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Protecting Human and Animal Health: The Road From Animal Models to New Approach Methods

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Running title: The Balance Between Use of Animal Models and NAMs

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Abbreviations:

3Rs	reduction, refinement, and replacement
AD	Alzheimer's Disease
AI	Artificial Intelligence
AOP	Adverse Outcome Pathway
CDER	Center for Drug Evaluation and Research (US FDA)
COVID-19	Coronavirus disease 2019
EPA	Environmental Protection Agency (US)
ECHA	European Chemicals Agency (EU)
FDA	Food and Drug Administration (US)
FDORA	Food and Drug Omnibus Reform Act of 2022 (US)
IATA	Integrated Approaches to Testing and Assessment
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ICH	International Council on Harmonisation
IVIVE	*in vitro*-to-*in vivo* extrapolation

iPSCs	induced pluripotent stem cells
MIE	Molecular initiation event
MPS	microphysiological systems
NAMs	New approach methods
NCI	National Cancer Institute
NCTR	National Center for Toxicological Research (NCTR)
NIH	National Institutes of Health
NTP	National Toxicology Program
OECD	Organization for Economic Cooperation and Development
OSHA	Occupational Safety and Health Administration (US)
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals (EU)
TSCA	Toxic Substances Control Act (US)
WoE	Weight of evidence

Abstract

Animals and animal models have been invaluable for our current understanding of human and animal biology, including physiology, pharmacology, biochemistry, and disease pathology. However, there are increasing concerns with continued use of animals in basic biomedical, pharmacological, and regulatory research to provide safety assessment for drugs and chemicals. There are concerns that animals do not provide sufficient information on toxicity and/or efficacy to protect the target population, so scientists are utilizing the principles of the 3Rs (replacement, reduction, and refinement) and increasing development and application of new approach methods (NAMs). NAMs are any technology, methodology, approach, or assay used to understand effects and mechanisms of drugs or chemicals with specific focus on applying the 3Rs. Although progress has been made in several areas with NAMs, complete replacement of animal models with NAMs is not yet attainable. The road to NAMs requires additional development, increased use, and for regulatory decision-making, usually formal validation. Moreover, it is likely that replacement of animal models with NAMs will require multiple assays to ensure sufficient biological coverage. The purpose of this manuscript is to provide a balanced view of the current state of use of animal models and NAMs as approaches to development, safety, efficacy, and toxicity testing of drugs and chemicals. Animals do not provide all needed information nor do NAMs, but each can elucidate key pieces of the puzzle of human and animal biology and contribute to the goal of protecting human and animal health.

Significance Statement: Data from traditional animal studies have predominantly been used to inform human health safety and efficacy. While it is unlikely that all animal studies will be able to be replaced, with the continued advancement in NAMs, it is possible that sometime in the future, NAMs will likely be an important component by which discovery, efficacy, and toxicity testing of drugs and chemicals is conducted and regulatory decisions are made.

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I. History of animal use, NAMs, and the 3Rs

The use of animals for scientific purposes dates back to the 6th century BC and their use throughout the intervening millennia continued to provide invaluable medical knowledge in human and animal anatomy, physiology, pathology, pharmacology, and medical devices (Choudhary and Ibdah, 2013; Ericsson et al., 2013; Miziara et al., 2012). Of course, early important scientific discoveries were made in animals since alternatives were not yet available (Dagnino, 2009). Importantly, many of these important discoveries have been translated to humans; some examples are provided in Table 1.

Although animals have provided significant contributions to modern medical understanding and advancement, there has been concern with their use for decades due to the lack of complete translation of findings to human application and efficacy and toxicity predictions, as well as ethical concerns about animal welfare issues, primarily pain and distress, numbers of animals used (Andersen and Winter, 2019; Joffe et al., 2016; Prabhakar, 2012; Robinson et al., 2019), and the concept of animal rights (Andersen and Winter, 2019). Specifically, there is concern with the number of animals that would be required to meet the challenge of testing product/chemical safety of the huge number of drugs and chemicals in commerce; it is estimated that there are 40,000-100,000 chemicals (EPA, 2016b; Wang et al., 2020). In addition to public concerns and those of funding agencies, animal use has come under even more scrutiny with the relatively recent adoption of Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) in the European Union (EU) and Frank R. Lautenberg Chemical Safety for the 21st Century Act in the US (Congress, 2016; EU, 2023).

The Center for Drug Evaluation and Research (CDER) at the US Food and Drug Administration (FDA) always welcomed non-animal tests, although it was clarified in the recent Food and Drug Omnibus Reform Act of 2022 (FDORA) (Congress, 2022). FDORA defined nonclinical tests as “a test conducted *in vitro*, *in silico*, or *in chemico*, or a non-human *in vivo*

test" that occurs before or during the clinical trial phase of the investigation of the safety and effectiveness of a drug. It specified that these include assessments such as cell-based assays, organ chips, or animal tests. FDA does not "require" animal studies for assessing toxicologic risk, rather the state of the science dictates whether an animal study is "warranted" as being the most relevant to assessing risk. In summary, FDORA did not change the science supporting the regulatory use of NAMs nor remove a "requirement" for animal safety studies as there was no such requirement to remove. It did provide greater clarity to stakeholders and may result in increased investment in developing and validating NAMs.

The adoption of these various laws and regulations is based in part on the 3Rs, a concept to replace, reduce and refine animal use. This concept, expressed by W.M.S. Russell and R.L. Burch, started in a project initiated in 1954 by the Universities Federation for Animal Welfare, which led to the publication in 1959 of The Principles of Humane Experimental Technique (Russel and Burch, 1959). Reduction is the use of fewer animals with no loss of useful information, refinement refers to efforts to reduce pain and suffering, and replacement refers to using non-animal methods. Replacement can be absolute, in which the animal is not used in any stage of the experiment; or relative, in which the animal is used as source of organ or tissue to prepare a primary culture. Thus, many alternatives are not animal free as products from the animals are necessary for the methods (e.g., fetal bovine serum, antibodies as detecting agents). Replacement can also involve the replacement of sentient animals (usually vertebrates) with less sentient animals (usually invertebrates such as worms) or bacteria. Reduction in animal use can be achieved by different strategies, including improved study design, method development and project coordination. *In silico* (aka computational modeling), *in vitro,* and *in vivo* methods all hold the potential for applying the reduction "R" and should be coordinated at a strategic level (Tornqvist et al., 2014). Good animal welfare is consistent with the 3Rs refinement goal, and the best animal welfare is essential for reliable results (Neville et al., 2022). Exploiting the latest *in vivo* technologies and animal welfare science will help to

reduce pain, suffering, distress, or lasting harm that animals experience across their lifetime. Interest is growing in the development of NAMs to conduct various studies, such as those to evaluate toxicity, investigate drug and chemical mechanisms, and determine drug efficacy. It should be noted that NAMs can be defined in a number of ways; however, for the purpose of this paper, NAMs will be defined as any technology, methodology, approach, or assay used to understand effects and mechanisms of drugs or chemicals with specific focus on applying the 3Rs.

