial differences in drug responsiveness between species can limit the effectiveness of predicting clinical outcome from animal toxicity testing" (del Alamo, Lemons and Serrano), and that there are many known species-related differences in contractile function and calcium handling (del Alamo, Lemons and Serrano). For example, there are differences in the ion channel composition and property in the hearts of rats, guinea pigs, rabbits, and dogs compared to humans. This makes the profile of ventricular repolarisation and susceptibility of arrhythmia different, and hence leads to varied drug responses. Gintant et al even refer to testing cardiotoxicity in animal models a "black box" approach (Gintant, Sager and Stockbridge).

Apart from differences in the ion channels, rodents also differ significantly in contractile protein and function. A recent meta-analysis evaluated 11 functional parameters of the heart comparing rodents with humans, and only one of the 11 measured parameters (systolic pressure) was within an acceptable range for comparison (Milani-Nejad and Janssen). It is also well-known that rodents are resistant to (often diet-induced) atherosclerosis, a major cause of many cardiovascular diseases, due to their lack of cholesteryl ester transfer protein (Barter and Rye). It is clear that human-relevant *in vitro* and *in silico* methods are much more suitable for cardiotoxicity testing and cardiovascular research in general.

For heart failure research specifically, "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]" (Chandrasekera and Pippin). There are major flaws in animal models from fruit flies to non-human primates, and the authors plead that "we must use the human subject as the quintessential animal model for 21st century heart failure research" (Chandrasekera and Pippin).

The success rate of preclinical stroke research is especially low. There have been well over a thousand treatments investigated in animal models, but so far there is only one drug on the market, a tissue plasminogen activator. 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" and "the ultimate proof that plain standardization of procedures in fact increases the rate of successful translation from bench to bedside in stroke research is still missing" (Sutherland, Minnerup and Balami). Indeed, the first Stroke Therapy Academic Industry Roundtable (STAIR) recommendations were published in 1999, but the success rate hasn't improved, and the drug NXY-059, which fulfilled the STAIR criteria, still failed (Sutherland, Minnerup and Balami). This illustrates the need to shift away from animal models and focus on human-centered methods.

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OBESITY AND DIABETES

Recommendation: End immediately

From 2004 to 2014, more than 9,800 papers were published describing the induction of obesity in animals via dietary manipulation (Lai, Barnard and Chandrasekera) and more than 50 papers were released every month over the last three decades examining rodent models of type 2 diabetes mellitus (T2DM) (Chandrasekera and Pippin). Diet- and genetically-induced obese animal models are being used in an effort to understand obesity and its related comorbidities such as T2DM. Considering these numbers, we now know a great deal about metabolic conditions in rodents, but "many details of human T2DM pathogenesis remain unclear, and means of preventing disease progression remain elusive" (Chandrasekera and Pippin).

Diet-induced obese animals are most often made obese with exposure to commercially manufactured pre-defined diets, ignoring the heterogeneity of the human experience with food. Following consumption of obesogenic diets, rodents show high resistance to the cardiovascular complications that constitute the leading causes of obesity-related mortality in humans (Lai, Barnard and Chandrasekera), underscoring the significant physiological differences between humans and mice. On the other hand, genetic models of obesity clearly lack construct validity: The observed phenotypes in these animals are only "secondary to genetic mutations that do not reflect disease etiology in humans" (Wang, Chandrasekera and Pippin). Most genetic models of T2DM are based on leptin- or leptin receptor-deficiency when neither of these represent an important contributor to T2DM in humans (Wang, Chandrasekera and Pippin).

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, to disease progression at the organism level, all the way to dramatic differences in environmental exposure and autonomy of lifestyle (Chandrasekera and Pippin). Despite these very clear discrepancies, obesity and diabetes research in animals continues while more relevant, human-based methods are often ignored.

A major confound in animal studies of metabolic disease is the "control" animals against which "treated" animals are compared. In the experimental setting, animals are sedentary, without environmental stimulation, overfed, and are at times already insulin resistant and at risk for premature death, even without obesogenic diets (Martin, Ji and Maudsley). In addition, general laboratory temperatures are far below thermoneutrality for rodents, contributing to metabolic stress, increasing food intake in both "control" and "treated" animals, and further skewing data (Lai, Barnard and Chandrasekera).

Human-relevant alternatives to the use of animals in obesity and diabetes research include human imaging, *in vitro* technologies using human heterologous cell lines, organotypic 3D cell culture, the use of human organs *ex vivo*, genome-wide association studies, and *in silico* modelling (Chandrasekera and Pippin; Carnell, Gibson and Benson; Andersen and Sandholt). In fact, the United States Food and Drug Administration has approved a closed-loop insulin pump developed using *in silico* modelling as a substitute for animal testing, providing just one example of how "Realistic 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" (Kovatchev, Breton and Man).

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NEURODEGENERATIVE DISEASE

Recommendation: End immediately

Neurodegenerative diseases such as Alzheimer's (AD), Parkinson's (PD), Huntington's (HD) and amyotrophic lateral sclerosis (ALS) are all human-specific. None of these diseases occurs naturally in other animals and no animal model has been developed that recapitulates all aspects of a particular disease (Potashkin, Blume and Runkle). Most clinical trials for therapies developed using animal models have failed (Lane and Dunnett; Pistollato, Ohayon and Lam; Burns, Li and Mehta). For AD research, the clinical failure rate for new drugs is 99.6 percent and there have been no new discoveries aimed at slowing the progression of the disease for 10 years (Pistollato, Ohayon and Lam).

In a recent bioinformatic analysis comparing transcriptional signatures of human AD, PD, HD, and ALS with mouse models of these diseases, Stanford scientists found that:

> ...most available mouse models of neurodegenerative disease fail to recapitulate the salient transcriptional alterations of human neurodegeneration and that 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. (Burns, Li and Mehta)

These molecular discrepancies underscore the unnatural ways in which these models are created. Physical and chemical lesioning and systemic administration of toxins are acute stressors and as such necessitate a response in these animals that is 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 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 (Lane and Dunnett), further reducing the likelihood that their observations will be of use to humans.

Genetically modified mouse models of neurodegenerative disease exhibit an inconsistent range of pathological and behavioural phenotypes, due in part to the transgenes used, inconsistencies

in transgene insertion and expression, and mouse background strains (Ehrnhoefer, Butland and Pouladi). The most commonly used genetic mouse model of ALS, the SOD1 model, is based on a gene that accounts for only three percent of ALS cases in the human population (Ehrnhoefer, Butland and Pouladi). Again, systematic reviews have shown 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" (Benatar), and that "animal models are not an ideal system for studying ALS or for developing drug therapies" (Clerc, Lipnick and Willett). 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" (Menache and Beuter).

