Optimising inhalation research:

Transitioning to human-relevant science

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Executive summary

Many countries are experiencing a scientific revolution involving a transition to human-relevant science that does not involve the use of animals. This is due to the consensus in both industry and academia that animal studies frequently fail to translate to new treatments. Despite innovation and development over the last ten years, methods that are available or emerging replacements for animal use in inhalation research are underutilised in Australia. This can be attributed in part to an industry-academia divide hampering progress toward the use of human-relevant models more broadly [1]. The potential gains to universities and research institutes – and ultimately human patients – of adopting these new models and methods, as has been demonstrated internationally, warrants a timely re-assessment and uptake of alternatives in Australia. To assist the adoption of these new models, a dedicated Alternatives Validation Centre, and associated funding stream is needed, which over 10 other countries have to date. An investment in new approach methodology as seen internationally is essential to bridge the translational gap from benchtop to bedside.

Part one

Inhalation research in animals continues to be conducted for acute inhalation toxicity testing of pharmacological or non-pharmacological compounds, despite it being unanimously accepted in regulatory science that it has little to no relevance to humans [2,3,4,5]. Similarly, inhalation research in animals in academia is still being conducted to study local toxicity and associated disease with little relevance to humans [6,7,8]. The consensus in both industry and academia is that animal studies fail to translate to new treatments, resulting in a "valley of death" for preclinical research [9].

In part one the scope of animal inhalation research currently being conducted at Australian universities and research institutes is reported to identify the potential to overcome translational barriers, however there are also clear and significant animal welfare and ethical considerations. The focus is on research that uses two specific methods that have originated from acute inhalation toxicity studies - nose-only or "forced inhalation" methods and whole-body "passive" methods – to expose rodents to toxic aerosols, such as those generated by cigarettes or e-cigarettes.

Part two

Species differences that account for the failure of animal studies to accurately predict responses to inhaled drugs in humans, have been well documented, and is investigated in part two, along with some of the complexities of animal inhalation study design, and of attempting to simplify human pathology [10,11,12]. Attempts to overcome species differences are often by genetically engineering animals to be more "human" – a time and resource intense process – and in many cases is simply not possible [13]. At best, transgenic animal models allow replication of a disease symptom, a single biological mechanism [14], however the reality will undeniably be, that the best model for human responses to treatment will be a human-based model. This report highlights that increased justification is required for the use of animal-models in inhalation research.

Part three

Due to the inability of many animal models to produce results that translate into improved health outcomes for humans, we have seen technological advances over the past 10 years with a focus on scientifically valid, human-relevant *in vitro* research [15,16,17,18,19]. Research commissioned by the National Health and Medical Research Council indicates Australia can do better at recognising new innovations – a requirement to successfully implement the 3Rs ("Replacement, Reduction, Refinement")[20]. Additionally, the Australian Technology Network of Universities acknowledges that collaboration between industry and universities is poor in Australia and strongly encourages growth in this area [1]. Therefore, in part three advancements are recognised in respiratory research, particularly *in vitro* microphysiological systems, also known as organ-on-a-chip technologies, with a focus on Lung-on-a-chip [18].

The Lung-on-a-chip microphysiological system technology originated over a decade ago and may be able to immediately replace or improve upon many animal models of lung biology or pathology studied with basic science in academia [19]. Global efforts are well underway to completely replace animal models in inhalation toxicology and pharmacology, with a multitude of funding initiatives, collaborative projects and global discussion across industry and academia, including the "Advancing New Alternatives Methodologies at the FDA" working group, and the Horizon 2020, "Organ-on-Chip In Development" project by the European Commission [3,21,22,23,24]. Part three of this report is of interest to any researcher seeking to gain insight from international developments and transition to human-relevant respiratory science. Part three is also of interest to members of animal ethics committees who are tasked with assessing whether proposed animal research could be conducted by non-animal methods.

Part four

A scientific revolution is underway - a paradigm shift toward human-relevant science [25]. Thomas Kuhn has been cited over 300,000 times for his work on scientific revolutions where he states that "when a scientific paradigm is replaced by a new one, albeit through a complex social process, the new one is always better, not just different" [26]. The benefits of transitioning to human-relevant (non-animal) research have been identified and are underway internationally [27]. Throughout this process it has been recognised that there are logistical, institutional, economic, and regulatory barriers to transitioning [25,27]. Part four identifies inhalation research utilising *in vitro* methods being conducted in Australia and overseas, to highlight the current capabilities for *in vitro* human-relevant inhalation research. Part four also identifies the top five barriers to transitioning and their associated antidotes, with the barriers being; 1) Lack of understanding of human-relevant technologies, 2) Status-quo bias, 3) Journal editorial policy, 4) Regulatory requirement, and 5) Avoiding sunk-costs. A roadmap is highlighted to guide the movement.

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Aims and method

This research aimed to:

1) Investigate the use of *in vitro*, human-relevant methods that could be used to replace acute inhalation toxicity methods in inhalation research, 2) identify the scope of academic research being currently conducted in Australia utilising methods that could be replaced with these new *in vitro* methods, 3) in comparison, identify the scope of *in vitro* inhalation research being conducted internationally, and in Australia, and, 4) identify the potential barriers and gains to Australian researchers of adopting these new methods.

Databases searched: Pubmed, Google

Search period: 2014-present.

Search terms used for each above aim:

1,3) inhalation toxicology, nose-only, *in vitro* toxicology, acute inhalation toxicity, alternatives, new approach methodology, 2) Australia, inhalation, e-cigarettes, COPD, 4) Thomas Hartung, Kathrin Hermman.

Reference list from identified papers were also utilised and lead/senior authors on studies were also searched. For aim one, three, and four publication lists from keynote presenters in attendance at The 11th World Congress on Alternatives and Animal use in the Life Sciences were searched.

Definition of scope

This research aimed to identify *in vitro* methods that could be used to replace animal studies only. We acknowledge that *in silico* computer modelling and simulation – primarily computational fluid dynamics and physiologically-based pharmacokinetics to study the absorption, distribution, metabolism and elimination of the drugs or toxins from the lungs and body – are used widely and accepted as a replacement in lieu of animal studies in respiratory research [22]. Use of computer modelling and simulation and model informed drug development are essential to the evolution of the health care system [29]. *In vitro* and *in silico* scientifically valid human-relevant approaches are being used currently at regulatory institutions such the United States Food and Drug Administration, the Environmental Protection Agency, and the European Medicines Association [22,23,30]. This report will refer to *in silico* research wherever it is relevant, however an in-depth explanation of the methodology and use as a replacement for animal studies is outside the scope of this report.

Similarly, we acknowledge that respiratory studies in human volunteers and/or patients are viable and referred to briefly in this report. Furthermore, new approach methodologies are constantly evolving, and we refer readers to a recent publication which has identified 284 human-relevant models or methods in respiratory research, that have the ability or the potential to replace animal-models [17].

Conflicts of Interest:

The author is a scientific outreach consultant for Humane Research Australia and editor is Chief Executive Officer of Humane Research Australia.

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Part one: The scope of inhalation toxicology research in Australian universities and research institutes

Introduction to commonly used research methods in inhalation toxicology

Many leading Australian universities and institutes are active in the inhalation research field. Understanding the potential for toxic aerosols such as cigarette smoke or bushfire smoke to negatively impact health is essential to reduce morbidity and mortality in the human population. Associated respiratory conditions such as chronic obstructive pulmonary disease (COPD) and lung cancer are amongst the top five leading causes of death in developed countries today [31]. Acute inhalation toxicity studies are used in regulatory (safety) science to investigate two primary end points: 1) local respiratory toxicity and 2) systemic toxicity (death), these results are always extrapolated to humans - usually by employing a magnitude of 10 "safety factor" to account for inaccuracies and species differences [22,32,33,34]. However, the methods employed in acute toxicity testing are also being used in basic science by universities and research institutes to investigate local effects and disease pathogenesis [32].

"The valley of death"

Toxicology has a long history of testing on animals and it is commonly thought to be a necessary evil. However, for over twenty years now there has been huge demand, effort, and funding – from both public and regulatory agencies – to replace animal methods with advanced, human-relevant, *in vitro* and *in silico* science [21,24,32,33]. The bench-to-bedside rift between basic research and clinical research is widening [9]. Despite promising results in animal studies, 30% of drugs fail in human clinical trials due to adverse effects and another 60% fail due to lack of efficacy – the so-called "valley of death" where promising drug candidates fail [9,12]. This is particularly relevant for drugs targeting respiratory disease [17]. These failures have been well documented for over a decade and underpin the movement toward improved *in vitro* human-relevant technologies [12]. Furthermore, human-relevant alternatives to animal testing in acute inhalation toxicity testing of both pharmaceutical and non-pharmaceutical compounds have been discussed globally in attempts for harmonisation [2,5,24,33,35]. As a result, there has been considerable progress. In 2017, "evident toxicity" rather than death became the endpoint for systemic toxicity in testing of pharmaceutical compounds, and in 2018 guidelines for computer modelling and simulation of physiologically-based pharmacokinetics were formalised [35,36].