Emblematic in this context, 'Toxicology in the 21st Century (Tox21)', is a US federal research collaboration aimed at developing alternative high throughput screening methods to quickly and efficiently test thousands of chemicals for potential biological targets (National Research Council, 2007). Tox21 aims to identify *in vitro* methods and computational approaches for testing chemicals, including drugs and food additives, and medical products to better understand and predict hazards to humans and the environment (van der Zalm et al., 2022). The 3Rs seek to ensure the rational and responsible use of laboratory animals and maintain an adequate protection in bioethical terms (Gorzalczany and Rodriguez Basso, 2021). Thus, the value of NAMs is three-fold: 1.) provide value for 3Rs approaches; 2.) scientifically make decisions in the human context for efficacy and safety; and 3.) obtain mechanistic information.

Animals and NAMs are used in both basic biomedical and regulatory research (States United for Biomedical Research, 2023). We continue to use mammalian and non-mammalian animal models in biomedical research with the incorporation of molecular and biochemical events to detail the steps that occur in any physiological process. However, NAMs are often used in biomedical research to address mechanisms by which drugs or chemicals act. Regulatory research, a form of applied research, is the development of tools to standardize product development methodologies to increase transparency and efficiency of the entire product life cycle from upstream innovation through the regulatory processes. It also allows for

early benchmarking and reduction of risk. Applied research can be conducted with animals (usually mammals but includes roundworms and zebrafish), nonanimal alternatives such as computer models or tissue cultures, or in some cases with humans (States United for Biomedical Research, 2023). The complexity of our biological systems and the need to be able to use a drug safely or know the safe level of exposure to a chemical or environmental agent requires an understanding of the toxicity of a drug versus its therapeutic use or knowing the level of exposure that might cause harm. Risk assessment incorporates both hazard and exposure and this can differ between drugs in which therapeutic value is important as compared to environmental chemicals. Thus, both animal research and NAMs are important for the development of safe and effective new drugs to treat human and animal diseases, and to assess the safety and toxicity risks posed by environmental hazards. It should be emphasized, though, that translational concerns persist with both safety and efficacy assessments regardless of whether animal models or NAMs are used and that challenges encountered in toxicological assessments are often the same as those for safety assessments (Allen et al., 2021; Hughes et al., 2013).

Progress towards reducing animal use and increasing NAMs use has been summarized in Figure 1 - there has been much success on the road to reducing animal use and using more NAMs, but at this time, the science does not support total replacement of all animal models with NAMs (FDA, 2023). Thus, the goals of this article will be to provide an understanding of animal use in basic biomedical research and regulation, a summary of the need to use well-designed animal models where still utilized, and a view of the road from animal models to NAMs, including how the 3Rs have been, or can be, applied to specific examples of animal models. The paper will also address advantages and limitations of both animal models and NAMs, thereby providing a balanced view of all approaches to drug development, efficacy, and toxicity testing. Finally, the paper will identify data gaps, needs, and future directions.

II. Animal models

An animal model is “a non-human species used in biomedical research because it can mimic aspects of a biological process or disease found in humans” (NIH, 2023). As outlined above, both mammalian and non-mammalian animals have been used to study basic physiology and develop models of human and animal disease. Choice of the appropriate animal model is tantamount for avoiding incorrect findings, and the unnecessary use of time, resources, and animal lives (Mukherjee et al., 2022; Varga et al., 2010). Although an animal model is developed as a surrogate for a human disease process, it might not always fully replicate human diseases or conditions with respect to etiology, pathobiology, biomarkers, or toxicity predictions (Prabhakar, 2012). In some instances, a single animal model might not mimic a human disease; in that case, the combination of several models can potentially recapitulate the disease to inform the testing strategy or illuminate the underlying biological pathways (Mukherjee et al., 2022).

Several types of animal models are available; their selection is largely dependent upon the scientific question, research goals and the ethical implications (Davidson et al., 1987). Approximately 95% of all animal research is conducted on mice, rats, and fish ((Speaking of Research, 2021); Table 2), although research over the last three decades has seen a steady increase in the number of species used for biomedical research (Bolker, 2017). While the focus of many involved in NAMs is to reduce the number of animals used in the regulatory process, more are used in basic and applied research (EU, 2020). Rodents have been especially useful to model human disease. Rats are physiologically and genetically closer to humans than mice (Szpirer, 2020); however, genetically modified mammals were initially produced in mice (Vandamme, 2014). For instance, the p53-deficient mouse was critical in identifying the role of *p53* as a tumor suppressor gene in cancer; its deletion in mice rendered them susceptible to developing spontaneous tumors (Donehower, 1996). Since that time, several rodent models of human disease have been developed, including genetically modified rats. Publicly available

databases are available listing rodent models that mimic human disease (Jackson Laboratory, 2023; Taconic, 2023)).

Translational research is the process of transforming discoveries in pathobiology of human disease and drug development into human application (Prabhakar, 2012). Several characteristics have been suggested when developing an animal model (or NAM) in order to maximize the validity and translational value of the model. They include the following: 1. Pathogenesis similar to human disease; 2. Similarity in histological and phenotypical characteristics; 3. Similar biomarkers of disease, 4. Reliable toxicity predictions; and 5. Similar response to proven therapies in human model. In this manner, non-human primates are sometimes used in animal research due to their close phylogenetic relationship to humans, with similarities in terms of genetics, behavioral and biochemical activities (Estes et al., 2018).

As a prime example of translational research, animal use has provided a long history of safely enabling Phase I clinical studies. Some severe safety issues in humans are not seen until late-stage premarket clinical studies or post-marketing, the latter meaning after human clinical trials have not shown safety concerns, illustrating that human individuals do not always predict population responses that occur at low frequency. Similarly, animal studies do not always predict all adverse effects, particularly effects that occur at very low frequency, which may then be seen when a large number of people are exposed to a new drug or chemical.

II.A. Selecting the appropriate animal model: successes and challenges

II.A.1. Thalidomide

In the 1950s, thalidomide was released and marketed outside the US as a non-addictive sedative considered very safe in humans and became one of the world's largest selling drugs (Vargesson, 2009; 2015). While the record of premarket testing for thalidomide is not clear, many (non-pregnant) people took thalidomide without side effects and thus it was considered very safe in humans. It was discovered to be an effective anti-nausea drug and was prescribed,

and in some cases given as samples, for nausea in pregnant people (Lenz, 1988). Thus, in the late 1950s and early 1960s, thalidomide was used in pregnant people to treat morning sickness in Europe, Australia, and other countries (Diggle, 2001). In the US, thalidomide had not been approved for use in pregnant people due a concern about the safety of the drug as it produced peripheral neuropathy in humans (FDA, 2018). In 1961, two clinicians (Burley and Lenz, 1962; McBride, 1961) independently confirmed that thalidomide was the cause of limb malformations during development (for review, see (Vargesson, 2015)). Thalidomide was withdrawn from the worldwide market and the epidemic of malformations subsided (Diggle, 2001; Lenz, 1962; McBride, 1961). Afterwards, testing in mice did not show the same syndrome, but further testing of thalidomide in pregnant rabbits showed limb defects as seen in humans. This became the impetus for recommendations to test new drugs for reproductive effects in one species and for teratology effects in two species (Kelsey, 1988). Interestingly, thalidomide has since been FDA-approved (US) for the treatment of leprosy and myeloma (Gao et al., 2020). The thalidomide episode demonstrated the value of premarket testing in animal models and in selecting the appropriate animal models in product safety testing.