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

As animal studies fall short, scientists and policy makers are coming to the realization that research strategies should be more relevant and human-based. 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 (iPSC) models, 'omic' technologies (genomics, proteomics, etc.), *in silico* models, neuroimaging, and epidemiological studies (Pistollato, Ohayon and Lam).

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NERVE REGENERATION / SPINAL CORD INJURY

Recommendations: End immediately

Many neuroprotective agents have been developed that are successful in treating spinal cord

injury (SCI) in animal models, but the clinical trials have been disappointing. There are 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" (Akhtar AZ, 2008). For example, Methylprednisolone (MP), a routinely used treatment for acute SCI, has generated inconsistent results. A systematic review examining 62 studies of MP on a wide range of species from rodents to monkeys found that 34 percent of the studies reported beneficial results, 58 percent of them no effect, and eight percent of them mixed findings. The results were inconsistent both among and within species, even within strains. Furthermore, even when many of the study design and procedure variables were controlled, the variability in results remained (Akhtar AZ, 2008). The authors pointed out numerous intrinsic differences between and limitations of each species/models and suggested that no human-relevant animal model can be developed due to these immutable inter-species and intra-species differences. They concluded that "research emphasis should be on the development and use of validated human-based methods" (Akhtar AZ, 2009).

Among the different species, rats are particularly unsuitable for nerve repair 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" (Kaplan, Mishra and Kohn). More specifically, the inconsistencies between animal models and clinical situation include: "(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" (Kaplan, Mishra and Kohn).

Human relevant methods such as human stem cells and clinical research can bypass these limitations and should be the focus.

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MEDICAL TRAINING

Recommendation: End immediately

Biomedical education has traditionally used animals to teach human physiology, study human anatomical form and function, and practice human surgical procedures. Yet, recent studies showing human patient simulators (HPS) and computer assisted learning (CAL) programs teach biomedical education as well as or better than animal dissection and

experimentation (Patronek 37-43), rising public opposition to animal use in laboratories (Goodman), animal laboratory cost burdens (Reznick), and a renewed focus by the medical community on improving patient safety and reducing clinical errors through simulation-based training (Kohn), have all contributed to a paradigm shift in biomedical education. Human simulation-based learning has become the gold standard. Now medical students in the U.S. and Canada learn without using animals during the curricula (Fears).

Medical experts have recommended the 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" (Hansen). 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 (Dua).

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 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" (Hall). The author published a separate letter in the 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" (Hall).

For military trauma training, non-animal methods are used instead of animals by nearly 80 percent of NATO allies (Gala), confirming that animal use for this purpose is neither necessary nor justified. Efforts to replace the military trauma training on animals with human simulators have gained many prominent supporters, including recently the New York Times Editorial Board (Editorial), and numerous medical and veterans organizations representing more than 255,000 physicians and doctors-in-training that have former U.S. Surgeons Generals among their leadership (Rep. Hank Johnson).

MICROSURGERY TRAINING

Regarding the field of microsurgery, there now exists an array of low- and high-fidelity non-animal methods that have been developed to effectively teach a wide range of basic and advanced microsurgical skills to novice and expert physicians and have been endorsed as replacements for live animal use. These methods include task trainers and perfused cadaver-based methods that can teach procedures such as anastomoses, resection of artificial tumours, bypasses, and aneurysm creation, dissection, and clipping.

For example, a study from the University of Toronto comparing the microsurgical anastomosis skills of surgical residents trained on live rats versus 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" (Grober).

A systematic review of microsurgical training methods supported these findings, noting, "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" (Ghanem).

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.

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TRAUMA AND SHOCK

Recommendation: End Immediately

After rodents, pigs are the most commonly used animal in trauma experimentation. However, notable species-specific differences between pigs and humans render results from this research unintelligible. For example, pigs have a different coagulation activity than humans, making it difficult to achieve a state of coagulopathy in pigs. In instances of human trauma, coagulopathy, or the inability to clot, represents part of the "lethal triad" for patients and is a great concern for researchers and physicians (Hildebrand, Andruszkow and Huber-Lang). However, this aspect cannot be modelled in animals without a pre-trauma intervention, rendering the experimental situation far from what is experienced in a clinical setting. In addition, there are differences in administration of mechanical

ventilation and drugs such as vasopressin and heparin in research and, as with mice, immune responses are different between pigs and humans (Hildebrand, Andruszkow and Huber-Lang; Stadlbauer, Wagner-Berger and Raedler).

Trauma is incredibly heterogeneous: Patients differ in age, gender, ethnicity, medical history, alcohol and drug use, and presence of other injuries, making the production of an appropriate animal model difficult (Tsukamoto and Pape), if not impossible. In studies of traumatic brain injury, all promising therapeutics identified in animals have failed in human clinical trials (Xiong, Mahmood and Chopp). 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. (Combes)

A major contention with studying trauma *in vivo* is that for sound ethical reasons, animals must be anesthetized during the trauma and subsequent treatment. As summarized in a review by Fülop and colleagues, "anaesthesia usually depresses respiration, reduces metabolic demand, influences the central nervous system and moderates cardiovascular competency mechanisms. Furthermore, certain anaesthetics change the immune function (production of cytokines and activity of natural killer cells) and may facilitate bacterial translocation" (Fulop, Turoczi and Garbaisz).

Importantly, it has been shown that computer simulation can accurately replicate real-life trauma and predict patient outcomes (Brown, Namas and Almahmoud) and clinical research remains invaluable in this field.

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FORENSIC SCIENCES

Recommendation: End immediately

Forensic science is a unique research area and deserves serious ethical scrutiny, as the goal

of the research is to understand crime-related issues, rather than improve human health or life conditions, and the experimental methods are often horrific and conducted without anaesthesia (see below). The authors 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." (Cattaneo, Maderna and Rendinelli)

The use of animals in forensic research was heavily criticized as early as 1992, when Bernard Knight pointed out that ''painful, sometimes mutilating experiments on conscious animals'' in order to obtain ''tenuous potential benefit to some medico-legal problem'' cannot be condoned, particularly if one considers that such works ''are not regularly used in routine forensic practice'' and just ''gather dust in university libraries'' (Knight). 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. (Knight)''

Since then the situation has worsened. In 2015, Cattaneo et al published a meta-analysis and review examining 404 forensic science articles and found that 69.1 percent of them "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" (Cattaneo, Maderna and Rendinelli). Cruelty aside, the authors also stressed that "the history of forensic sciences has provided us with much evidence of the inapplicability of data obtained from studies performed on animal models" given the anatomical, physiological, and genetic differences between species. On the other hand, there are a plethora of alternative methods such as manikins, simulators, artificial materials, and *in vitro* technologies, and "applying alternative methods rather than using animals has provided, in the forensic field, important and reproducible results" (Cattaneo, Maderna and Rendinelli). Together with the abundant and readily available alternative methods, the ethical problems, and the scientific and practical issues associated with animal experimentation, forensic research is a prime area for animal use to end immediately.