Human-relevant research is now accepted in-lieu of animal studies in many aspects of regulatory science, being called "model-informed drug development" (particularly for *in silico* study), but to date there is no method that has been accepted as a full replacement for acute inhalation toxicity testing in animals for regulatory purposes [5,16,22,32]. *In vitro* and *in silico* species-specific studies are able to bridge the translational gap and reduce animal use [12,29]. For example, where human *in vivo* data is absent to validate *in vitro* and *in silico* human-relevant technologies, animal (species)-relevant versions of the same *in vitro* or *in silico* human-relevant technology are utilised – which can be validated *in vivo*, thereby validating the human technologies [22]. There is no legal requirement to use animal models in basic research, yet despite validated *in vitro* and *in silico* technology being available to transition to human-relevant inhalation research and ongoing demand for movement toward non-animal methods, animal models are still frequently used in inhalation toxicology today for this purpose [32,35].

Animal exposure methods

Mice and rats are the most commonly used species in inhalation toxicology [37]. When exposing rodents to inhaled substances the experimental set-up is designed in an attempt to recreate the aerosol delivery environment typical to humans. However, it is impossible to expose rodents to aerosols through the same mechanisms as humans, not least because rodents are obligate nose-breathers [30].

Two exposure methods are typically employed, 1) Nose-only dosing and 2) Whole-body dosing, Figure 1 (a) and (b) respectively [37]. Both methods have disadvantages, and the details of each and ethical considerations of such exposure systems have been summarised previously [38,39]. The scope-of-use of each of these exposure systems in Australian universities and research institutions iis detailed in Table 1 and Table 2, for inhalation nose-only and whole-body exposure systems respectively.

Figure 1. Aerosol exposure methods.
(A) Nose-only ("forced inhalation"), and (B) Whole-body ("passive inhalation")

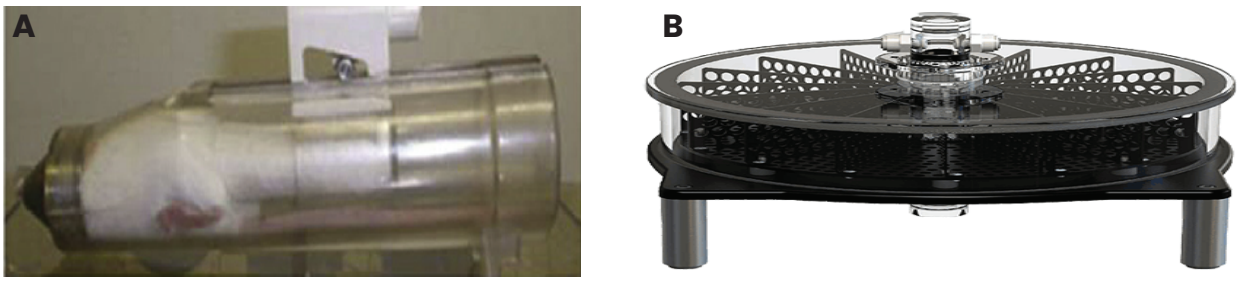


Image 1 (a) retreived from: A new short-term mouse model of chronic obstructive pulmonary disease identifies a role for mast cell tryptase in pathogenesis. Beckett, Emma L. et al. Journal of Allergy and Clinical Immunology, Volume 131, Issue 3, 752 - 762.e7 https://www.jacionline.org/article/S0091-6749(12)02642-5/fulltext. Image 1 (b) retreived from: SCIREQ website: https://www.scireq.com/inexpose/ accessed October 2021.

Table 1.
A range of recent animal studies conducted in Australia that utilise nose-only exposure methods.

Author affiliations: 1= University of Newcastle, Callaghan, NSW, 2= Hunter Medical Research Institute, Newcastle, NSW, 3= Monash University, Clayton, VIC, 4= Monash Institute of Medical Research, Clayton VIC, 5= University of New South Wales, Sydney, NSW, 6= University of Sydney, Sydney, NSW, 7= University of Technology Sydney, Sydney, NSW, 8= World Health Organization Collaborating Centre for Reference and Research on Influenza, Melbourne, Victoria, 9= John Hunter Hospital, Newcastle, NSW, 10= Woolcock Institute of Medical Research, University of Sydney, Sydney, NSW, 11= University of Queensland, Brisbane, QLD, 12= Queensland Institute of Medical Research, 13= University of Melbourne, VIC, 14=Centenary Institute

Damaging legacy: maternal cigarette smoking has long-term consequences for male offspring fertility [40]	
Approach:	54 female mice were exposed to smoke of 12 cigarettes twice daily over 60 minutes, five times a week, for up to 18 weeks (n=27) or room air for the same periods (n=27; control)
Key finding:	Maternal exposure to cigarette smoke negatively impacted male offspring fertility in a mouse model
Affiliations:	1,2,3,4

Table 1 continued.
A range of recent animal studies conducted in Australia that utilise nose-only exposure methods.

Enhancing tristetraprolin activity reduces the severity of cigarette smoke-induced COPD in mice [41]	
Approach:	Female mice (5-8 in each group; eight groups) were exposed to normal air or cigarette smoke equivalent to a pack-a-day smoker through the nose only for four ("acute exposure") or eight weeks ("chronic exposure"). There were four different groups evaluated acutely and chronically.
Key finding:	Inducing tristetraprolin has therapeutic potential for COPD in a mouse model
Affiliations:	1,2,5,6,7
Targeting PI3K-p110α Suppresses Influenza Virus Infection in Chronic Obstructive Pulmonary Disease [42]	
Approach:	Alongside *in vitro* human cell cultures, an unspecified number of female mice were exposed to: smoke of 12 cigarettes twice daily, five times a week, for eight weeks ('experimental COPD mouse model') vs control (air only). On the final day of exposure, the mice were anaesthetised, and influenza virus introduced nasally.
Key finding:	Both *in vitro* and *in vivo* studies identified a pathway with greater activity in COPD models that increased susceptibility to infection.
Affiliations:	1, 2,8,9
Chronic cigarette smoke exposure induces systemic hypoxia that drives intestinal dysfunction [43]	
Approach:	An unclear number of female mice were exposed to: • Smoke of 12 cigarettes twice daily, 5 times a week, for 12 weeks ('experimental COPD mouse model') • Normal air for the same periods (control) • Smoke of 12 cigarettes twice daily, 5 times a week, for 8 weeks, then normal air for the remaining exposure periods (smoking cessation mouse model)
Key finding:	In an experimental mouse model, chronic smoke exposure led to damage and pathological changes at the critical site in the gastrointestinal tract of Crohn's disease.
Affiliations:	1,2,10,7,11,12
Time-resolved proteomic profiling of cigarette smoke-induced experimental chronic obstructive pulmonary disease [44]	
Approach:	Investigation of the smoke-induced chronic obstructive pulmonary disease (COPD) pulmonary proteome. Exposure of mice to cigarette smoke for eight weeks using forced-inhalation method.
Key finding:	Identification of changes to the proteome associated with [mouse model] smoke-induced COPD
Affiliations:	1,2,7,12,13,14

Table 2.
A range of recent animal studies conducted in Australia that utilise whole-body exposure methods.

Author affiliations: 1= University of Technology Sydney, Sydney, NSW, 2= Woolcock Institute of Medical Research, University of Sydney, Sydney, NSW, 3= University of Newcastle, Newcastle, NSW, 4= Royal Melbourne IT University, Bundoora, VIC, 5= University of Melbourne, Parkville, VIC, 6= Royal Adelaide Hospital, Adelaide, SA, 7= University of Adelaide, Adelaide, SA, 8= Monash University, Melbourne, VIC, 9= South Australian Health and Medical Research Institute, Adelaide, SA, 10= University of Sydney, Sydney, NSW, 11= SA Pathology, Adelaide, SA, 12= University of Western Australia, Subiaco, WA, 13= Curtin University, Perth, WA, 14= Department of Health, Shenton Park, Perth, WA.

Neurological effects in the offspring after switching from tobacco cigarettes to e-cigarettes during pregnancy in a mouse model [45]	
Approach:	24 pregnant mice were exposed to three different interventions in a custom-made 9 L chamber: • Smoke of 2 cigarettes twice daily from 6 weeks prior to pregnancy through lactaction using the inExpose cigarette system; n=8; • Ambient air for the same periods (n=8; ambient air, 'sham') • Smoke of 2 cigarettes twice daily from 6 weeks prior to pregnancy using the inExpose cigarette system, switching to e-cigarette aerosols (Tobacco flavour; 18mg nicotine) using the KangerTech NEBOX e-cigarette device (KangerTech, Shenzhen, China) from gestation through lactation (n=8; 'switch')
Key finding:	Compared to the sham offspring, both intervention offspring groups demonstrated reduced birth weights, increased activity, reduced anxiety, and greater genetic changes. The switch group was the only offspring demonstrating short-term memory deficits.
Affiliations:	1
Evidence from a mouse model on the dangers of thirdhand electronic cigarette exposure during early life [46]	
Approach:	An unspecified number of 4-week-old male mice (n=36?) were placed in a 9 L chamber for 2 hours daily with a newly exposed towel for 8 consecutive days. The towel had been exposed to 1 of 3 interventions; 1) Nicotine-containing e-vapour, 2) Non-nicotine-containing e-vapour and 3) Neither of the above (unspecified; 'sham')
Key finding:	Despite short-term exposure to nicotine-containing e-vapour, nicotine metabolites in the mice sera were equivalent to that of a light cigarette smoker; lung function and brain development were also impacted. The non-nicotine e-vapour group also demonstrated immune system suppression, indicating biological relevance of e-vapour constituents.
Affiliations:	1,2,3

Table 2 continued.
A range of recent animal studies conducted in Australia that utilise whole-body exposure methods.