II.A.2 COVID-19

Because the COVID-19 pandemic spread so rapidly, there was an immediate need to identify an appropriate animal model for learning about the disease process, developing treatments and therapies, and developing and testing vaccines. A number of animal models were used to compare which were most likely to recapitulate human disease (Fan et al., 2022; Munoz-Fontela et al., 2020; Pandey et al., 2021; Zhao et al., 2022). Mice were one of the first animal models examined for COVID-19 research but unlike humans, mice are not spontaneously susceptible to COVID-19. To overcome this, mice were genetically modified to express human angiotensin converting enzyme 2 (hACE-2), the protein to which the SARS-CoV-2 virus attaches to the cell for infection. Mice expressing hACE-2 are susceptible to SARS-

CoV-2 infection showing COVID-19 symptoms and disease similar to humans (Jia et al., 2020; Zhao et al., 2022).

Other species examined as COVID-19 models include Syrian and Roborovski dwarf hamsters and ferrets. Both species of hamster have disease that closely resembles COVID-19 in humans (Gruber et al., 2022), although the Roborovski dwarf hamster is more susceptible to COVID-19 at lower doses, exhibits more severe disease, and more closely mimics human COVID-19 in individuals with predisposing conditions (Gruber et al., 2022). Ferrets are naturally susceptible to COVID-19 and have been used as animal models of aerosol infectious agents (for review, see (Fan et al., 2022)). Detection of Sars-CoV-2 in the upper respiratory tract and nasal cavities of ferrets (Kim et al., 2020), showed that ferrets may be a good model for development and testing of mucosal vaccines. Non-human primates are often used in non-clinical trials for new drug candidates and vaccines. Advantages of non-human primates include that they are outbred and have an immune response similar to humans (Lu et al., 2020). Additionally, clinically relevant vaccine doses can be used in non-human primates, which is not the case for smaller animals (Li et al., 2022). Non-human primates demonstrated how COVID-19 could be transmitted and the effects of aging on severity (Yu et al., 2020) as well as understanding re-infection and efficacy of drugs, vaccines and antibodies (Corbett et al., 2020; Furuyama et al., 2022). Animal models played important roles in protecting human health from the worst pandemic in 100 years.

II.A.3 Osteosarcoma

One difficulty in finding appropriate models and treatments for cancer is the numerous different kinds of cancer. Cancers found in dogs and humans share similar characteristics including age of onset, presentation of symptoms, response to treatment and outcomes (Ostrander et al., 2019). Indeed, the National Cancer Institute (NCI) is using companion dogs for osteosarcoma research (NCI, 2019; Oh and Cho, 2023; Ostrander et al., 2019). Osteosarcoma is a cancer generally in the long bones of extremities in both humans and dogs and is

particularly invasive in both (for review, see (Makielski et al., 2019)). The ability to enroll large numbers of patients in clinical trials and compare genetic changes between humans and dogs, the similar environments in which the companion dogs and humans live, as well as the physiology, size and ability to tolerate drugs are just a few of the advantages of using companion animals such as dogs (LeBlanc and Mazcko, 2020; Leonardi et al., 2021; Ostrander et al., 2019; Simpson et al., 2022). The large number of spontaneous osteosarcoma cases in companion dogs each year has resulted in a well-characterized disease in dogs with pathological, biological and clinical similarities to human osteosarcoma (Tarone et al., 2022) (Leonardi et al., 2021). Studies in companion dogs have led to a better understanding of mutations, copy alterations, and pathway dysregulations (Gardner et al., 2019; LeBlanc and Mazcko, 2020; Megquier et al., 2022). Many of these alterations are also found in human osteosarcoma and result in similar symptoms, tumor progression, immune evasion and often recurrences and metastases (Moukengue et al., 2022).

Therapies are currently being developed and tested in dogs and some early trials have begun in humans. Such therapies have the promise of helping both species (NCI, 2019). This use of companion dogs in research that is needed for both human and canine disease is an example of translational research, comparative oncology, and application of the 3Rs - refinement and reduction. It also illustrates the need to conduct research in models that most closely resemble human disease, and in this example to the benefit of both humans and companion animals.

III. The road to NAMs: Refinement and Reduction

Several avenues to reduce animal testing are being explored including using legacy animal data, if available, to develop control groups for traditional animal studies. Additionally, shorter, more targeted animal testing is encouraged rather than long term testing, and researchers are encouraged to do both the animal study in partnership with the NAM(s) to

compare the results for certain chemical domains. Two recent papers from CDER/FDA provide insight into the opportunities and challenges of using NAMs and key areas where current approaches are less than optimal that might benefit from alternative methods (Avila et al., 2020; Avila et al., 2023).

III.A Refinement of animal models of Alzheimer's Disease

Alzheimer's Disease (AD) is a complex progressive neurological condition with behavioral and neurochemical manifestations that has proven to be resistant to current therapies. To address the multifaceted nature of AD, many animal models have been developed to understand the pathology and progression of the disease. These include both transgenic animals as well as natural, non-transgenic models of AD. Species used have included mice and rats, rabbits and the marmoset and cynomolgus monkey. However, these models do not spontaneously induce tangles, plaques or biochemical or cellular changes seen in AD (McKean et al., 2021), so most animal models currently used in AD research are transgenic mice that demonstrate the hallmarks of this disease including memory issues. Because spontaneous cognitive decline is common in older animals several species including the rat are used as models of age-related mild cognitive impairment or prodromal AD. Several higher order species such as aging canines more closely recapitulate the neuropathology seen in humans with AD. The dog for example shows spontaneous age-related cognitive decline and, progressive accumulation of Aβ plaques and tauopathy (Abey et al., 2021; Head, 2013).

NAMs have been developed to study AD and a recent review article describes progress in this field to model the pathogenesis of AD (Blanchard et al., 2022). Human-derived induced pluripotent stem cells are used to create brain organoid models of AD (Lagomarsino et al., 2021). Recent advances in this area combine blood vessel models with brain organoid models to overcome one of the major limitations of this model (Chen et al., 2021; Sun et al., 2022). However, brain organoid models have yet to fully recapitulate the parcellation into distinct cortices (i.e., prefrontal, visual, or somatosensory) seen in the human brain which continues to

limit their translational value (Andrews and Kriegstein, 2022). Despite the limitations, these models do provide complimentary data which can be used to further reduce and refine the use of animal models in AD research.

III.B Reduction of animal use with the carcinogenicity bioassay

A recent revision to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Guideline S1B - Testing for Carcinogenicity of Pharmaceuticals (published August 2022) is on its way to implementation, which would lead to a reduction in animal usage in the assessment of carcinogenicity for pharmaceuticals (ICH, 2022). The new S1B(R1) Addendum introduces an additional approach for assessing the human carcinogenic risk of pharmaceuticals that evaluates specific weight of evidence (WoE) criteria to inform whether a 2-year rat study is likely to add value to a human carcinogenicity risk assessment. Two-year rodent studies are intended to investigate the cancer risk of lifetime exposure to chemicals. In cases where the rat study is not determined to be of value, the carcinogenicity WoE assessment can be accepted in lieu of the 2-year rat study.