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INFLAMMATION AND IMMUNE RESPONSE

Recommendation: Immediate end, particularly for mice

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 humans, and that there are certain fields in particular where 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 (Mestas and Hughes). Since then several other analyses have been published detailing

the vast differences between human and mouse immunology. In 2013, a large and collaborative statistical analysis showed that the responses of mice following acute inflammatory stressors such as burn, trauma, endotoxin exposure, and sepsis were "close to random in matching their human counterparts" and supported the "higher priority for translational medical research to focus on the more complex human conditions rather than relying on mouse models to study human inflammatory disease" (Seok, Warren and Cuenca).

A 2014 study found fundamental differences in the innate immune response between the species, stating that "While in human blood mechanisms of immune resistance are highly prevailed, tolerance mechanisms dominate for the defense against pathogenic microorganisms in mouse blood" (Zschaler, Schlorke and Arnhold). Logically these differences make sense: We humans "do not live with our heads a half-inch off the ground" (Mestas and Hughes) and we have considerably longer lifespans and a larger body size than do mice (Mestas and Hughes; Zschaler, Schlorke and Arnhold). As concisely stated by Leist and Hartung, "humans are definitely no 70-kg mice" (Leist and Hartung). Despite the glaring contention, mice continue to be used for immunological research.

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. A recent 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, 3D organotype systems, and organ-on-chip technologies will "enable human models of well-controlled complexity, yielding detailed, reliable data; thus providing a fitting solution for the drug development process" (Bergers, Reijnders and van den Broek). In addition, advances in data collection and computer analyses have allowed for the development of 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" (Cappuccio, Tieri and Castiglione).

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NEUROPSYCHIATRIC DISORDERS

Recommendation: End immediately

Animals "models" of neuropsychiatric disorders, such as depression, schizophrenia, bipolar disorder, and anxiety, lack two critical aspects of model validity: 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 face validity, meaning that animals lack the ability to "recapitulate important anatomical, biochemical, neuropathological, or behavioural features of a human disease" (Nestler and Hyman). No one animal model is able to replicate all aspects of a particular condition, and features of human behaviour representing hallmarks of these disorders cannot be produced or properly assessed in animals (Nestler and Hyman; van der Staay, Arndt and Nordquist; Kato, Kasahara and Kubota-Sakashita).

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 behavioural test is the forced swim test. In the forced swim test, a rat or mouse is placed in a container of water with no way to escape nor any place to rest out of the water. Naturally, the rodent will spend some time swimming and trying to find a way to get out of the stressful situation in which they find themselves but will eventually become immobile and float. The time spent swimming may be extended by giving the animal an antidepressant drug, a finding which led some scientists to assert that less time spent immobile was a sign of the animal being less "depressed" and more time spent immobile meant that the animal was more "depressed," as if they had "given up" and were in despair. However, as Molendijk and de Kloet point out, immobility in the forced swim test is simply an adaptation to the situation and should not be used to determine an animal's mood (Molendijk and de Kloet). Animals that are quicker to float also save their energy and are less likely to sink, meaning that animals who more rapidly pick up on this reality are simply learning this adaptive behaviour more readily. Even the inventor of the test, Porsolt, stated that the forced swim tests could not be used as a model for depression; nevertheless, more than 2000 papers published over the past decade have ignored this warning (Molendijk and de Kloet).

Importantly, despite the suffering endured by animals induced to display symptoms of neuropsychiatric disorders through experiences such as severe stress due to uncontrollable pain, isolation, social defeat, or maternal deprivation, or through surgical or genetic manipulation, little of this work is relevant to humans. In a survey of 121 animal studies claiming to investigate Attention Deficit Hyperactivity Disorder (ADHD), only five of these studies were found to be in any way relevant to the hypotheses of the human medical papers in which they were cited. The authors of this survey concluded that "animal research has contributed very little to contemporary understanding of ADHD" (Carvalho, Crespo and Bastos). A similar failure of animal studies to translate into a clinical setting has been noted with bipolar depression (Kato, Kasahara and Kubota-Sakashita) and animal studies have been cited as the primary source of attrition in neurobehavioral clinical trials (Garner). Significant differences in physiology between humans and other animals likely accounts 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 (Jin, Romano and Marshall). Misregulation of tyrosine hydroxylase has been implicated in several psychiatric illnesses, such as bipolar disorder and schizophrenia.

Due to the psychological distress inherent in animals induced to display neuropsychiatric disease tendencies and the inapplicability of such studies to humans, we recommend that the use of animals in neuropsychiatric disorders be ended immediately.

To quote 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" (van der Staay, Arndt and Nordquist). This group also pointed

out that in all cases, "benefits must outweigh the ethical costs of the animals. These costs include pain and suffering, distress and death," (van der Staay, Arndt and Nordquist). Funds should be allocated towards more relevant, human-based experimental models, such as computational modelling using already well-defined biomarkers (Siekmeier) and the use of patient-specific stem cells for personalized medicine, which "affords the ability to general neuronal cell-based models that recapitulate key aspects of human disease" (Haggarty, Silva and Cross).

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ADDICTION STUDIES

Recommendation: End immediately

Nonhuman animal "models," primarily rats, are the most widely-used method for studying human addiction. However, there are several fundamental aspects of nonhuman animals that make them inappropriate for this purpose. 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 (Tzschentke). It has been argued that attempts to model human disorders such as addiction in nonhuman animals, especially rodents, is "overambitious" and that the "'validity' of such models if often limited to superficial similarities, referred to as 'face validity' that reflect quite different underlying phenomena and biological processes from the clinical situation" (Stephens, Crombag and Duka).

Second, the pharmacokinetic actions of drugs are different among species. For example, "the rate of metabolism of MDMA [street name: Ecstasy or E] and its major metabolites is slower in humans than rats or monkeys, potentially allowing endogenous neuroprotective mechanisms to function in a species-specific manner" (Green, King and Shortall). 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 (Green, King and Shortall). Since MDMA is being explored not only because of its illegal use as a recreational drug, but 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 drug user chooses to consume the abusive substance over other methods of obtaining a feeling of reward. Laboratory animals are not given this option. When they are, the vast majority of animals will choose an alternative reward, such as sugar, over the drug of abuse (Ahmed). This holds true for primates as well as rodents (Ahmed). Even in animals with very heavy previous drug use, only about 10

percent would continue to give themselves a drug when they had the option to make another rewarding choice. Two reviews assert that the lack of choice provided to animals in these experiments elicits "serious doubt" about "the interpretation of drug use in experimental animals" and the author predicted that "taking the full measure of this situation could lead to a validation crisis that in turn will lead to a change in the way animal models of addiction are conceived and validated" (Ahmed). The non-human animal has been called "the most reluctant collaborator" in studying alcohol addiction and noted to have a "determined sobriety" that the researcher must fight against in order to overcome "their consistent failure to replicate the volitional consumption of ethanol to the point of physical dependency" (Ramsden). Indeed others have reasoned that "it is difficult to argue that it truly models compulsion, when the alternative to self-administration is solitude in a shoebox cage" (Hyman and Malenka).