Ebselen prevents cigarette smoke-induced gastrointestinal dysfunction in mice [47]	
Approach:	Male mice were placed in a 18 L chamber and exposed to: • 9 cigarettes daily (3 cigarettes over 3 sessions for 1 hour each time) for 2 months (n=28) or 6 months (n=20); a subgroup from the 2-month cigarette group had a 10-day cessation of exposure to mimic cessation (n=14 of the 28) • Room air for the same periods for 2 months (n=28) or 6 months (n=20) • Separate cohorts of mice were additionally given ebselen (an antioxidant and anti-inflammatory) or a vehicle placebo (numbers not specified)
Key finding:	In mice, cigarette exposure increased gut motility and constricted the colon similar to an inflammatory response; ebselen reversed the constriction. Anatomical changes to the mice' gastrointestinal tracts following 2 and 6 months cigarette exposure was irreversible.
Affiliations:	4,5
Interventional low-dose azithromycin attenuates cigarette smoke-induced emphysema and lung inflammation in mice [48]	
Approach:	Female mice were placed in the inExpose robot cigarette and whole-body chamber system (Figures 2A and 2B) and exposed to: • 9 cigarettes twice daily for 1 hour, 5 days a week, for 12 weeks (n=5) • The same, with azithromycin via nebulisation after their second cigarette exposure each day from Weeks 7-12 (n=5) • Fresh air at the same schedule as the cigarette exposure (n=5; 'sham')
Key finding:	Low-dose arithromycin reduced emphysema-like changes in the lungs of mice exposed to cigarette smoke, compared to cigarette smoke-exposed mice not given arithromycin.
Affiliations:	6,7,8,9,10,11
The effects of electronic cigarette aerosol exposure on inflammation and lung function in mice [49]	
Approach:	Female mice were placed in 27 L chamber and exposed to: • 3 cigarettes for 1 hour/day, 5 days a week, for 7 weeks via the inExpose cigarette-smoking machine (Figure 2B), increased to 1 hour twice daily from Week 8 (n=12) • 4 different types of e-cigarette 'juice' (each differing in nicotine:no nicotine and glycerin:propylene glycol as the main excipient) at the same schedule as the cigarette smoke-exposed group using a custom-designed computer-controlled syringe pump connected to an Innokin iTaste MVP2.0 aerosoliser (Innokin Technology, Shenzhen, China; n=48 (12 per group)) • Medical air at the same schedule as the cigarette exposure (n=12)
Key finding:	Mice exposed to e-cigarettes did not have increased lung inflammation (as seen in the cigarette smoke-exposed group) but demonstrated impaired lung function.
Affiliations:	12,13,14

Part two:
How animal studies can fail to translate to human outcomes

Species differences account for a large proportion of the failures in translation of respiratory medicines [4,17]. Additionally, unblinded studies and human-led animal selection can skew data through bias, as can poor study design [50,51].

Animal inhalation study design complexity

Due to species differences, it is difficult to design an animal inhalation study that will provide human-relevant results. Inhalation toxicology computational models have been validated in animals and report these anatomical differences between animals and humans, and the basic differences are shown in Table 3 [22,34,52,54]. The first consideration for any animal inhalation study should be whether a computational model been validated already for the animal or exposure, as many now exist for rats and monkeys [22,34,52]. Computational modelling can either replace animal studies, reduce the number of animals used in inhalation experiments, or at the very least, inform the design of the inhalation study. If there is no available computational model, it is strongly advisable to consult an *in silico* expert to inform study design. Study design then needs to be considered carefully after seeking advice from aerosol scientists, toxicologists, and animal experts. Some of the minimum considerations required for inhalation toxicology study designs are presented here to give an idea of the complexity encountered with this type of research [39].

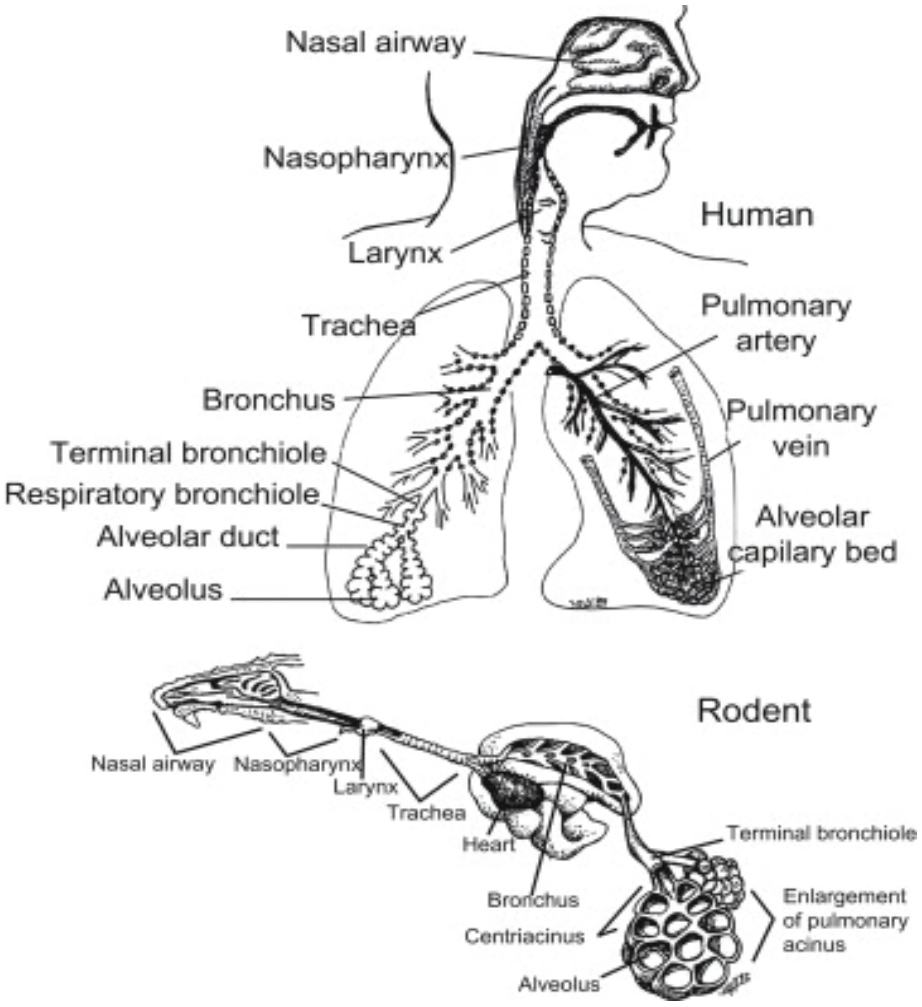
- Should inhalation be nose-only or whole-body? (Figure 1 A and B respectively)
- Has the aerosol been characterised so that dose rate in mg/kg is known, and this can then be attributed to a dose rate per square centimetre of lung epithelium, after accounting for losses to nose breathing?
- Is the animal's inhalation rate and heart rate able to be monitored during the experiment to account for increased minute volume on outcomes?
- If using a nose-only chamber, has the animal been trained, and are not suffering?
- If using whole-body exposure – would ingesting the experimental agent through fur cleaning impact outcomes?
- Can chamber concentration and conditions be controlled and adequately maintained? Has this been confirmed prior to animal exposure?
- Does the exposure system comply with international standards or guidelines for the field, e.g. the CORESTA method for cigarette smoking [53]?
- Are animals being monitored appropriately for signs of toxicity that lead to death [35]?
- Will key anatomical differences (Table 3 and Figure 2) negatively impact on outcomes?
- Has the humanised model been confirmed to recapitulate the hallmark lung features of disease?

Table 3.
Comparative Gross and Microscopic Anatomy of Human and Mouse Respiratory Tracts.

Adapted from: Aeffner F, Bolon B, Davis IC. Mouse Models of Acute Respiratory Distress Syndrome: A Review of Analytical Approaches, Pathologic Features, and Common Measurements. Toxicologic Pathology. 2015;43(8):1074-1092. doi:10.1177/0192623315598399

Feature	**Human**	**Mouse**
Nasal breathing	Optional	Obligate
Body posture	Biped	Quadruped
Total lung capacity	6,000 mL	1 mL
Respiratory rate	12–16 bpm (adult)	250–300 bpm (adult)
Lung lobation	Right: 3 lobes Left: 2 lobes	Right: 4 lobes Left: 1 lobe
Branching of conducting airways	• 17 to 21 generations • Dichotomous branching pattern	• 13 to 17 generations • Monopodial branching pattern
Thickness of blood–gas barrier	0.62 µm	0.32 µm
Alveolar diameter	210 µm mean linear intercept	80 µm mean linear intercept

Figure 2.
The human and rat nasal passages



Reprinted and adapted under creative commons license https://creativecommons.org/licenses/by-nc-nd/4.0/ from [35] Clippinger AJ, Allen D, Jarabek AM, Corvaro M, Gaça M, Gehen S, et al. Alternative approaches for acute inhalation toxicity testing to address global regulatory and non-regulatory data requirements: An international workshop report. Toxicol In Vitro. 2018;48:53-70.

The inadequacies of "humanised" and non-humanised animal use

Despite the many limitations of animal disease models of COPD and other respiratory disorders being acknowledged, they are still considered necessary by some [55]. This is primarily due to animals being easily accessed, and a perceived benefit of testing on a whole organism, despite overwhelming consensus to the contrary in many areas of science [4,7,9,12,13,14,27,28,34,55,56,57,58]. In other words, it is tradition and not science, opinion and not evidence, that is the basis of animal model selection [25,28].