The key WoE criteria identified in the S1B(R1) Addendum were selected following a prospective study, which includes data that inform the carcinogenic potential based on drug target biology and primary pharmacologic mechanism; results from secondary pharmacology screens that inform selectivity and off-target potential; histopathology data from repeated-dose toxicology studies with an emphasis on the 6-month rat study; evidence for hormonal perturbation and immune modulation; and genetic toxicology study data. While all criteria contribute to the integrated analysis, the relative importance of each factor may vary depending on the pharmaceutical under consideration. Evaluation of these factors may be sufficient to conclude whether a 2-year rat study would add value to the assessment of human carcinogenic risk of a pharmaceutical. However, if any factors are deemed inconclusive, or if a concern is identified, a sponsor can conduct additional investigative studies or evaluate clinical data to further inform human mechanistic relevance of the concerning findings. These additional studies

could include NAMs that provide information to clarify the mechanism of action and clinical relevance of a concerning finding. The Addendum encourages the reduced use of animals by recommending that a short-term mouse study in a transgenic model, using approximately one-half the animals compared to a 2-year study, is prioritized over a traditional 2-year study in mice unless there is a scientific rationale for conducting the 2-year study.

III.C Reduction of animal use through repeated measures

One method to reduce animal use is the re-use of the same animals throughout an assessment without the need for multiple groups sacrificed at varying time points. Identification and prevention of human toxicological effects throughout the lifespan of an individual has proven a challenging and complex task, and clearly multiple and new approaches are needed to make continued progress in this area. Repeated assessments of blood, cerebral spinal fluid (CSF), and urine for endpoints/biomarkers coupled with targeted biological imaging can generate a useful profile of toxicity and are most useful if linked to concurrent traditional histopathological analyses collected in the same animals at the end of the experimental period. As an example, repeated assessments of blood, CSF, and urine for candidate biochemical markers coupled with targeted MRI and magnetic resonance spectroscopy can generate a useful, time-course profile of fluid and imaging biomarkers indicative of the neurotoxicity induced by the prototype compound, trimethyl tin (Imam et al., 2018). In other studies, the repeated use of imaging in a longitudinal study design has provided critical data on the assessment of several neurotoxicants using MRI (Anklam et al., 2022) and the therapeutic, methylphenidate, using PET/CT (Zhang et al., 2023). Using neurotoxicity as an example, Table 3 provides some examples of longitudinal and minimally invasive approaches that can be used in the same animal (Roberts et al., 2015). It is important to note that use of multiple endpoints in the same animal is a concept that can be broadly applied to reduce the number of animals necessary for an assay and may add value to the standard approach of using different groups of animals.

III.D Examples of *In vitro* methods used for COVID-19 vaccine development

The rapid development of vaccines for COVID-19 was done in part with *in vitro* methods, which allowed for a reduction in animal use. Three approaches to developing a COVID-19 vaccine were based on creating antibodies to a harmless version of spike protein found on the surface of COVID-19 (Li et al., 2022). When injected in human muscle, the spike protein was incorporated into cells and antibodies produced. The differences in the vaccines were in how they produced or delivered the spike protein: a) mRNA vaccine, used a genetically engineered messenger RNA based on the spike protein that when injected would incorporate into cells stimulating the production of antibodies to the COVID-19 spike protein; b) viral vector vaccine, the spike protein was inserted into a viral vector which when injected into a human would produce antibodies; and c) protein subunit vaccine, the spike protein was inserted into bacteria, yeast or animal cells to produce more spike proteins and then combined with substances such as adjuvants which would boost antibody production when injected into humans (Li et al., 2022). A review in 2022 describes the use of primary cell cultures, organoids, and MPS to study all aspects of infection, drug discovery, and drug repurposing (Pandamooz et al., 2022).

IV. The road to NAMs: Replacement

IV.A NAMs: *In vitro* replacements

In vitro methods have and will continue to play an important role in basic biomedical and regulatory research by allowing the investigation of a single or limited series of effects of a substance or an action in isolation and offer high sensitivity without interference from other biological phenomena, such as hormones or immune responses. Understanding single effects has been shown to be useful in identifying the various events that can be used to form an Adverse Outcome Pathway (AOP) with the first step in any pathway being a Molecular Initiating Event (MIE) and then a series of key events leading to an outcome (Jeong and Choi, 2017). AOPs provide a potential description of how an agent moves from a MIE to an outcome that you might not be able to achieve in an animal study. However, there are many different pathways

from a MIE to adverse outcome and there is no potency information in the AOP. Until quantitative aspects are added, they might not be as useful in a regulatory context. The classical *in vitro* (e.g., two dimensional cultures), microphysiological systems (MPS), organoids, tissue or organ evaluations provide valuable information improving our understanding of a toxic response while generating data faster and many times, at a far lower cost than methods using live animals. These *in vitro* tests can help identify single pathways that may be impacted before using whole animal studies. However, the complexity of the processes that occur even in a single cell and the numerous pathway interactions that can either enhance or reduce effects, whole animal studies are, at this time, necessary to identify potential effects in humans (Juberg et al., 2017; Knudsen et al., 2021; Knudsen et al., 2015; Rowlands et al., 2014).

It should be stressed that using *in vitro* assays to address the 3Rs has been a long-term investment in academia, companies, and regulatory agencies and great progress has been made (Clippinger et al., 2021). Progress and strategies have been addressed over the past two decades to increase the applicability, implementation, and acceptance of modern animal-free methods for efficiently and credibly evaluating chemical toxicity, drug efficacy, and safety assessment (Luechtefeld et al., 2018; Mahony et al., 2020; Methods, 2018). Publicly available high throughput screening data sets are now available for broad *in vitro* profiling of bioactivities across large inventories of chemicals (Thomas et al., 2019). Coupling this vast amount of mechanistic data with a deeper understanding of biology lays the groundwork for using NAMs. Leveraging advancements in such approaches and the accompanying efficiencies to detecting potential health hazards are unifying concepts toward implementing NAMs for decision-making in an animal-free zone. For example, the Frank R. Lautenberg Chemical Safety for the 21st Century Act that amended the Toxic Substances Control Act (TSCA) requires the US Environmental Protection Agency (EPA) to encourage and facilitate "… the use of scientifically valid test methods and strategies that reduce or replace the use of vertebrate animals while providing information of equivalent or better scientific quality and relevance that will support

regulatory decisions …" and also consider the impacts of chemicals and chemical mixtures to subpopulations who "…may be at greater risk than the general population of adverse health effects from exposure to a chemical substance or mixture, such as infants, children, pregnant women, workers, or the elderly" (EPA, 2016a). The US EPA convened a conference in 2019 to discuss NAMs for achieving reduced animal testing in chemical safety research and issued a guidance in February 2020 for meeting its animal testing reduction goals (EPA, 2023a). Complex NAMs that accurately predict the potential for human toxicity are needed to succeed or supersede conventional testing of chemicals in mammalian animal studies.