Despite the prevalence of addiction research, "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" (Tzschentke). The data from animal studies was promising in certain drug classes, but these have either failed to be effective in human trials or have not been tolerated well by humans, a negative outcome that was not predicted by animal trials (Tzschentke). The funds used to support ineffective and wasteful animal addiction studies could instead be used to aid effective and directly human-relevant drug prevention, rehabilitation, and mental healthcare programs. In addition, non-invasive human research methods such as imaging could provide us with answers to the questions that nonhuman animals, in their distaste for drugs of abuse, cannot possibly answer.

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TOBACCO TESTING

Recommendation: Immediately eliminate the use of animals for developing and testing tobacco products.

Non-Animal Approaches Available Now

The United States should immediately prohibit animal testing for existing tobacco products and for the development of new tobacco products. While the Family Smoking Prevention and Tobacco Control Act does not require animal tests for tobacco products, the Food and Drug Administration lists *in vivo* studies among the types of nonclinical studies that may be used to support marketing applications for new tobacco products (FDA). Internationally, the European Commission Scientific Committee on Health, Environmental and Emerging Risks appropriately states that, following the EU policy to ban animal studies for chemicals to be used in voluntary products, animal studies are not endorsed to assess the safety of tobacco additives (SCHEER). In addition, Belgium, Estonia, Germany, Slovakia, and the United Kingdom already prohibit animal tests for tobacco products due to ethical concerns (Brepoels; Parve; The German Government; Glasa; Home Office). The hazard assessment of tobacco products increasingly employs innovative non-animal

methods including cell and tissue cultures exposed to whole cigarette smoke or e-cigarette vapour at the air–liquid interface, cell transformation assays, and genomic analyses (Behrsing, Raabe and Manuppello; Manuppello and Sullivan). These methods have been used to investigate cytotoxicity, genotoxicity, inflammation, and gene expression. They are more relevant to actual human exposure than animal studies that have historically under-predicted the hazards of tobacco

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PYROGENICITY

Recommendation: Immediately eliminate the use of animals for pyrogenicity assessment.

Non-Animal Approaches Available Now

Before drugs and medical devices can be marketed, regulators require testing to demonstrate the absence of contamination 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.

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 (EDQM 2010) (European Directorate for the Quality of Medicines). The MAT 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 is compatible with drugs and medical devices (Fennrich, Hennig and Toliashvili).

The rabbit pyrogen test (RPT) has never been formally validated for its relevance to humans, in part because of species-specific differences in pyrogen sensitivity, and it is incompatible with certain drug classes (Hartung, Borel and Schmitz). The RPT requires that rabbits be injected with a test substance and subsequently restrained for three hours, during which changes in their body temperatures are monitored rectally. In Europe alone, more than 100,000 rabbits are used each year in the RPT (Daneshian, Akbarsha and Blaauboer). The *Limulus* amoebocyte lysate test (LAL), sensitive only to bacterial endotoxins and no other pyrogens, requires the use of haemolymph bled from captured horseshoe crabs. After bleeding, up to 30 percent of horseshoe crabs die and those who survive the initial bleeding are less likely to survive in the wild (Anderson, Watson and Chabot). A synthetic version of the LAL is available that replaces haemolymph with a recombinant reagent (the recombinant factor C assay), but sensitivity is still limited to bacterial endotoxins. The MAT avoids these problems, and case studies document instances in which the MAT detected pyrogen contamination in products that passed the RPT and LAL but caused fever in human patients (Hasiwa, Daneshian and Bruegger).

Although the MAT is accepted in the U.S and E.U. following product specific validation, tests that use animals are still used despite their well-documented limitations (U.S. Food and

Drug Administration). Eliminating the use of animals in pyrogen tests requires additional effort by regulatory authorities and standards organizations (1) to integrate and harmonize a preference for the MAT in international testing requirements, and (2) to encourage drug and device manufacturers to use and submit data from the MAT in their product dossiers. Following a survey of pyrogen test users, the European Directorate for the Quality of Medicines and HealthCare (EDQM) revised the Ph. Eur. general chapter on the MAT to improve the method's usability and to emphasize that the MAT is considered a replacement for animal-based pyrogen tests (EDQM 2016a; 2016b) (European Directorate for the Quality of Medicines and Health Care) (EDQM). This is repeated in statements from the European Medicines Agency (European Medicines Agency Committee for Medicinal products for Veterinary Use). The International Organization for Standardisation (ISO) is revising its guidance to allow use of MAT when evaluating medical device pyrogen contamination, but the revision process has moved slowly (Fennrich, Hennig and Toliashvili). 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 ambiguity is resolved.

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ANTIBODY PRODUCTION

Recommendation: Immediately eliminate the use of animal-derived antibodies in scientific applications.

Non-Animal Approaches Available Now

Affinity reagents, such as antibodies, are essential tools used in research to bind to a molecule to identify it or influence its activity. Animals used in antibody production are subjected to a number of invasive and painful procedures, including antigen injection, injection of a priming solution to induce an immune response, and repeated blood or ascites collection, before being killed. Additionally, there is a growing concern about the lack of quality and reproducibility of animal-derived antibodies, which often show poor specificity or fail to recognize their targets. 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, and provide scientific and economic advantages (Groff; Gray). They can be developed by researchers in their laboratories and are commercially available. The U.S. should introduce a ban on *in vivo* production of monoclonal antibodies using the ascites method, such as one that has been in place in the Netherlands for more than 20 years, and should further move to eliminate the use of animals in the hybridoma method and the import of animal-derived monoclonal antibodies (Marx, Embleton and Rischer). Furthermore, because only 0.5–5 percent of the

antibodies in a polyclonal rea¬gent 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 (Bradbury and Pluckthun). For scientific and ethical reasons, the U.S. should immediately move to end the use of animal-derived antibodies in scientific applications.

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FETAL BOVINE SERUM

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

Non-Animal Approaches Available Now

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 cows are found to be pregnant during slaughter, a large gauge needle is used to draw the blood from the beating heart of the fetus. Because the unborn calves are not anesthetized at the time of blood collection, they likely experience pain when blood is drawn. In 1995, it was estimated that 500,000 liters of FBS are produced worldwide each year (Hodgson). While this number has likely increased since 1995, this amount of FBS translates to more than one million bovine fetuses being used for this purpose each year (Jochems, van der Valk and Stafleu). 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 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 (van der Valk, Mellor and Brands; van der Valk, Brunner and De Smet). The U.S. should expediently move to restrict the production and use of FBS when non-animal media or supplements are available (www.piscltd.org.uk/fbs) for list of several commercially-available products). For cell types in which non-animal supplement concentrations have not yet been optimized, the U.S. should require exemptions to be obtained to produce or use FBS. To obtain exemptions, measures taken to seek non-animal alternatives and a plan to transition to non-animal media or supplements should be required.