For academic research, mice are commonly engineered in attempts to mimic a certain biological mechanism/symptom that constitutes a disease hallmark in humans. The most commonly used "humanised" animal models to study toxological effects of cigarette smoke are COPD [41,42,44] and Chron's disease [43]. These animal models of disease can only replicate some key symptoms and do not reproduce pathology or accurately predict response in humans, and responses to human smoking interventions vary between species [6,7,14].

For example, rats demonstrate signs of disease two months post-exposure and mice are actually resistant to some smoke-induced pathologies observed in humans, thus, murine models have been cited as inadequate at capturing smoke-induced human pathological mechanisms [55]. Cigarette smoking is the leading cause of COPD worldwide, and smoking is a harmful addictive behaviour whereby a persons dysregulated nervous system seeks to inhale a toxic substance (nicotine) in attempts to self-soothe [59,60]. The underlying cause of addictive behaviours is a heavily debated area and it is essential to stay up to date in this field as it evolves [61]. It is clearly inaccurate to simplify the extremely complex COPD disease process in humans by the "humanisation" of a mouse, and therefore any research project applications must provide justification for why an animal model of disease is chosen over others [25].

Respiratory research scientist Arno Gutleb, PhD, from Luxembourg Institute for Science and Technology has carefully considered the respiratory variation between humans and rodents.

He notes that – aside from anatomical disparities – the upright stance of humans means the aerodynamics of a human lung are *"completely different"* to a quadruped such as a mouse. This changes the way inspirated agents move along the respiratory system, which likely alter the route and sites of exposure between species.

Media release: RTL Today: Science in Luxembourg. A lung model replaces the use of animals for experiments. 14 September 2020. Available: https://today.rtl.lu/news/science-in-luxembourg/a/1508815.html?fbclid=IwAR3_xTs7I6qK8idmnhejCCdckq1whJO-7KPteqLoKaL3e831ymTHosWT1Yw Accessed: January 2021

The complexities of human behaviour and a "systems" approach

Humans are an incredibly complex, social and emotional species. Over the past 30 years there have been many advances in the fields of relational neuroscience (interpersonal neurobiology), developmental trauma and emotional intelligence [62,63,64,65,66,67]. These research fields show a distinct connection between the human nervous system and our social/interpersonal environment, our "everyday behaviour", including addictive behaviours such as smoking, at all stages of life [68,69].

Similarly, there have been many advances in systems biology, showing a clear interaction of the nervous system - the brain - and immune system, which has distinct implications for its role in disease development and progression [70,71]. For example, massive neural networks are continuing to be characterised between the gut-brain, gut-immune and gut-lung axis [72,73,74,75]. It is increasingly clear that species differences cannot and should not be overcome, and instead a "systems" approach should be taken to bring toxicology into the 21st century [21,23]. During research project application stages it is therefore essential that systematic reviews or meta-analysis are identified or conducted that have reviewed the disease causes/progression in humans, which should include prevention, diagnosis and treatments [25].

Alternative pathways to translation - lessons learned from regulatory science

We have been moving away from the reductionist paradigm and into a systems paradigm for over 20 years now, as is evident by the Human Genome project, the Human Proteome project and now the Virtual Physiological Human Initiative[76,77,78]. In 2018, a large consortium of regulatory bodies, industry and academia acknowledged that legislation in place for regulating healthcare products predated the existence of computer modelling and simulation and was no longer fit for purpose [29]. Regulatory science is leading the way in alternatives validation, with new standards and guidelines being developed over the last decade to increase acceptance of human-relevant *in vitro* and *in silico* technologies and tools that can be used to replace animal use and animal-model use [24,32].

There is much that academia can learn from developments in regulatory science and it is necessary in Australia in particular to bridge the gap between industry and academia [1]. To ensure research can bridge the translational gap from benchtop-to-bedside, the most current understanding of alternative methods to animal-use in regulatory science, must be demonstrated prior to animal experimentation. Specifically, systematic reviews, meta-analysis or specialised reviews should be identified or conducted for alternative (*in vitro* or *in silico*) methods in the field of study. This includes searching all known international databases, and wiki pages, such as those hosted by international agencies for Alternatives Validation, such as ECVAM - The European Centre for the Validation of Alternative Methods and ICVAAM (USA) - Interagency Coordinating Committee on the Validation of Alternative Methods, or that being added to through the "ontology-driven and artificial intelligence-based repeated dose toxicity testing of chemicals for next generation risk assessment" (ONTOX) program [16,17]. Research project applications should therefore include identification of all known clinically relevant diagnostic or prognostic markers and updates on what alternatives are available in the field of study [25].

"Many promising drug candidates successfully tested in preclinical models on rodents have failed when tested in humans due to differences between the species and in the expression of a lung disease. … This is why, in the long term, we aim to reduce animal testing and provide more patient-relevant systems for drug screening with the possibility of tailoring models to specific patients (by seeding organs-on-chip with their own cells)."

Guenat O, ARTORG Center, University of Bern, Switzerland. 8 February 2021.

Media release: Bernese researchers create sophisticated lung-on-chip. University of Bern and Insel Gruppe. 8 February 2021. Available: https://www.unibe.ch/news/media_news/media_relations_e/media_releases/2021/media_releases_2021/bernese_researchers_create_sophisticated_lung_on_chip/index_eng.html Accessed: February 2021

Research commissioned by the National Health and Medical Research Council on the 3 R's indicates we can do better in Australia at recognising new innovations

Australian codes and practice guidelines for preclinical research recognise the limitations with preclinical animal research [20,79,80].

Part three:
Human-relevant inhalation models

"The best model for human is human."[132]

The high failure rate in development of initially promising pharmaceuticals, including inhaled drug-products can be largely attributed to the failure of preclinical animal studies to reliably predict outcomes, in subsequent human clinical trials - proving that human-relevant research is required to replace animal studies, in order to facilitate translation [4,15,17,58]. As a result of the limitations of preclinical animal studies being identified over two decades ago, we have seen huge advancements in human-relevant *in vitro* technologies [2,12,19,21,33]. These advancements include the movement from 2D to 3D cell cultures, from perfusion to organoids right through to the "organ-on-a-chip" microphysiological system technologies of today [18,19,81]. In a recent report, the European Commission Joint Research Centre has identified 284 human-relevant models or methods in respiratory research, that have the ability or the potential to replace animal-models in respiratory research, with 27 being applicable to the study of COPD [17]. "Organ-on-a-chip" technology, has been identified, with the overwhelming consensus of international experts, as being the most promising *in vitro* method to replace toxicology testing for regulatory purposes, and also the most suited of all the *in vitro* models to model aspects of COPD, and smoke-induced disease [17,82,83]. Therefore, the Lung-Chip will be discussed exclusively here.

Studies in humans

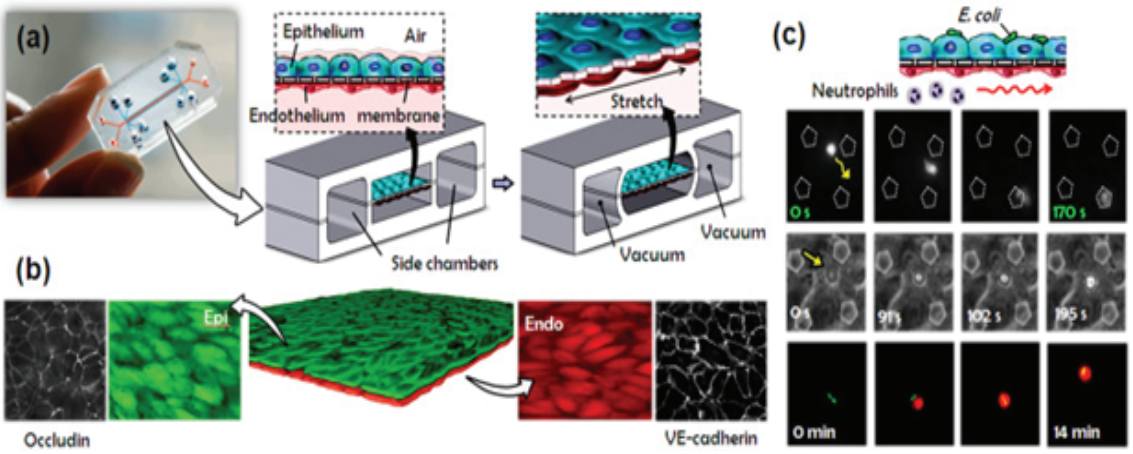
Respiratory studies to investigate disease pathogenesis are possible and ethical in many naturally occurring human populations, and increasingly necessary to validate *in vitro* systems data. A recent study published in Nature has detailed the human gut microbiome associated with COPD [116], to complement previous investigation of the gut-lung axis in COPD [117,118]. Additionally, University of Queensland has launched a world first study to explore the impact of e-cigarettes on lung health and exercise in humans.