The use of NAMs in industrial chemical safety assessments varies. For example, cosmetic ingredients sold in the EU can no longer be tested in animals to evaluate product safety (EU, 2009). In these cases, companies use *in silico*/read across, *in vitro* and physiologically based kinetic models to conduct a Next Generation Risk Assessments using, for example, a 'margin of safety' approach (i.e., the margin between the lowest concentration causing bioactivity in *in vitro* assays and the estimated *in vivo* concentration of the ingredient under normal conditions of use) (Baltazar et al., 2020; EU, 2021). The idea is not to identify adverse effects, but rather support the premise that cosmetic ingredient exposures that are too low to cause bioactivity in *in vitro* assays will not cause toxicity to consumers. Challenges for these assessments include the incorporation of more integrated endpoints (e.g., systemic or developmental toxicity), ensuring that sufficient biological space has been examined to identify the lowest bioactive concentration, and adequately evaluating metabolites that may not form in all *in vitro* test systems. In other chemical regulatory programs (e.g., REACH for industrial chemicals), some NAMs can be used but animal data are still required. Animal testing, which identifies doses causing adverse effects for use in risk assessments, lessens concerns about examining complex biological processes and assessing metabolite toxicity; however, recently there has been unease about the variability of animal data and the extrapolation of these data to human toxicity/safety (National Academy of Sciences, 2022). Given issues related to number of

chemical assessments needed, duration/cost of animal testing, and ethical issues, some stakeholders are questioning whether NAMs should play a more prominent role in industrial chemical safety assessments, in which compounds often are not designed to be biologically active, and exposures are generally unintended and at lower levels.

IV.B NAMs: examples of assays they replaced

Studies performed *in vitro* offer certain benefits over *in vivo* approaches such as controlling the exact exposure conditions and identifying specific cell type responses. One goal of *in vitro* studies is to address the 3Rs and examples adopted by US and/or EU regulatory agencies can be found at (NTP, 2023; EU, 2006). A table entitled “Alternative Methods Accepted by US Agencies” published by the National Toxicology Program reveals 50 replacement assays, 43 that reduce animal use, and 17 for refinement (NTP, 2023). Some of the assays embrace both replacement and reduction (e.g., acute inhalation toxicity) so may occur in several of the lists in the above referenced table.

Central to the application of the 3Rs and NAMs is that the methods must provide data that lead to equivalent (or better) quality of the decision, which in toxicology means proper toxicity assessment, hazard identification, and characterization. Likewise, it will be difficult to rely on NAMs for pharmacology and efficacy assessment if they are less reliable than the currently-used methods. What differentiates drug versus chemical is that benefits for human can be part of the equation for the medical products but not for other chemicals. Regulators might accept alternatives to animal tests in toxicology if they allow them to classify and label chemicals, drugs, or food additives in the same way as the current tests. The principle always remains ‘safety first’, which is the final goal of toxicological assessments, and it is often forgotten that there were good reasons for introducing animal experiments in these assessments. One of the best examples is the Draize test, which was introduced to avoid ocular and skin corrosion and severe irritation in humans, following the numerous cases of blindness and disfigurement resulting from the presence of a synthetic aniline, used as a dye in Lash-

Lure, applied by operators in beauty salons to darken eyebrows and eyelashes (Greenbaum, 1933). The Draize test is rarely used today for assessing eye irritation and has been replaced by an Organization for Economic Cooperation and Development (OECD) test guideline in the Ocular Corrosivity and Irritation area (Test No. 494: Vitrigel-Eye Irritancy Test Method for Identifying Chemicals Not Requiring Classification and Labelling for Eye Irritation or Serious Eye Damage (OECD, 2021)) in which human cornea epithelium are used. In fact there are a number of NAMs that assess eye irritation and there also exists a defined approach that describes how the different methods can be used and interpreted (OECD, 2022). In some cases, there is the possibility of having tests waived; for instance, EPA has a guidance that describes how one might request a waiver of acute dermal toxicity tests for pesticides (EPA, 2023a). Although not truly a NAM, it is certainly aligned with the 3Rs.

IV.C NAMs: Computational Modeling

Computational (computer-based, aka *in silico*) models use structural information to predict chemical properties (e.g., health or environmental hazards) based on the premise that a specific interaction between a chemical and a protein target requires certain structural features. Quantitative structure activity relationships (QSAR) computational models can limit animal use in safety assessments by filling data gaps and contributing to more effective chemical screening, weight-of-evidence assessments, mode-of-action determinations, or integrated approaches to testing and assessment (IATAs). For example, “read across” is based on structure-activity relationships allowing data from a source compound to be used to fill data gaps for a related target chemical, thereby alleviating the need to conduct additional animal studies. Similarly, computational screening can be used to identify candidate chemicals with better safety profiles during new product development (e.g., select away from reactive chemicals that may have genotoxic/sensitization hazards) and thereby, avoid animal testing for chemicals that subsequently will not be commercialized. Computational models are especially

powerful when combined with other data streams. Mechanistic models can identify specific sub-/cellular targets, allowing specific follow-up screening/testing to confirm a potential mode of action. Lastly, computational models also can be used to predict toxicokinetic parameters (e.g., metabolite predictions, distribution, absorption), including *in vitro*-to-*in vivo* extrapolation (IVIVE), which provides dosimetry context to *in vitro* bioactivity data and thus, avoids animal testing for bioactivity that is not relevant based on exposure levels. Several recent publications describe protocols to illustrate how computational models can be used with other data streams to evaluate chemical hazards (Crofton et al., 2022; Hasselgren et al., 2019; Myatt et al., 2018) for more efficient and effective animal use. The summary from the FutureTox-IV workshop covered the diverse and specific types of computational models for developmental and reproductive toxicity from a broad perspective, including engineered microsystems, small model organisms, and computer simulation (Knudsen et al., 2021).

IV.C.1 Examples of computational models

The Collaborative Estrogen Receptor Activity Prediction Project (CERAPP) model (Mansouri et al., 2016) may predict interaction with the estrogen receptor (ER). This information can be used to prioritize *in vitro* estrogen receptor screening assessments and if warranted, an *in vivo* study designed to examine estrogen-related adverse effects (e.g., (EPA, 2022)). Both the CERAPP model and the corresponding androgen receptor model (CoMPARA; (Mansouri et al., 2016)) can be used to prioritize/deprioritize endocrine assessments and contribute to WoE evaluations (EPA, 2022), resulting in fewer animal studies.

In silico approaches are being developed and used to identify chemical-induced biological effects in human cells. Typically, these are high-throughput or high-content screening tools applied to thousands of chemicals used in commerce or found in the environment that provide information on how chemicals affect living systems (Knudsen et al., 2020). Information that can be obtained on these chemicals include bioactivity profiling, *in silico* dosimetry, and predictive toxicology (Knudsen et al., 2020). As one specific example, the CompTox Chemical

Dashboard maintained by the US EPA provides access to over a million concentration-response curves (EPA, 2023b).

Many scientists are also working on *in silico* approaches to address the 3Rs and improve the prediction of adverse events and efficacy of FDA regulated products. One such program (AL4Tox; Artificial Intelligence | FDA) is being conducted at the National Center for Toxicological Research (NCTR). Two of the four initiatives: 1) AnimalGAN and 2) SafetAI may impact the use of animals. ToxGAN (one of the models developed under AnimalGAN initiative) strives to generate animal study results of new compounds through the use of AI methods that are based on existing animal toxicogenomics data without testing the new compound in animals (Chen et al., 2022). They have built a liver model that can potentially predict a novel compound's gene expression changes in the rat liver and its effect(s) on biological pathways to assess the potential to cause liver damage. Many such read-across applications rely solely on the concept that compounds with similar chemical structures cause comparable toxic effects, which is not always true. Instead, this system infers gene expression changes that would be seen with a novel compound to predict liver toxicity. This might be useful to identify hepatotoxic compounds prior to animal testing. To put this in context, to replace animal testing such models would be needed to assess the more than 40 organs and tissues examined in a classical nonclinical toxicity assay. Unfortunately, toxicogenomic data do not exist for this extended list of target cells at this time.