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SKIN IRRITATION / CORROSION

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

Non-Animal Approaches Available Now

The Organisation for Economic Cooperation and Development (OECD) has developed step-wise guidance for an integrated testing strategy using *in vitro* skin irritation methods that avoids or minimizes animal use (OECD 2014).

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) category 2, category 3 or non-classified chemicals. May be used as a stand-alone test or in a tiered testing strategy.

a) **OECD Test No. 430: *In Vitro* Skin Corrosion: Transcutaneous Electrical Resistance Test Method (TER).** Allows the identification of non-corrosive and corrosive test chemicals in accordance with the UN GHS but does not allow the sub-categorization of corrosive substances and mixtures.
b) **OECD Test No. 431: *In Vitro* Skin Corrosion: RHE Test Method.** Allows the identification of corrosive chemical substances and mixtures and enables the identification of non-corrosive substances and mixtures when supported by a weight of evidence determination using other existing information. The test protocol may also provide an indication of the distinction between severe and less severe skin corrosives.
c) **OECD Test No. 435: *In Vitro* Membrane Barrier Test Method for Skin Corrosion.** Allows for subcategorization of corrosive chemicals into the three UN GHS sub-categories of corrosivity.

Methods are generally validated for use with cosmetics and industrial chemicals registered under the European Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH) regulation. Efforts are ongoing to validate the reconstructed human epidermis (RHE) method as an acceptable alternative to the ISO 10993-required rabbit skin irritation test for assessing medical device biocompatibility (Casas, Lewerenz and Rankin). Likewise, some of the above methods are currently undergoing evaluation in a joint effort by the US Environmental Protection Agency (EPA), industry, and the US NTP Interagency Centre for the Evaluation of Alternative Toxicological Methods (NICEATM) for use with pesticide products. This consists of side-by-side comparison and analysis of existing *in vitro/in vivo* data generated by pesticide companies for their products. *Depending on the outcome of these efforts, additional work may be needed to further validate 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 OECD Guidance document on considerations for waiving or bridging of mammalian acute toxicity tests (OECD 2016).

Cited References:

Casas, J W, et al. "In vitro human skin irritation test for evaluation of medical device extracts." *Toxicology In Vitro* 27.8 (2013): 2175-2183.
OECD 2014. "OECD Environment, Health and Safety Publications Series on Testing and Assessment No. 203: Guidance Document on an Integrated Approach On Testing and Assessment (IATA) for Skin Corrosion and Irritation." 2014. http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2014)19&doclanguage=en.
OECD 2016. "Guidance Document on Considerations for Waiving or Bridging of Mammalian Acute Toxicity Tests. Series on Testing & Assessment No. 237." 2016. http://www.oecd.org/env/ehs/testing/mono%202016%2032.pdf.

EYE IRRITATION / CORROSION

Recommendation: Immediately eliminate the use of animals for eye corrosion / irritation testing and validate a non-animal method that can directly predict GHS category 2 (irritant) substances for use in a regulatory setting.

Non-Animal Approaches Available Now

The general consensus is that no single *in vitro* alternative test can be used to replace the *in vivo* rabbit eye test to predict across the full range of serious eye damage / eye irritation

responses for different chemical classes. However, by employing combinations of alternative test methods used in a tiered testing strategy replacement may be accomplished. 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. A guidance document on an integrated approach to testing and assessment (IATA) of serious eye damage and irritation is underway at the OECD (OECD 2016a).

a) **OECD Test No. 491: Short Time Exposure (STE) Test Method.** This method can identify, without further testing, either a chemical causing serious eye damage (GHS category 1) or one not requiring classification (GHS No category). The test guideline states that it cannot be used to define GHS category 2 substances (moderate/mild irritants) and further testing for definitive classification is needed. **However, this test method has been shown to be capable of classifying irritants as minimal, moderate, or severe, although results are not accepted for regulatory use** (Institue for In Vitro Sciences). *Additional work in this area may allow this method to be acceptable for classifying GHS category 2 irritants for regulatory purposes and should be pursued.*
b) **OECD Test No. 492: Reconstructed human Cornea-like Epithelium (RhCE) Test Method (EpiOcular™, MatTek, Corp.).** This method can identify those chemicals not classified for eye irritation or causing serious eye damage (GHS No category), but cannot differentiate between GHS category 1 and GHS category 2, and thus a positive finding would require additional testing.
c) **OECD Test No. 460: Fluorescein Leakage Test Method.** This method can identify, without further testing, either a chemical causing serious eye damage (GHS category 1) or one not requiring classification (GHS No category). It is recommended as an initial step within a top-down approach to identify ocular corrosives / severe irritants, specifically for limited types of chemicals (i.e., water soluble substances and mixtures).
d) **OECD Test No. 437: Bovine Corneal Opacity and Permeability (BCOP) Test Method.** This method has undergone international validation by the US Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the European Union Reference Library for alternatives to animal testing (EURL ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM). The method is capable of accurately predicting chemicals (both substances and mixtures) that induce serious eye damage (GHS category 1) as well as those not requiring classification for eye irritation or serious eye damage (GHS No category) without further testing. OECD does not recommend its use to classify category 2 substances. However, the EPA has worked with industry and a private *in vitro* contract laboratory to develop a system whereby the BCOP can be used to identify moderate / mild irritants in AMCPs as defined by EPA's system of classification, which is similar but not completely analogous to the GHS. *Further work in this area may lead to acceptable use of this method to classify category 2 irritants.*
e) **OECD Test No. 438: Isolated Chicken Eye Test Method.** This method has undergone international validation by ICCVAM, EURL ECVAM and JaCVAM. The method is capable of accurately predicting chemicals (both substances and mixtures) that induce serious eye damage (GHS category 1) as well as those not requiring classification for eye irritation or serious eye damage (GHS No category) without further testing. It is recommended as the first step within a top-down or a

bottom-up testing strategy approach. It cannot be used to classify category 2 substances.

Methods are generally validated for use with cosmetics and industrial (REACH) chemicals, and there may be limitations with some methods with certain types of chemicals (e.g., surfactants, solids, etc.). None of the current OECD-approved assays is recommended to be used to directly determine category 2 eye irritants in a regulatory setting (i.e., most have been validated to determine GHS 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 determination of eye irritation / corrosion when classifying pesticidal anti-bacterial cleaning products (AMCPs) and has published a guidance document that describes the testing framework industry can use for this endpoint (USEPA). Also, the EPA in collaboration with NICEATM and industry members is currently engaged in evaluating these methods for use with conventional pesticides through a side-by-side comparison of *in vitro/in vivo* data for representative pesticide chemical classes. A report on this effort is expected within the next six months.