University of Queensland media release, Available:
https://www.usq.edu.au/news/2020/09/ecigarettes-exercise-study Accessed: May 2021

Lung-on-a-chip: microphysiological systems

The Lung-Chip (Lung-Alveolus-Chip) was the first of all the organ-chips to be created and validated over a decade ago, and now there are more than 15 different Organ-Chip models, including liver, intestine and kidney chips [18,19]. The Lung-Chip combines tissue engineering, stem cell biology, microfluidics and microfabrication techniques from the microchip industry (Figure 3) [18]. Microfluidics is the study of manipulating small amounts (10-9 to 10-18 litres) and microfabrication techniques include photolithography, replica moulding, and microcontact printing [84,18,8]. These "chip" technologies have now advanced beyond lab-based prototypes to commercial manufacturing and are available in both industry and academia – for all researchers to either purchase or create themselves [8,85,86]. In fact, there are many different companies in play, and a resulting need for standardisation [87].

Figure 3.
A human breathing lung-on-a-chip [35].

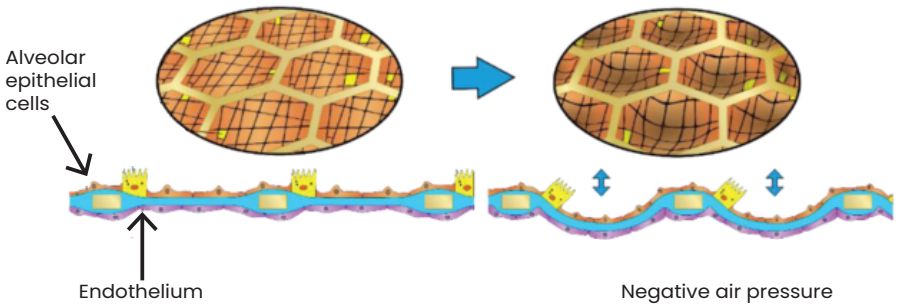


From [35] "(a) The microfabricated lung mimic device recreates physiological breathing movements by applying vacuum to the side chambers and causing mechanical stretching of the PDMS membrane forming the alveolar-capillary barrier. (b) Long-term microfluidic co-culture produces a tissue-tissue interface consisting of a single layer of the alveolar epithelium (Epi; green) closely apposed to a monolayer of the microvascular endothelium (Endo; red), both of which express intercellular junctional structures such as occludin or VE-cadherin. (c) Neutrophils flowing in the lower vascular channel adhere to the endothelium activated by E. coli in the alveolar chamber, transmigrate (top row), emigrate into the alveolar space (middle row), and engulf the bacteria (bottom row)."

The Lung-Chip recapitulates or mimics biochemical and mechanical characteristics of lung physiology and pathophysiology – it recreates the microphysiological system (MPS) of the organ and are hence also known as MPSs. These models can be either static or dynamic, although dynamic Lung-Chip models have been validated to more accurately recapitulate (mimic) lung characteristics [88]. Since the dynamic Lung-Chip was first invented, validated dynamic Lung-Chip models of MPS have evolved to include; pulmonary oedema [88], vascular thrombosis [89], small airways [90], smoke-induced COPD [90,91,92], lung cancer [93], influenza [94,95], sars-CoV2 [96] and more recently, cystic fibrosis [97]. The area is rapidly evolving, and second-generation Lung-Chips have recently been reported that better recapitulate the alveolar-basal-membrane, overcoming the first generation issue of using PDMS, (Figure 4) [12,98]. Lung-Chip models have applications for disease modelling, drug development and personalised medicine [8].

Applications for drug development and personalised medicine are made possible because the Lung-Chip can be combined with other organ-on-a-chip systems, to create a more complete organ-system or human "body-on-a-chip" systems (Figure 5). The "body-on-a-chip" aims to recapitulate human physiologically-based pharmacokinetics that can then be validated against pharmacokinetic and pharmacodynamic studies in humans [99,100]. There were four early "body-on-a-chip" designs, the first being the German system, with the initial project funded in 2010 and proof of concept achievement in 2013 [101], which now has the spin-off company "TissUse GmbH" [82,83]. Other "body-chip" systems were conceptualised shortly after with various multi-million dollar funding schemes; the Russian "Homunculus" Body-chip program was awarded 5.7 Million Euros in 2011, The European Body-Chip program was awarded 1.4 million Euros in 2012, and a United States project was awarded 140 million USD in 2012 [82]. The end goal of these MPS Body-Chip systems is to mimic human homeostasis, and a true Body-chip should have the "Universal Physiological Template" shown in figure [83]. The template can then be used to investigate a human-relevant mode-of-action or adverse outcome pathways of a particular environmental or pharmaceutical compound [83].

Figure 4.
A second generation lung-on-a-chip [98].



> *"The new lung-on-chip reproduces an array of alveoli with* in vivo *like dimensions. It is based on a thin, stretchable membrane, made with molecules naturally found in the lung: collagen and elastin. The membrane is stable, can be cultured on both sides for weeks, is biodegradable and its elastic properties allow mimicking respiratory motions by mechanically stretching the cells."*
> Pauline Zamprogno, Model developer. Organs-on-Chip Technologies Laboratory, ARTORG Center, University of Bern, Switzerland.
>
> Quote: media release, 8 February 2021, accessed: https://www.unibe.ch/news/media_news/media_relations_e/media_releases/2021/media_releases_2021/bernese_researchers_create_sophisticated_lung_on_chip/index_eng.html Accessed: February 2021.
>
> Image: reprinted and adapted under creative commons license https://creativecommons.org/licenses/by/4.0/ from: Zamprogno P, Wüthrich S, Achenbach S, Thoma G, Stucki JD, Hobi N, et al. Second-generation lung-on-a-chip with an array of stretchable alveoli made with a biological membrane. Communications Biology. 2021;4(1):168 [98].

Limitations of Lung-Chip technology

There are technical and other challenges associated with Lung-Chip (and all organ-chip) technology, specifically to do with characterisation, qualification and standardisation, as identified by the Horizon 2020 FET-Open project, "Organ-on-Chip In Development (ORCHID) and the Transatlantic Think Tank on Toxicology, summarised in Figure 6 [83,87]. Particular barriers for the Lung-chip that need to be tackled by research and development include; Barrier function for kinetics, especially in the alveoli, better alveolar models, and mucocillary clearance [102]. The technical challenges of finding an alternative for the polydimethylsiloxane material may have been recently overcome with the invention of the "second generation" Lung-Chip [98]. Broadly, limitations can be overcome with education and training, political engagement and legislative change, redeployment of funds, and scientific collaboration [103].

Logistical challenges for Australia

There are logistical challenges in Australia for importing either Lung-Chip technology and equipment, or cells for use in the Lung-Chip systems from overseas, which puts emphasis on the need for increased internal resources or providers/importers of these technologies and resources within Australia. For example, cells from overseas may not survive the long journey to Australia, or access to technology may be waitlisted or priority given to the country in which they are produced in. An Australian company, Minifab has been identified by ORCHID group as a manufacturer of Lab-on-a-chip technology [87]. Furthermore, Australia may benefit from collaboration with Asia-Pacific Region universities, such as universities in Tokyo or Beijing, many whom are actively publishing in the area, and leading in engineering approaches to emulate the lung tissues in particular [87]. The many companies that provide organ chip and associated technology, and universities actively publishing in the area have been outlined by the ORCHID workshop report [87].

Figure 5.
The minimum requirements for a "Body-Chip"

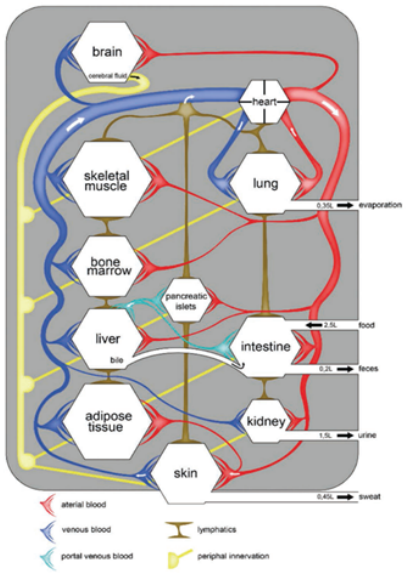


Image from Marx U, Akabane T, Andersson TB, et al. Biology-inspired microphysiological systems to advance patient benefit and animal welfare in drug development. ALTEX. 2020;37(3):365-394. doi:10.14573/altex.2001241. Reprinted with permissions under creative commons license: http://creativecommons.org/licenses/by/4.0/),

Opportunities for basic science

Part two of this report identifies that animal disease models fail to translate to human health outcomes [51]. Whilst regulatory science pursues the technical challenges of creating guidelines for use of microphysiological systems data in regulatory applications, researchers, in a relatively unregulated environment, have the opportunities to explore the unmet need for disease model development with microphysiological systems [83]. Disease model development poses is a unique opportunity that could further global efforts to harmonise the assessment of microphysiological systems data at the regulatory level, and advance human-relevant science [83]. Additionally, microphysiological systems have the capability to investigate human mechanisms and this is particularly important where there are gross species differences – for example with the immune system – which underpins all aspects of disease [83].

In silico computer modelling can complement *in vitro* study

Computer modelling and simulation is used by the Environmental Protection Agency and the applicants to the Federal Drug Administration or European Medicines Agency regulators to assess the safety and efficacy of an inhaled substance. These models combine computational fluid dynamics (CFD) and physiologically-based pharmaco-kinetics (PBPK) to assess the lung deposition, absorption, distribution, metabolism and excretion of an inhaled substance, from the human body. The models are based on complex mathematics and physics and use high performance computing or "supercomputing" resources to complete the simulations. Whilst these models do require an experienced user, software is openly, and often freely available.