Another approach (SafetAI) being used by NCTR is to identify safety issues related to drugs. This is a collaborative effort being led by CDER investigators with the help of NCTR. The latter is working on developing *in silico* models of five safety endpoints including carcinogenicity (DeepCarc) (Li et al., 2021). Previous computational models tend to be restricted to certain chemical classes. This model uses a deep learning approach based on data found in NCTR's liver cancer database containing results from mice and rats tested with 863 compounds and is

publicly available (Li, 2023). They propose this model be used in early developmental screening for drugs to remove potential carcinogens from animal testing.

V. Advantages and limitations of animal use and NAMs

With any assay comes areas of usefulness and limitations. As noted above, animals do not provide all needed information, nor do NAMs. A well-known example of a failure in nonclinical assessment of safety occurred with fialuridine, a treatment for chronic hepatitis B, in 1995. This drug caused severe hepatotoxicity in some patients during the clinical trial resulting in its immediate discontinuation (McKenzie et al., 1995). Woodchucks are a commonly used model for hepatitis research. A study performed in 1998 with fialuridine found hepatotoxicity in the woodchuck but reported that others studying this drug in monkeys, rats, or dogs did not see such evidence (Tennant et al., 1998).

There are advantages and limitations for animal models and NAMs for both toxicity and efficacy (Table 4). It should be noted that even when using humans (i.e., phase I clinical trials) or human cells, assays are often conducted in healthy humans, cells derived from healthy humans or cell lines, and therefore might not reflect a compromised human. Likewise, often ages or life stages have not been studied sufficiently in animals and might not be mimicked using NAMs. Finally, there is no way currently to model effects of chemicals, drugs, or other substances on language or emotion, which can have an impact on human disease states. The complexity associated with understanding the toxicity of complex substances/mixtures is still being investigated with both animal models and NAMs. For example, a recent paper noted that, at this time, brain development cannot be assessed only with current *in vitro* or NAMs approaches (Juberg et al., 2023), especially when evaluating neurobehavioral endpoints.

VI. Identification of data gaps, needs, and future directions

The road to NAMs might lead, in the distant future, to a balanced approach for both basic biomedical and regulatory research, in which the use of NAMs outweighs the use of animals and animal models (Figure 1). There are several areas in which animal use might continue. First, in various diseases that occur in both animals and humans (i.e., osteosarcoma) any information obtained in either humans or animals (in this case, dogs) will inform mechanisms and/or treatments for both species. Second, animals will continue to be used to study animal diseases in veterinary medicine. Third, it might be valuable to continue to test chemicals in animal models if disease outcomes or if adverse pregnancy outcomes are not faithfully recapitulated using NAMs. Fourth, at least currently, as sophisticated as computer simulations and *in vitro* methods are today, they cannot generate sufficiently reliable data about how a substance affects a real living being - a complex, interactive system made up of dozens of organs, hundreds of biological messengers, thousands of enzymes, and hundreds of thousands, if not millions, of different proteins, many of them not even identified. This includes the need to genetically match cell type for some assessments (i.e., T cell haplotype match). Fifth, some animal products are necessary even in *in vitro* assays or NAMs (i.e., antibodies, primary cells from animals, fetal bovine serum, bovine serum albumin). Sixth, validation and acceptance strategies have not been implemented for all regulatory use. The current criteria are that NAMs should be good or better than the currently accepted assay.

There is no doubt that NAMs should and will continue to be developed and refined to use in both basic biomedical and regulatory research. It is unlikely that a single NAM will be sufficient; instead, a series of different approaches will be needed. Novel testing platforms and computational models have emerged that cover multiple levels of biological organization, to be combined with toxicokinetic parameters essential in supporting IVIVE. For example, research in the field of human-relevant organotypic culture models and engineered microsystems has exploded in recent years due to advances in directed differentiation of human induced pluripotent stem cells (iPSCs), bioprinting, microfluidics, microengineering, and materials

science. This has enabled complex tissue constructs that recapitulate some of the microarchitecture and function of human tissues and organs for *in vitro* testing and mechanistic understanding of drug efficacy and chemical toxicity (Rusyn et al., 2022).

Although use of NAMs for higher level regulatory decision making is still on the horizon, the regulatory coexistence with animal models requires sufficient complexity to establish performance metrics for predictivity and biological plausibility (Middleton et al., 2022). These studies show the pressing need for computational models that offer quantitative value in establishing a point of departure for hazard evaluation and classification of chemicals by critical effects. Assessing confidence in the models is key for regulatory acceptance, especially for reference compounds where traditional animal testing has failed to predict human hazard. While Frank R. Lautenberg Chemical Safety for the 21st Century Act sets the bar for use of best available science, NAMs have to be as good or better than traditional test methods especially where data gaps cannot be filled using animal models, because human relevance is unclear.

In thinking about future development of NAMs, assays that employ human cells and the application of computational (AI) methods will be valuable. It is conceivable that AI will be able to integrate information from different NAMs or even integrate legacy animal data with NAMs. It is also possible that AI will be able to compare effects in healthy individuals to those that are immunocompromised or have various diseases. Health digital twins are being developed to represent real individuals and can be used to simulate diseases, comorbidities, drug safety and efficacy (Venkatesh et al., 2023), and potentially other biological targets for safety and toxicity testing. The increased reliability on AI, however, is not without concerns, including how the model is generated (i.e., how is it trained, how it is tested, will there be ethical issues) and its stability. These future computational tools also need to be stable, allowing faithful, reliable and regularly applied software updates that allow model refinement as new information is revealed.

VII. Conclusions

Data from traditional animal studies have predominantly been used to inform human health safety and efficacy. While it is unlikely that all animal studies will be able to be replaced, with the continued advancement in NAMs, it is possible that sometime in the future, NAMs will likely be an important component of drug development, and from which efficacy determinations and toxicity testing of drugs and chemicals is conducted and regulatory decisions are made. NAMs-based *in vitro* assays and *in silico* models are aimed at the 3Rs as a whole, and replacement, which might be desired, is constrained by the state of the science, and the need for validation for those assays used to make regulatory decisions. Moreover, NAMs developers and regulators have considered whether a new framework for establishing confidence in NAMs data is needed, although that framework is still being defined (van der Zalm et al., 2022). Part of the challenge lies in identifying the acceptable variability in NAMs. The participation of multi-disciplinary groups including biologists, computational experts, toxicologists, and veterinarians looking at alternatives from a multifactorial perspective (i.e., the combination of approaches) will propel the field forward likely using both animal models in some instances, and NAMs. As always, moving into a new area takes funding and a collaborative effort among all stakeholders, which will also be the case to accelerate progress in protecting human and animal health.