Additionally, there are opportunities available to waive these tests based on criteria described in OECD Guidance document on considerations for waiving or bridging of mammalian acute toxicity tests (OECD 2016b).

Cited References:
OECD 2016a. "Work plan for the Test Guidelines Programme." 2016. http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2013)6&doclanguage=en.
OECD 2016b. "Guidance Document on Considerations for Waiving or Bridging of Mammalian Acute Toxicity Tests. Series on Testing & Assessment No. 237." 2016. http://www.oecd.org/env/ehs/testing/mono%202016%2032.pdf.

SKIN SENSITIZATION

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

Non-Animal Approaches Available Now

Testing on animals for skin sensitization can be fully replaced with three *in vitro / in chemico* assays that each address a different key event in the adverse outcome pathway for this endpoint (OECD 2012). The methods distinguish between sensitizers and non-sensitizers and are recommended to be used in an integrated approach to testing and assessment.

a) **OECD Test No. 442C: *In Chemico* Skin Sensitization Direct Peptide Reactivity Assay (DPRA).** The DPRA addresses the molecular initiating event of the skin sensitisation AOP.
b) **OECD Test No. 442D: *In Vitro* Skin Sensitization ARE-Nrf2 Luciferase (KeratinoSens) Test Method.** This method addresses the second key event of the skin sensitization AOP.
c) **OECD Test No. 442E: *In Vitro* Skin Sensitization Human Cell Line Activation Test (h-CLAT).** This method addresses the third key event of the skin sensitization AOP. While none of the methods is endorsed for potency determination, the h-CLAT shows promise in this regard. *Further efforts are required to explore this potential.*

In general, the methods can be used to test industrial chemicals and cosmetics although they may not be suitable for testing some substance types. There is an ongoing effort to validate non-animal skin sensitization methods to replace the ISO 10993-required guinea pig skin

sensitization test for assessing medical device biocompatibility (Coleman, McNamara and Grailer; McKim, Keller and Gorski). In the US, an integrated testing strategy that draws on *in silico*, *in chemico*, and *in vitro* approaches has been developed by ICCVAM, in collaboration with Procter and Gamble, to determine skin sensitization potential (ICCVAM). The EPA is currently collecting and analysing paired i*n vitro/in vivo* skin sensitization data to assist in moving towards non-animal approaches to evaluating pesticides (USEPA). With a successful outcome, this work could lead to regulatory acceptance by the EPA within the next two years. Additionally, there are opportunities available to waive these tests based on criteria described in OECD Guidance document on considerations for waiving or bridging of mammalian acute toxicity tests (OECD 2016).

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ACUTE SYSTEMIC TOXICITY

Acute systemic toxicity testing includes acute oral, dermal, and inhalation toxicity testing. Several international efforts have focused on non-animal replacements for acute systemic toxicity testing including the Multicenter Evaluation of In Vitro Cytotoxicity (MEIC) program (Ekwall, Barile and Castano) and the EU Framework Programme (FP) 6 ACuteTox project (http://www.acutetox.eu/). In addition, a 2015 series of webinars and a workshop hosted by the PETA International Science Consortium Ltd., the Physicians Committee for Responsible Medicine, and NICEATM presented a strategy for replacing acute systemic toxicity testing (Hamm, Sullivan and Clippinger; PISC 2016a). A 2016 series of webinars and a workshop hosted by the PETA International Science Consortium Ltd. and NICEATM focused specifically on alternative approaches for acute inhalation toxicity testing (workshop report draft in progress, PISC 2016a). As a result of these and other efforts, there are alternative approaches that can be currently used to reduce/ replace or waive these tests, and other approaches undergoing further development.

Non-Animal Approaches Available Now

GENERAL WAIVING OF ACUTE TOXICITY TESTS

Waivers for acute toxicity testing in animals may be issued by regulatory authorities if certain criteria can be met (this includes the three topical skin and eye endpoints discussed above as well as acute systemic toxicity testing). The EPA issued guidance for waiving or bridging acute toxicity tests for pesticides or pesticide products (USEPA 2016a) and the OECD recently issued guidance for waiving or bridging acute toxicity testing (OECD 2016). The guidance includes use of existing data for read-across and the consideration of the physicochemical properties of the test substance.

Another approach that can be currently used to avoid certain acute toxicity testing is the use of the GHS Additivity Equation for classifying formulations and mixtures for acute systemic toxicity tests (United Nations). This is a method that essentially "adds" the toxicities of known ingredients in pesticide mixtures without the need for animal testing of those mixtures. It has

been demonstrated to accurately predict acute systemic toxicity for agrochemical formulations (Corvaro et al.) The GHS Additivity Equation is currently usable as stand-alone replacement method in some geographies, such as for the purposes of the European Classification, Labelling and Packaging regulation. The EPA is currently involved in a pilot study to evaluate use of this method in its pesticide registration program (USEPA 2016b).

Retrospective analyses of data that have been traditionally required by regulatory agencies are needed to determine how the data generated are actually being used. This analysis will provide evidence of redundant or valueless animal tests that can be waived or completely eliminated without endangering public health and safety. Information on mechanisms of acute systemic toxicity will improve the development of assays for key events, predictive models, and integrated approaches to testing and assessment. The OECD's AOP framework can facilitate the systematic reporting, curating, and integrating of this information (OECD 2013).

ACUTE DERMAL TOXICITY

Recommendation: Acute dermal toxicity testing should be avoided.

Non-Animal Approaches Available Now

Acute dermal toxicity testing is often a regulatory requirement in addition to acute oral and acute inhalation toxicity testing and it focuses on determination of lethal doses through the dermal route. There has been general consensus that testing through the dermal route is essentially redundant if there is available data on oral toxicity. EPA and NICEATM analyzed the relative contribution of data from acute oral and dermal toxicity tests to pesticide hazard classification and labelling. Data were collected from about 600 paired acute lethality dermal and oral toxicity studies in rats used to assess pesticide formulations. Finding that the dermal study for formulations provided little to no added value in regulatory decision making, EPA has issued guidance allowing registrants to submit waiver requests for this study (USEPA 2016c).

Further assessments of existing data are needed for other classes of chemicals to determine if acute dermal toxicity testing can be subject to waiver or be eliminated altogether.

Furthermore, there were recommendations to waive dermal studies for substances that are non-classified by the oral route as well as testing dermal absorption prior to conducting acute dermal toxicity studies. As a result, in Europe REACH Annex VIII has been amended so that substances that are non-classified by the oral route do not require dermal data (The European Commission).

ACUTE INHALATION TOXICITY

Recommendation: Acute inhalation toxicity testing should be avoided through waivers or use of the GHS Additivity Equation; further work is needed to develop non-animal testing approaches.