Free software includes MPPD https://www.ara.com/mppd/ and OpenFoam https://openfoam.org/, and PkSim https://www.open-systems-pharmacology.org/. Many other paid softwares exist for either CFD or PBPK, and some have "on-demand" use which also incorporates supercomputing resources into the fee paid, such as STAR-CCM+ has "Power On Demand" option (https://www.dex.siemens.com/plm/simcenter-on-the-cloud/simcenter-star-ccm-power-on-demand). High performance computing centres, such as Pawsey in Western Australia or National Computing Infrastructure in Canberra will often have computational fluid dynamics software on their systems and have software specialists who can assist.

Brief overview:
Cell cultures for use in microphysiological systems

The cellular make-up of the human lung surface is complex, and to mimic its structure and activity multiple cell types must be grown together. Lung and immune-derived cell types and lines most commonly used to "seed" chips have been discussed and reviewed recently [12,81]. Some of the advantages of chip or microphysiological system technology over other methods of culturing cells include: 1) passive perfusion, and associated decrease in culture time-frame compared to static culture 2) the ability to mimic breathing and associated enhancement of inflammatory and immune signalling [18,104], 3) functional readout - real time monitoring with biosensors, and associated improvements such as device portability and reproducibility 4) ability to expose cells to aerosols under conditions of physiological flow and associated improvements [12,81]. The Lung-Chip system therefore does not sacrifice the benefits of 3D cell cultures but improves on it [81].

Figure 5.
Challenges and unmet needs for organ-chip technology

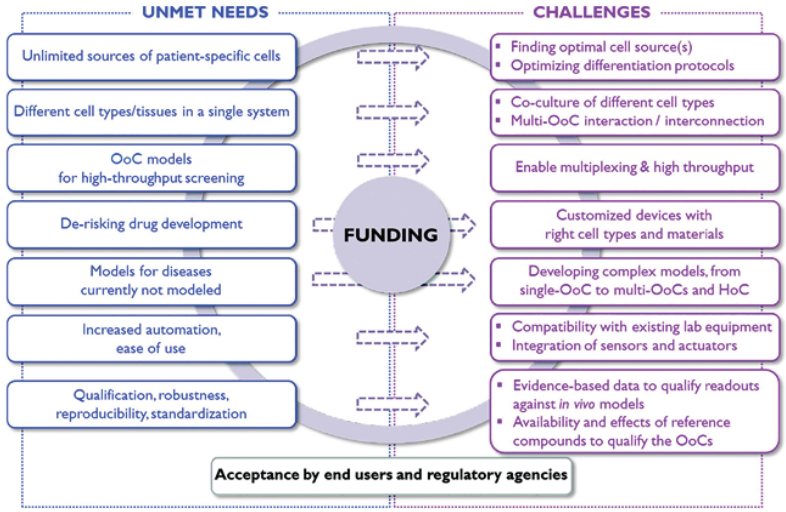


Image: reprinted under creative commons license, http://creativecommons.org/licenses/by/4.0/, from: Mastrangeli, M., Millet, S., ORCHID partners, T. and van den Eijnden-van Raaij, J. (2019) "Organ-on-chip in development: Towards a roadmap for organs-on-chip", ALTEX - Alternatives to animal experimentation, 36(4), pp. 650-668. doi: 10.14573/altex.1908271.

Brief overview: organoids

Organoids represent a fully differentiated 3D tissue structure, they consist of "a collection of organ-specific cell types that develops from stem cells or organ progenitors and self-organizes through cell sorting and spatially restricted lineage commitment in a manner similar to *in vivo*" [105]. At present, microphysiological systems technologies represent a better tool to study airways biology and pathology than organoids [8]. Developments in airway organoids are reported by Sachs et al (2019) [106] and the general differences between organoids and microphysiological systems have been reviewed recently by Bai & Wang (2020) [107] and limitations discussed with relevance to toxicology testing [8,14].

Aerosol expsoure systems

Key considerations of aerosol delivery systems will be discussed here. Whole-smoke (i.e. containing the complete mass, range of compounds and particle sizes of the real product) exposure systems will always more accurately represent the human exposure to cigarettes or e-cigarettes, however frequently other methods are used. Often, to deliver smoke, exhaust, or e-cigarette vapor to cells, dilution is required to accurately mimic the exposure in humans, and this is usually, but not always to do with the difficulties of exposing cells to the aerosol [108]. For example, in e-cigarette aerosol research, "smoke-extract" or just e-liquid rather than the aerosol produced by the e-cigarette device, is used, and most studies sample only a small component of the total mass of aerosol produced from an exposure [109,110]. Aerosol exposure to cell cultures should be informed by *in silico* modelling to ensure accurate mass of aerosol, and concentration of toxicants, is delivered as would be received *in vivo* [111]. The dose delivered to the lungs must be able to be accurately measured, if to properly simulate real-world delivery of aerosol to the lung epithelium at relevant concentrations [111]. The physics of doing so and the good success so far of *in silico* physiologically-based pharmacokinetic models of lung absorption, distribution, metabolism and elimination have been reviewed recently in detail [111].

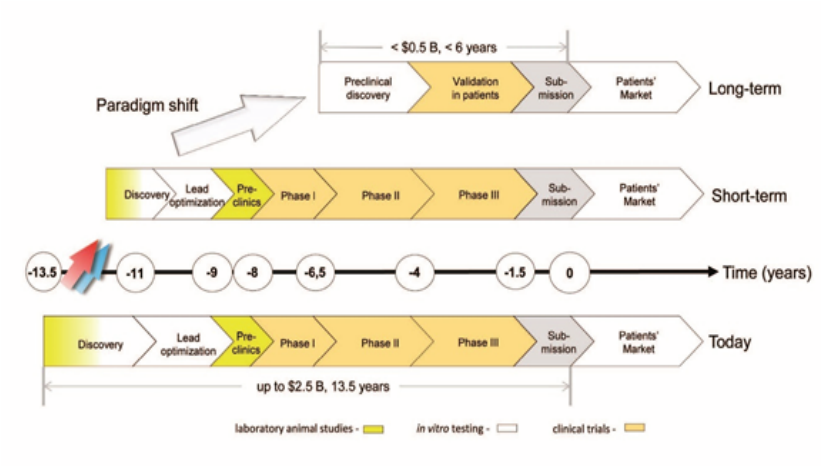
In efforts to harmonise whole-smoke (cigarette) aerosol exposure systems, the Cooperation Centre for Scientific Research Relative to Tobacco recently reported, "the first study to comprehensively survey over 40 parameters from aerosol generation, dilution, biological methodology, data analysis and dosimetry approaches, across eight independent laboratories, using a cytotoxicity endpoint" [112]. It was concluded that whilst there were many differences in methodology hindering or preventing comparison between laboratories, some aerosol delivery systems were commonly used, including the "Vitro Cell VC 10", and the "Borgwalt RM20S" [112].

Similarly in efforts to harmonise e-cigarette exposure systems and testing methods, a report commissioned by the European Union has been recently released with an overview of all types of testing conducted [110]. However, both cigarette and e-cigarette markets are rapidly evolving, making it extremely difficult for researchers to keep up with the vast array of products on the market, thus harmonisation is a huge and ongoing challenge. There have been some recent attempts to create "universal" testing methods, such as use of databases to prevent overlap of testing, although this is an ongoing task [113,114]. Additionally, the inventors of the Lung-Chip report a method to re-create their "Smoking Robot" [94]. Regardless of the system used, if aerosol delivered to cells can be physically characterised by chemical type and aerosol particle size and mass, and if the study is informed by *in silico* modelling, it is more likely to be successfully interpreted, and recreated by other laboratories.

Part four:
Transitioning to human-relevant science

While many international initiatives are aiming to drive research towards non-animal methods, barriers remain [8,17,79,115,82,83]. Some key road-maps have been proposed by experts from academia, industry and regulatory agencies, at the "Transatlantic Thinktank for Toxicology Workshop" to drive forward progress on microphysiological systems (Figure 7).

Figure 7.
Road Map detailing what progress looks like toward drug development; bottom=today, middle=in the short term and top=long term, post paradigm shift.



Red and blue arrows indicates use of single and multi-organ systems, and the white arrow indicate what progress will look like with human body-on-a-chip systems. Image: reprinted under creative commons license http://creativecommons.org/licenses/by/4.0/ from; Marx, U., Akabane, T., Andersson, T. B., Baker, E., Beilmann, M., Beken, S., Brendler-Schwaab, S., Cirit, M., David, R., Dehne, E. M., Durieux, I., Ewart, L., Fitzpatrick, S. C., Frey, O., Fuchs, F., Griffith, L. G., Hamilton, G. A., Hartung, T., Hoeng, J., Hogberg, H., … Roth, A. (2020). Biology-inspired microphysiological systems to advance patient benefit and animal welfare in drug development. ALTEX, 37(3), 365–394. https://doi.org/10.14573/altex.2001241

Overview of Australian studies utilising non-animal inhalation models

To demonstrate the capability in Australia for *in vitro* inhalation research we have highlighted some of the scientific findings recently published from Australian research institutes and universities (Table 4) and a few international examples for comparison (Table 5). Studies were excluded where research was a combination of both animal and *in vitro* models.