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Figure Caption

Figure 1. The Road to NAMs. Early animal use included a wide variety of animals to investigate physiology, pharmacology, putative therapies and medical devices. The 3Rs concept increased awareness of animal use and encouraged replacement, reduction, and refinement of animals. As more advanced technologies were developed (i.e., genetic modification of rodents) and adoption of 3Rs became widespread, some animal use decreased (as indicated by blue arrowhead). There was also increased use of lower phylogenetic species (i.e., worms, flies) and development of in silico models at this time. NAMs were introduced and continue to be developed, used, and validated. In the future, it is likely that NAMs use will continue to increase while decreasing, although not eliminating, animal use.

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Table 1. Examples of biomedical advances made with animals and animal models

Year	Discovery *indicates Nobel Prize awarded	Species Used	Researcher(s) to whom Nobel Prize was awarded
1881	Germ theory of disease	sheep	
1905	Studies of pathogenesis of tuberculosis*	cow, sheep	R. Koch
1915	Blood transfusion	dog	
1923	Discovery of insulin and mechanism of diabetes*	dog, rabbit, fish	F. G. Banting J.J.R. Macleod
1936	Chemical transmission of nerve impulses*	cat, frog, bird, reptile	O. Loewi H. H. Dale
1937	Heparin used as an anti-coagulant	dog, guinea pig, mouse, rabbit	
1945	Discovery of penicillin and its curing various diseases*	mouse	A. Fleming E.B. Chain H.W. Forey
1955	Polio vaccine	mouse, primates	
1977	Smallpox eradicated in humans	cow	
1990	Organ transplant techniques*	dog	J.E. Murray E. D. Thomas
1996	Cloning of Dolly the sheep	sheep	
2008	Discovery of Human Immunodeficiency virus* Discovery of Human papilloma viruses causing cervical cancer*	monkey, chimpanzee, mice hamster, mouse, cow	F. Barre-Sinoussi L. Montagnier H. zur Hausen
2011	Antiretroviral drug therapy for HIV	non-human primate	
2013	CRISPR-Cas9 gene editing	mouse	
2015	Novel therapy for malaria* Novel therapy for infections from roundworm parasites*	mouse, monkey mouse, dog, sheep, cattle, chicken, monkey	Y. Tu W.C. Campbell S. Omura
2017	Approval of CAR-T-cell therapy for cancer	mouse, non-human primate	
2018	Gene therapy for humans and animals	dogs	
2018	Treatment of cancer by inhibition of negative regulation of immunity*	mouse and murine cell lines	J.P. Allison T. Honjo
2019	Gene therapy for sickle cell anemia	non-human primate	
2019	Ebola vaccine	mouse, non-human primate	
2020	Discovery of hepatitis C virus*	chimpanzee	H.J. Alter M. Houghton C.M. Rice
2020	COVID-19 vaccine	hamster, ferret, llama, non-human primate	

(FASEB, 2023; Nobel Prize, 2023)
Table 2. Animal Models

Species Used in Biomedical Research		
Rodents	Rat Mouse Guinea pig Hamster	
Non-rodents	Bird (quail, finch, pigeon) Rabbit Cat Dog (e.g., beagle) Ferret Sheep Cow Non-human primate	
Non-mammalian	Zebrafish *Drosophila melanogaster* (fruit fly) *Caenorhabditis elegans* (nematode) Frog	
Types of Animal Models Used in Biomedical Research		
Type	Description	Example
Normal	Organisms without any observable deficits (can be used as controls)	Any
Negative/Non-reactive	Organisms in which a certain disease does not develop	Opossum - resistant to rabies Rhesus monkeys - resistant to hepatitis B, Gerbils - resistant to radiation Rabbits – resistant to transmissible spongiform encephalopathy
Spontaneous	Animals with naturally occurring pathological conditions, which mimic human disease	Rats - Spontaneously hypertensive Doberman Pincher – von Willebrand's disease Dogs - spontaneous model for prostate cancer, osteosarcoma, breast cancer, aging
Disease-induced/Experimental	Animal models in which the experimentally reproduced condition mimics a human disease	Rodent - induce diabetes with streptozotocin Rodent and non-human primate – induce Parkinson-like disease with neurotoxicant, MPTP

Genetic	Result of selective sibling breeding for a specific trait	Wistar Kyoto (WKY) rat for hypertension Athymic nude mouse - result of a natural mutation that lacks T cells
Genetically-engineered	Organisms in which genes have been modified to delete or enhance gene expression	See below
Genetically-engineered Animals (mouse, rat, primate)		
Transgenic	Organism in which the genome is modified by the artificial insertion of foreign DNA (trans-gene) into every cell	2D2 mice express T cell receptors that recognize proteins involved in a multiple sclerosis model
Knock-out	Organism in which foreign genetic information is expressed in the nucleus of embryonic cells, thereby inhibiting expression of certain gene(s)	AKT2 gene deletion to examine glucose uptake in diabetes
Knock-in	Organism in which generated specific mutations or exogenous genes are introduced into specific sites of a target gene through homologous recombination, so that the expression of the gene knock-in may be tracked through the expression of a reporter gene	Erbb2 (HER-2) overexpressed in mice to examine its role in cancer
Humanized	Organism (typically a mouse) that carries functioning human genes, cells, tissues, and/or organs	Express human ACE-2 protein in mice to study SARS-CoV2 infection

Table 3. Examples of Potential Neurotoxicity Biomarkers used in Longitudinal Studies

Fluid Based - Direct analysis of plasma, serum, urine, or CSF – longitudinal and minimally invasive		
Biomarker	**Endpoint**	**Comments**
F_2-IsoPs (F_2-iso prostanes)	Indirect measurement of oxidative injury	Used clinically as biomarker of exposure
		Not specific for neurotoxicity
GFAP (glial fibrillary acidic protein)	Biomarker of all types of neural (neuronal and glial) damage	ELISA already developed
		GFAP is a sensitive and specific marker of astrogliosis (indicative of all types of CNS damage)
MAP-2 (microtubule-associated protein)	Biomarker of dendritic injury	ELISA already developed
MBP (myelin basic protein)	Biomarker of myelin disruption	Immunoassay developed, but not widely used
Microtubule-associated protein tau (total tau, phosphorylated tau, and cleaved tau)	Biomarker of neurodegeneration/axonal injury	ELISAs developed
Neurofilament (light chain and phosphorylated heavy chain)	Biomarkers of axonal injury	ELISA exists
Spectrin breakdown product (SBDP-145)	Found in the CSF as a biomarker for neurodegeneration (apoptosis and necrosis)	Recently reported
TSPO (translocator protein)	Biomarker of activated glia	Has been validated in a variety of preclinical models of neurotoxicity including preclinical and clinical imaging
UCH-L1 (ubiquitin C-terminal hydrolase)	Biomarker of cell body injury	Immunoassay developed
Imaging - less invasive, longitudinal analysis in living animals, high-resolution in postmortem fixed animals		
MRI T_2 relaxation	Detects edema, hemorrhage, water redistribution, cellular disruption, cellular density, infiltration, blood flow changes, and temperature changes	Data obtained using T2 relaxation is quantitative Correlation to pathology can be achieved via digital analysis
Electroencephalography and *in vivo* electrophysiology	Permits repeated measurements of neural activity and dose response effects within subject	Invasive

		Electrical, electrode, muscle, and movement artifact
Magnetic resonance spectroscopy (MRS)	Non-invasive Permits within subject repeated measurements of brain metabolites associated with toxicity	
Positron Emission Tomography (PET)	Minimally invasive *in vivo* imaging	
MicroPET	Molecular level view of biochemical, physiological, pathological, and pharmacological processes *in vivo*	Tags for specific neurotransmitter receptor systems can be used Resolution less than MRI needs specific short-lived radiolabeled ligand to probe the function of interest

Abbreviations: CFS, cerebrospinal fluid; CNS, central nervous system; ELISA, enzyme-linked immunosorbent assay; MRI, magnetic resonance imaging; PET, positron emission tomography.