Non-Animal Approaches Available Now

As described above, the EPA and OECD have guidance documents for waiving or bridging acute toxicity testing, including acute inhalation toxicity (USEPA 2016a) (OECD 2016). For example, in the case of acute inhalation toxicity, if the substance demonstrates low volatility, is not aerosolized or otherwise made inhalable as a gas or vapor under conditions of use, storage, handling, or transport then the test can be waived.

Non-Animal Approaches Likely to be Available Within 2-5 Years

Numerous promising research efforts are underway in both the US and Europe to develop non-animal methods for acute inhalation toxicity. A recent series of webinars and a workshop

hosted by the PETA International Science Consortium and NICEATM presented several approaches that could eventually replace animal testing for this endpoint, including use of human "lung-on-a-chip" and "Metabo-Lung" models as well as QSARs and read-across predictors (PISC 2016b). The drafting of a workshop report and establishment of specific working groups to carry out workshop recommendations are underway. Developing an integrated approach to replacing animals in inhalation toxicity testing will likely be needed and include the use of *in silico*, *in chemico*, and *in vitro* methods. Continued development of AOPs and increased understanding of toxicity mechanisms will be important in defining human-relevant testing batteries. *For use by regulatory authorities, more research, proof of concept, evaluation with different classes of chemicals, and validation is needed.*

ACUTE ORAL TOXICITY

Recommendation: Acute oral toxicity testing should be avoided through waivers or use of the GHS Additivity Equation; however, further work is needed to develop non-animal testing approaches.

Non-Animal Approaches Available Now

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 cytotoxicity assay, which can be used in a weight-of-evidence approach to support the identification of non-classified substances (acute oral LD50>2000mg/kg b.w.) (EURL ECVAM). *In vitro* tests such as 3T3 NRU and normal human keratinocyte (NHK) assays that measure basal cytotoxicity can also be useful in determining starting doses in animal tests, but cannot be used to fully assess systemic toxicity as a stand-alone test.

Non-Animal Approaches Likely to be Available Within 2-5 Years

EURL ECVAM is currently working to improve confidence in the 3T3 NRU through the use of quantitative structure-activity relationships (QSARs), and by accounting for target organ information and the lack of metabolism in 3T3 cells (Hamm, Sullivan and Clippinger; Prieto). QSAR models have been developed for predicting rodent oral toxicity (The National Academies of Sciences, Engineering and Medicine). Information on repeated dose toxicity, if available, can also be used to predict acute effects. EURL ECVAM has proposed an approach to identify non-classified substances using information from 28-day repeated dose toxicity studies, thereby avoiding acute systemic toxicity testing (Bulgheroni, Kinser-Overskainen and Hoffmann; Graepel, Asturiol and Prieto).

Promising approaches are under development that involve use of both *in silico* and *in vitro* methods. One approach that uses mitochondrial membrane potential inhibition as measured in the ToxCast™ suite of assays has been investigated for 1,800 compounds as a predictor of mammalian and fish acute toxicity (USEPA 2016d) (Bhhatarai, Wilson and Bartels). When compared to curated data from regulatory and literature studies, the method did well for predicting fish toxicity and rat intravenous toxicity, but did not perform well for rat oral toxicity. After inclusion of a model that first accounted for bioavailability after passage through the gut, the ToxCast results had much better correlation to rat oral data.

Further work, particularly at the international level, is needed to develop a reliable non-animal approach to predicting acute oral toxicity.

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GENOTOXICITY

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

Non-Animal Approaches Available Now

Currently, the assessment of genotoxicity typically follows a step-wise approach, beginning with a core battery of *in vitro* tests. The major endpoints that must be evaluated are gene mutation, structural chromosome aberrations and numerical chromosome 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 chromosome aberrations (EURL ECVAM). 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 these *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 (Corvi and Madia). These considerations have been incorporated into recent revisions of OECD Test Guidelines.

To better assess 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 Testing Cosmetic Ingredients and Their Safety Evaluation, the SCCS recommends using a micronucleus test on 3D-reconstructed human skin or a comet assay in either mammalian cells or on 3D-reconstructed human skin (SCCS). However, negative results produced in these alternative tests do not necessarily rule out genotoxic potential. In such cases, expert judgement as well as mechanistic investigations may be helpful to evaluate the weight–of-evidence. For example, *in vitro* toxicogenomics-based tests can provide information on the mode of action of potential genotoxicants by identifying global gene expression changes.

Non-Animal Approaches Likely to be Available within 2-5 Years

Validation studies of the micronucleus test and comet assay on 3D-reconstructed human skin are currently being conducted by Cosmetics Europe providing further opportunities for phasing-out the use of animals for genotoxicity testing (Pfuhler).

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CARCINOGENICITY

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

Non-Animal Approaches Likely to be Available within 2-5 Years

a) Mouse carcinogenicity study:

It is essential that data from animal tests undergo systematic review for their value in protecting human health and the environment. In an assessment of 202 pesticide evaluations from the European Union review program, Billington et al. (2010) demonstrated the mouse carcinogenicity study contributed little or nothing to either derivation of an acceptable daily intake (ADI) for assessment of chronic risk to humans, or hazard classification for labelling purposes. In terms of pesticide approvals, the authors showed the mouse study did not influence a single outcome. *Additional reviews of this kind may show that the mouse carcinogenicity study provides little value in assessment of other classes of chemicals as well. Further investigation into this area could yield results that would argue for elimination of this study entirely.*

b) Cell transformation assays:

In vitro cell transformation assays (CTAs) recapitulate a multistage process that closely models *in vivo* carcinogenesis and have the potential to detect both genotoxic and non-genotoxic carcinogens. In its recommendation on the CTA based on the Bhas 42 cell line, EURL ECVAM notes that information on the transforming potential of substances generated by CTAs may be sufficient for decision-making (EURL ECVAM). The Bhas 42 cell line was developed from BALB/c 3T3 cells through transfection with a Harvey ras sarcoma viral mutated oncogene homolog (v-Ha-ras). Since Ha-ras is involved in multistage carcinogenesis, Bhas 42 cells are more predisposed to transformation than the original cells BALB/c 3T3. In a validation study, the Bhas 42 CTA was tested with 98 substances including carcinogens and non-carcinogens. For predicting carcinogenicity, its performance was equivalent or superior to conventional genotoxicity assays (Hayashi, Kojima and Corvi). 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 the assay 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 (OECD 2015). The structural alerts (SAs) rulebase has recently been expanded with a

large number of new SAs for non-genotoxic carcinogenicity and has been implemented into the OECD (Q)SAR Toolbox version (Benigni, Bossa and Tcheremenskaia). The identification of DNA-reactive chemicals with the Ames test or genotoxic SAs can be combined with the identification of non-genotoxic carcinogens with non-genotoxic SAs leaving CTAs to model most of what is left unexplained.