> *"Using non-animal models in biomedical research makes scientific sense. We really hope this knowledge [data] base will inspire scientists who currently rely on animal models for their research – we want to stimulate healthy scientific debate, to challenge mind-sets, and to pave the way for doing better and more human relevant science without animals."*
>
> Maurice W-helan, JRC scientist and head of EURL ECVAM. September 2020
>
> Media release: Tackling respiratory diseases with advanced non-animal models. 18 September 2020. Available: https://ec.europa.eu/jrc/en/science-update/tackling-respiratory-diseasesadvanced-non-animal-models Accessed: May 2021

Table 4
Published Australian studies using non-animal inhalation models.

Author affiliations: 1= Lung Research Unit, Hanson Institute, 2= Department of Thoracic Medicine, Royal Adelaide Hospital, Adelaide, 3= Department of Medicine, University of Adelaide, Adelaide, 4= Department of Occupational and Environmental Health, School of Public Health, University of Adelaide, Adelaide, 5= Telethon Kids Institute, Western Australia, 6= Walyan Respiratory Research Center, 7= Curtin University, Western Australia, 8= University of the Sunshine Coast, Queensland, Australia, 9= Department of Primary Industries and Regional Development, Western Australia, 10= Chemical Safety and Applied Toxicology (CSAT) Laboratories, School of Safety Science, The University of New South Wales, Sydney, Australia

Effects of E-cigarette E-liquid components on bronchial epithelial cells: Demonstration of dysfunctional efferocytosis [109]	
Approach:	"This study assessed the effect of E-cigarette constituents, 3 E-liquid apple flavours, nicotine, vegetable glycerine and propylene glycol, on bronchial epithelial cell viability, apoptosis and cytokine secretion and macrophage phagocytosis of apoptotic airway cells and phagocytic recognition molecules"
Exposure method:	Cigarette smoke extract infused cell media, method developed by Su, 1998. "Based on the average users' puff duration of 2.6 s,28 50 × 3 s puffs with 5 s in between were bubbled through 10 mL of culture medium … to create E-cigarette Extract"
Conclusion:	• E-cigarettes can cause bronchial epithelial apoptosis and macrophage efferocytosis dysfunction via reduced expression of apoptotic cell recognition receptors. These data further show that E-cigarettes should not be considered harmless to non-smokers and their effects may go far beyond cytotoxicity to cells.
Affiliations:	1,2,3,4
Fuel feedstock determines biodiesel exhaust toxicity in a human airway epithelial cell exposure model [108]	
Approach:	"To compare the exhaust exposure health impacts of a wide range of biodiesels made from different feedstocks and relate these effects with the corresponding exhaust characteristics"
Exposure method:	"Primary airway epithelial cells were exposed to diluted exhaust from an engine running on conventional diesel and biodiesel made from Soy, Canola, Waste Cooking Oil, Tallow, Palm and Cottonseed"
Conclusion:	• "This study … shows that exposure to different exhausts results in a spectrum of toxic effects in vitro when combusted under identical conditions"
Affiliations:	5,6,7,8,9

Table 4 continued.
Published Australian studies using non-animal inhalation models.

A novel in vitro exposure technique for toxicity testing of selected volatile organic compounds [119]	
Approach:	"The aim of this study was to develop a practical and reproducible in vitro exposure technique for toxicity testing of VOCs"
Exposure method:	"Human cells including: A549-lung derived cell lines, HepG2-liver derived cell lines and skin fibroblasts, were grown in porous membranes and exposed to various airborne concentrations of selected VOCs directly at the air/liquid interface for 1 h at 37 °C"
Findings:	Cigarette smoke exposure resulted in: significant decrease in tissue viability and barrier function, and a significant increase in the secretion of inflammatory cytokines and a marker of DNA damage. e-cigarette aerosol results did not differ from air controls.
Conclusion:	"Our findings suggest that static direct exposure at the air/liquid interface is a practical and reproducible technique for toxicity testing of VOCs. Further, this technique can be used for inhalational and dermal toxicity studies of volatile chemicals in vitro as the exposure pattern in vivo is closely simulated by this method"
Affiliations:	10
Soy Biodiesel Exhaust is More Toxic than Mineral Diesel Exhaust in Primary Human Airway Epithelial Cells [120]	
Exposure method:	"Using human airway epithelial cells obtained from young children, we compared the effects of exposure to exhaust generated by a diesel engine with Euro V/VI emission controls running on conventional diesel (ultra-low-sulfur mineral diesel, ULSD), soy biodiesel (B100), or a 20% blend of soy biodiesel with diesel (B20)"
Findings:	The exhaust output of biodiesel was found to contain significantly more respiratory irritants, including NOx, CO, and CO2, and a larger overall particle mass
Conclusion:	Exposure to biodiesel exhaust resulted in significantly greater cell death and a greater release of immune mediators compared to both air controls and ULSD exhaust
Affiliations:	5,6,7,9

Table 5:
Examples of published International studies using non-animal inhalation models.

Author affiliations: 1= Department of Genetic Toxicology and Nanotoxicology, Institute of Experimental Medicine of the CAS, 2= Department of Physiology, Faculty of Science, Charles University, 3= Center of Vehicles for Sustainable Mobility, Faculty of Mechanical Engineering, Czech Technical University in Prague, 4= Department of Vehicles and Ground Transport, Czech University of Life Sciences in Prague, 5= Department of Computer Science, Czech Technical University in Prague, 6= Department of Chemistry and Toxicology, Veterinary Research Institute, 7= Division of Hygiene and Medical Microbiology, Medical University of Innsbruck, Innsbruck, Austria, 8= Experimental Orthopedics, Medical University of Innsbruck, Innsbruck, Austria, 9= Department of Anaesthesiology and Critical Care Medicine, Medical University Innsbruck, 10= Central Institute for Blood Transfusion & Immunological Department, Medical University of Innsbruck

The Biological Effects of Complete Gasoline Engine Emissions Exposure in a 3D Human Airway Model (MucilAir(TM)) and in Human Bronchial Epithelial Cells (BEAS-2B) [121]	
Findings:	The 3D model showed 'weak' biological impact from exposure, with minimal effect on cell integrity. Gene expression in the MucilAir model altered in response to exposure, whereas the same measures altered in relation to time of treatment and not the exposure itself. The 3D model was, therefore, best suited for longer treatment assessments as there were not the confounding protocol-imposed effects observed with the cell culture.
Models *(Developer/ Institute)* used:	• 3D human lung tissue model MucilAir from disease-free male *(Epithelix, Switzerland)* • Human bronchial epithelial cell line (BEAS-2B) from disease-free male
Affiliations:	1,2,3,4,5,6
Fast-track development of an in vitro 3D lung/immune cell model to study Aspergillus infections [122]	
Findings:	Fast-track culturing of normal human bronchial or small airway epithelial cells under ALI and perfusion resulted in a significantly accelerated development of the lung epithelia associated with higher ciliogenesis, cilia movement, mucus-production and improved barrier function compared to growth under static conditions. Immune responses to fungi exposure were efficient, with dendritic cells and macrophages demonstrating full functionality, mimicking *in vivo* activity.
Models *(Developer/ Institute)* used:	• Primary normal human bronchial epithelial or small airway cells and healthy donor monocytes were grown in a Quasi-Vivo QV600 ALI perfusion chamber bioreactor (*Kirkstall, UK*)
Affiliations:	7,8,9,10

Transitioning to human-relevant science - the top five barriers and solutions

Australian researchers are underfunded in comparison to other countries, with UNESCEO Institute for Statistics data showing that Australia spends 2.2% of gross domestic profit on research and development, ranking as 13th in research and development spending. Research and development spending is required to increase by 2030, if to achieve the 17 Sustainable Development Goals, outlined in the "2030 Goals for Sustainable Development" agreement that all United Nations countries signed to in 2015 [123]. However, Australia is off track to achieve these goals [124].

There are considerable economic advantages to transitioning to human-relevant methods in all areas of regulatory science, and there is a resultant shift underway [27]. For example, the market for animal testing was valued at 10.74 billion USD in 2019 and expected to grow at a compound annual growth rate of 4.27% between 2019-2025; in comparison, the alternatives testing market was valued at 1.11 billion USD in 2019 and expected to grow at twice that rate - 10.4% - in the same time period [125].

> The top five recently identified barriers at the 2021 11th World Congress for Alternatives and Animal Use in the Life Sciences were:
>
> 1) Lack of understanding of how advanced human-based technologies work
> 2) Status-quo bias
> 3) Journal editorial policy
> 4) Regulatory requirement
> 5) Avoiding sunk-costs
>
> [Session titled:"Proof in animals":Has journal editorial policy fallen behind human-based approaches]

Conversely, the antidote to these barriers, or the way towards human-relevant science was also reported at the World Congress as being: 1) open-mindedness – breaking the "lock-in" of tradition and opinion, with scientific evidence [25,28], 2) focus of education and training in alternative methodologies, 3) scientific dialogue and collaboration, 4) informing the public and politicians, 5) redeployment of funds [103]. This report will address each of these five antidotes in detail below.

1. Open-mindedness - breaking the "lock-in" of tradition and opinion with scientific evidence

Medical research is in the midst of a paradigm shift, a scientific revolution toward human-relevant *in vitro* and *in silico* science [25,26,28,34,115,82,83]. In contrast, status-quo bias - a cause of "lock-in", is an emotional bias a "preference for the current state of affairs", and a barrier to any transition in life. Embracing new methods requires a change in mindset by researchers, regulators, peer reviewers, grant makers, animal ethics committees and journal editors alike. According to Thomas Kuhn, a paradigm shift is part of any scientific revolution, and requires a complex series of social changes that occur in different phases [26].