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Table 4. Advantages and Limitations of Animal Models and NAMs

	Advantages	**Limitations**
Animal Models	effect on entire body assessed (i.e., histology, clinical chemistry)	translation issue; does not identify all adverse events seen in humans nor drugs that prove to be non-efficacious in humans
	good safety record for Phase I clinical trials	optimal test species and strain not always clear
	many disease models exist for efficacy assessment	some models do not replicate human diseases accurately and this can lead to clinical failures
	identify mechanistic issues	cost and throughput an issue for classical toxicology studies, to model efficacy in disease models and when a fast assessment is needed (e.g., prepared food on the market potentially showing unexpected adverse events)
	can study developmental stages although may not mirror human totally	limited genetic variability in inbred strains and genetic drift in animal colonies
	enables studies of medical devices	irreproducibility is sometimes an issue
NAMs	may address the 3Rs	animals may need to be euthanized to provide cells for *in vitro* systems or other NAMs
in vitro assays	use of human cells may provide better prediction of human responses	translation issue; does not identify all types of injury within a tissue, adaptive responses, or interactions among body systems
	may enable precision medicine by studying donors with unique characteristics	may reflect the response of an individual donor versus population; must investigate how many donors required; *in vivo* human studies do not predict all other humans
	can control the test environment (e.g., dose of drug, duration of exposure)	may be difficult to keep cells differentiated particularly if trying to mimic an *in vivo* chronic study
	can be easier to study mechanistic questions of toxicity and efficacy	fresh human cells may be difficult to obtain particularly for complex platforms with multiple cell types (e.g., liver)
	faster and can be less expensive than *in vivo* studies	cost and throughput may depend on question being asked; complex NAMS are expensive, usually just address one organ/tissue type and are often low throughput
	may enable toxicity and efficacy testing in disease models	may be difficult to replicate disease models *in vitro*
	a relatively small amount of test materials is needed	difficult to replicate responses that involve multiple cell types (e.g., immune cells and liver cells)
		at this time, cannot study all organs/tissues in the body

		irreproducibility is an issue
		do not replicate complexity of human system
		difficult to replicate pregnancy and developmental stages
		domain of applicability might be limited
		physical/chemical properties of substances might not be compatible with assay
		not able to replicate complex human/animal traits like behavior
	can identify a bioactivity point of departure	unclear how many cell types are needed to provide sensitivity/confidence that toxicity has been adequately evaluated
		limited assessment over time versus *in vivo* studies (e.g., disease progression)
in silico assays	might avoid the need for any new biological testing	critical that models are trained and tested accurately
	fast and expensive once models are built	may be difficult to obtain sufficient data
	flexible in terms of what models can be built (e.g., disease, normal)	may be limited to chemical structure space
		cannot always predict metabolic breakdown of compounds
		at this time, models do not exist for all organs and tissues
		do not replicate complexity of human systems

Fig. 1

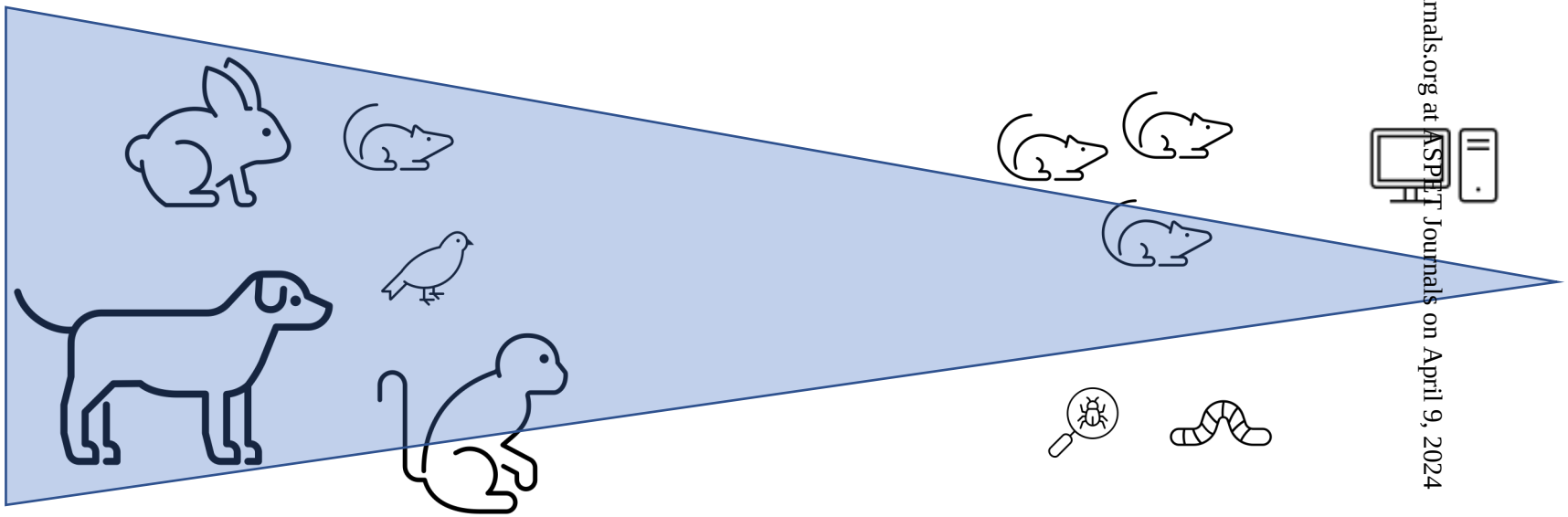
The Road to NAMs

Before and early 20th Century:
- Many different animals
- Mostly *in vivo*

Mid 20th Century:
3Rs

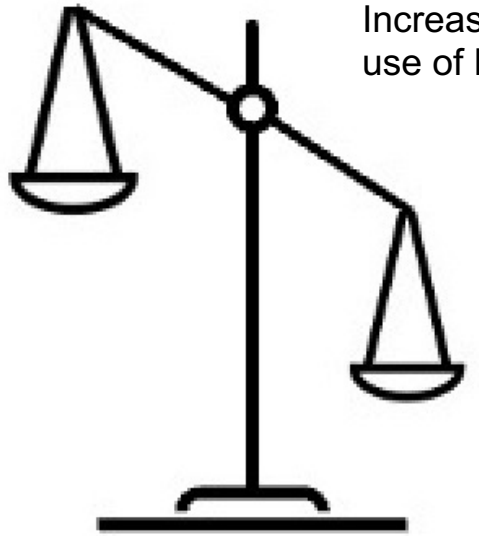
Late 20th Century:
- Increased use of
- (genetically-modified) rodents
- Increased use of *in vitro* with *in vivo*
- Increased use of lower phylogenetic species
- *In silico* methods

Late 20th Century and Early 21st Century:
NAMs



Future:

Decreased
Use of animals



Increased
use of NAMs