There is an effort underway at the OECD level to generate an integrated approach to testing and assessment for non-genotoxic carcinogens (OECD 2016).

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ENDOCRINE DISRUPTION

Recommendation: In light of existing non-animal methods and weight-of-evidence approaches, the use of animals in endocrine testing can be substantially reduced.

Non-Animal Approaches Available Now

ToxCast for Oestrogen and Androgen Pathways:

EPA's Toxicity Forecaster (ToxCast) uses more than 700 high-throughput screening assays, which cover a range of high-level cell responses and approximately 300 signalling pathways, and computational toxicology approaches to rank and prioritize chemicals. Data have already been generated on thousands of chemicals of interest to the EPA.

A subset of the ToxCast assays are devoted to evaluating possible effects on various hormone pathways including the oestrogen, androgen, and thyroid systems. These are being used successfully in the EPA's Endocrine Disruption Screening Program (EDSP) to rank and prioritize chemicals. After a comparative study of ToxCast oestrogen pathway assay results to uterotrophic assay results (Browne, Judson and Casey), EPA has 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 oestrogen pathway (USEPA).

EPA recently published results of work using ToxCast and a computational model to screen chemicals for effects on the androgen pathway (Kleinstreuer et al. 2016) and is working on use of this method as an alternative for the rat Hershberger assay that is currently used to screen for androgen effects. Work is expected to be completed within the coming year.

Non-Animal Approaches Likely to be Available Within 2-5 Years

ToxCast and other approaches for the Thyroid Pathway:

The thyroid pathway has more complexity than either the oestrogen or androgen pathways. Though ToxCast is showing promising results, more research is required in this area and use of this system to replace tests in 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 signalling pathway was published in 2014 (OECD). 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 / use.
More research and development is needed in this area to obtain non-animal approaches to screening for thyroid disruption potential in humans and wildlife populations.

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DEVELOPMENTAL AND REPRODUCTIVE TOXICITY

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

Reproductive and developmental toxicity testing is not just one of the most animal intensive areas of regulatory toxicology, where just one test can consume more than a thousand animals, but it is also time- and cost-intensive. None of the *in vivo* methods used for testing reproductive and developmental toxicity have been validated for their relevance to humans (Rovida, Longo and Rabbit). There are considerable limitations surrounding the *in vivo* methods, with a predictivity of only around 60 per cent and large interspecies variations (Hartung; Bouvier d'Yvoire, Bremer and Casati).

There are many promising ongoing efforts within the area of development and reproductive toxicity, and as such, it is important that the U.S. immediately connects with the relevant institutes and organizations involved. In particular, EU-ToxRisk is an integrated European 'Flagship' program-driving mechanism-based toxicity testing and risk assessment for the 21st Century (EU-ToxRisk). Currently, the Johns Hopkins Center for Alternatives to Animal Testing (CAAT) is the only U.S.-based partner in the EU ToxRisk program. U.S. federal agencies would be well served to become a project partner within the program as well.

Additionally, the EPA is moving forward with 21st century toxicology methods to screen and prioritize chemicals for endocrine-disrupting potential (see above section) (EDSP). Data from this effort should be used along with other existing data to evaluate aspects of reproductive toxicity in a weight of evidence approach (Martin, Knusden and Reif).

Non-Animal Approaches Likely to be Available within 5-10 Years
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 (Rolaki, Nepelska and Bremer; AOP Wiki). The EU FP6 project, ReProTect, has also investigated possible strategies to cover the entire mammalian reproductive cycle, resulting in a series of published works (ReProTect). Furthermore, the ChemScreen FP7 project has been designed to generate a rapid screening system that is relatively simple and cost-effective (van der Burg, Bay Wedebye and Dietrich). The US 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 integrate *in vitro* and *in silico* modelling approaches (USEPA). While the field is gradually moving toward integrated testing and assessment strategies in order to cover the majority of possible mechanisms driving a broader range of

potential adverse outcomes, the U.S. could accelerate work in this area by promoting and funding projects aimed at developing additional AOPs of developmental and reproductive toxicity, developing rapid validation testing procedures and by promoting regulatory acceptance of non-animal testing strategies.

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BIOLOGIC DRUGS

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

Non-Animal Approaches Likely to be Available within 5-8 Years

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 death of the animals. New technologies have enabled the production and testing of biologic quality, identity, and safety without animals, but experience has shown that validation and regulatory acceptance of these alternatives has not guaranteed that they are used in place of animals (Dozier, Brown and Currie; Draayer; Bristow, Schulster and Jeffcoate; European Directorate for the Quality of Medicines and HealthCare). Activities intended to phase out the use of animals in this context must ensure that regulatory authorities and industry commit to (1) transition to non-animal biologic production platforms, (2) ensure that available non-animal methods are consistently used in place of animal-based tests, and (3) develop 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 hyperimmunising horses and isolating the resulting immunoglobulins from animals' blood. These equine-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 (Unkauf, Miethe and Führer). All biologics of animal origin, including antibodies (described below), can be replaced in a similar fashion with adequate funding and support from regulators.

Non-animal quality tests are available that replace animals, but no formal mechanism exists to ensure that barriers to their implementation are resolved in a timely manner (Dozier, Brown and Currie). In some instances, manufacturers report difficulty meeting the technical criteria for using validated alternative methods (as with the *in vitro* Leptospira vaccine potency tests) (Stokes, Srinivas and McFarland). In other instances, international

regulators have yet to agree on technical criteria for using alternative methods (as with the *in vitro* rabies vaccine potency test) (Stokes, McFarland and Kulpa-Eddy). In the absence of oversight of the implementation process, these barriers are left to be resolved informally, through workshops and decentralized problem solving. For companies seeking to use validated alternatives, this approach is prohibitively expensive and slow. As a consequence, industry adoption of alternative testing approaches remains limited despite the documented reduction in animal use these approaches achieve when they succeed (Veterinary Medicines Directorate). Additional barriers to the implementation of currently available animal replacement tests have been discussed at length in the literature for these vaccines; for erysipelas, clostridial, tetanus, vaccines; and for recombinant therapeutic hormones (International Alliance for Biological Standardisation). Accelerating and formalizing processes that facilitate 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 (De Mattia, Chapsal and Descamps; De Mattia, Hendriksen and Buchheit).

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EXPOSURE-BASED ASSESSMENT

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

Non-Animal Approaches Likely to be Available within 5-10 Years

This approach to reducing animal testing focuses on shifting from hazard-based approaches to exposure-driven approaches for regulatory decision making. It promotes "fit-for-concern" assessments rather than "box-checking" regulatory testing approaches to human safety assessment. It explores safety based on real concern rather than characterizing hazard(s) that will not be relevant for human safety assessment. There is also movement in the pesticide industry to explore ways to further the approach of exposure-based assessment efforts.

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

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