Editorial policy is one example of a necessary complex social change. In practice, this could be a requirement to change journal editorial policy that often requires testing in animals [8]. Some journals are already addressing this, for example, Nature Biomedical Engineering employs an editorial process where any reviewer's report can be requested to be published by the submitting authors. This opens the way for increased transparency and openness of the review process that enables researchers submitting to journals a fairer path to publication, and increased justification and visibility of any requests by reviewers for animal testing.

2. Focus on education and training in alternative methodologies

Alternatives Validation Centres provide education, a bridge between industry and academia, opportunities for funding, and much more. Australia urgently needs an alternatives centre - over 10 centres have has been established internationally. Two key centres are; the Center for Alternatives to Animal Testing (CAAT), John Hopkins University, United States, and the European CAAT, at University of Konstanz in Germany. In addition, the European Union Reference Laboratory for Alternatives to Animal Testing is an integral part of the European Commission Joint Research Centre. Similarly, the Interagency Coordinating Committee on the Validation of Alternatives Methods is an integral part of the National Toxicology Program in the United States. Alternatives centres follow the OECD guidance: "Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment" when validating alternative methods. A list of key alternatives centres can be found in our resource section.

Funding for education and training in alternative methods is essential to ease transitioning and avoid sunk-costs. Sunk-costs when transitioning to animal research refer to costs that cannot be recovered by any means, for example - purchase of equipment that cannot be on-sold, or the time spent previously refining animal-models. Additionally, there is an urgent need for the next-generation of researchers to be trained in alternative methods, or fields that support alternatives, such as Biomedical Engineering or Biotechnology, to ensure their employment, and the economic growth of Australia. Human-relevant science is the future of medicine and medical research.

3. Scientific dialogue and collaboration

Partnering with industry to gain insight is crucial to recognise scientific developments. The Australian Cooperative Research Centres, National Innovation System and the Australian Governments Global Innovation Strategy can be better promoted and funded as part of this process [126]. Additionally scientific dialogue and collaboration can be encouraged through creation of working group parties as seen in the United States and the European Union [2,3,5] or strengthening involvement on global harmonisation committees [35].

As always, attendance at international conferences, particularly those with a focus on alternatives, give insight on worldwide developments and provide opportunities to collaborate. One example is the World Congress on Alternatives and Animal Use in the Life Sciences. Collaboration with the scientific arm of animal advocacy groups is also a way to be involved. For example, the PETA Science Consortium International, or BioMed 21, who are associated with Humane Society International. Avicenna Alliance – the Association for Predictive Medicine, actively engages with academia, industry and regulatory agencies through various working groups to further projects such as the Virtual Physiological Human Initiative.

Engagement between researchers who use animals and those participating in *in vitro* and *in silico* human-relevant research is essential to bridge the gap between regulatory bodies, industry and academia and encourage uptake and funding of new methods. For example, attendance at conferences such as Australian and New Zealand Council for the Care of Animals in Research and Teaching (ANZCCART). Engagement can also involve collaboration with engineering departments at universities who develop new technologies, or engagement in industry events, such as Biodesign [127], or participation at AusMedTech conferences.

Open and engaged communication is essential with animal ethics committees and regulatory bodies to highlight innovation in the field, address necessary improvements and independently monitor scientific developments. This will ensure that the 3Rs can truly start with: 1) Replacement.

4. Informing the public and politicians

In 2018, only 10% of respondents to a national Australian survey commissioned by Humane Research Australia indicated that the annual animal use in research in Australia – 7 million animals per year – was acceptable [128]. Furthermore, nearly ~70% of respondents supported allocating public funds to alternatives to animal use, and 80% of respondents were unaware there were alternatives to animal testing in research for human medicines [128].

Transparency is key to the future of non-animal, human-relevant research and is essential to build trust in science and the ethical framework surrounding research. For example, in July 2021, ANZCCART launched the "Openness Agreement on Animal Research and Teaching for New Zealand", where the objective of the agreement is to: "… ensure that the public are well informed about what animal research involves, the role it plays in the overall process of scientific discovery, how such research is regulated in New Zealand, and what researchers and animal care staff do to promote welfare, reduce animal usage and minimise suffering and harm to the animals" [129]. This is in alignment with several other countries who have already implemented, or are working on, openness agreements, with the first adopter being the United Kingdom in 2014. Listening to the public is key to ascertaining whether there is a social license-to-operate – or public approval – for such animal research, particularly for research utilising highly invasive methods such as those in acute inhalation toxicity studies, and especially where there are known alternatives available. Consumer and community engagement is encouraged to further this.

The National Health and Medical Research Council (NHMRC) recognises consumer and community involvement to be an essential requirement prior to any research project being undertaken. The NHMRC offers a toolkit on their website to help researchers throughout Australia connect with the various state or territory bodies to facilitate this. Other funding bodies are likely to have similar requirements. The commitment by the NHMRC to consumer engagement has ensured that infrastructure is already in place to enable researchers to conduct community engagement throughout Australia.

5. Re-deployment of funds

Funding initiatives are necessary from both government and industry bodies, such as have been established previously in the European Union and United States: ONTOX (launched 2021), The Innovative Medicines Initiative, e-TOX, and many other programs with a focus on implementing 21st Century toxicology [82,83,21]. Funding has been used internationally to develop roadmaps and strategies that make alternatives possible in practice [5,12,16]. The Unites States implemented their "21st Century Cures" Act in 2016, in alignment with regulatory agencies worldwide making reforms to their own medical devices regulations, where the focus for the Food and Drug Administration was to "bring new innovations and advances to patients who need them faster and more efficiently" [130].

Humane Research Australia notes that there is already considerable support from Australian researchers for the provision of funding to develop alternatives [125]. However, funding for projects that promote the 3Rs is limited, and funding specifically for the replacement of animals extremely rare - despite the recommendation by the Australian Senate Select Committee in 1989 to have a dedicated funding program for alternatives, it has never been established [125]. Furthermore, scholarships for developments of alternatives should be created by industry, government and academia in Australia to encourage new generations of researchers in the use of alternative methods.

To ensure the next generation of scientists have a sustainable research career, and that the research sector prospers, it is necessary to look to the future, and the potential of new approach methodologies. Vast sums of money have been deployed over the last 10 years in the European Union and the United States purely for the human organ-chip and body-chip programs [82,83]. This effort has been hastened with the development of the Sars-CoV2 pandemic, and will be increasingly necessary, with a rapidly growing population requiring solutions at a rate that only can only be achieved with computation and technology, and not with conventional animal science.

Currently, there is a unique opportunity to redivert funds from the proposed Animal Resource Centre closure in Western Australia, into alternatives funding. Similarly, the United Kingdom announced the closure of their animal genomics research facility in 2019, where they closed primarily to pursue alternatives [131].

The way towards human-relevant science

Transitioning to something new can seem an insurmountable endeavour, but stagnation is undesirable also. Collaboration within academia with those who have developed or are using alternative models can fill the void of knowledge that makes the journey appear so mountainous. Therefore, a list of researchers who are active in human-relevant (non-animal) research is provided in the Resource section.

Arno Gutleb, Luxembourg Institute for Science and Technology, Luxembourg

"I've been working in the field of in vitro *models for the respiratory system for more than a decade, and began my research with much simpler models than we use today.*

"Since then, I've seen the progression of our field. We have come quite far when it comes to the development of New Approach Methodologies (NAMs). These models can be based on human cell lines or primary cells and allow the culture of cells at the air-liquid interphase which is an absolute necessity for cells from the respiratory system. Such complex models have been shown to have a high similarity with the human lung applying biological network models of active genes.

"Today we also have adequate exposure systems that allow exact dosimetry, which is needed to benchmark effects against what is observed in vivo in humans. The combination of complex in vitro *models for the respiratory system with state-of-the-art exposure systems allow us to mimic the* in vivo *situation in humans to a very high degree, allowing for comparability of the* in vitro *with the* in vivo *data.*

"I encourage Australian research institutes to explore the possibility of using NAMs for more human-relevant research."

Personal communication with Humane Research Australia, June 2021.

In closing

It is clear from this report that human-relevant science is the way forward and a transition to human-relevant research methods as adopted internationally must occur in order to provide better healthcare for Australians. However, in the absence of a dedicated Alternatives Validation Centre, or a dedicated 3R's funding stream as recommended by a Senate Committee to the Australian Government in 1989, many Australian researchers are still using animal methods in inhalation research. Based on the limitations discussed, it is clear that whilst non-animal methods may not immediately replace all existing animal inhalation studies, it is an evolving field, in comparison with animal models - which despite modification attempts, will never be able to truly replicate human biology. Therefore, researchers, regulators, funders and animal ethics committee members are encouraged to consider the information and resources provided and commit to a transition to human-relevant research.

> **During the interim transition period increased scrutiny is required for the use of animal models or animals in research. Research applications should now include the reporting of:**
>
> **1) Thorough and up-to-date investigation of the disease or pathology in humans for which prevention, diagnosis or treatment is intended;**
>
> **2) A search for validated alternatives and justification for why a specific model is chosen over others, and;**
>
> **3) Up-to-date assessment of all clniically relevant diagnostic or prognostic markers.**

Barriers faced to this transition could be overcome by increased government and industry support that can be provided by funding initiatives, scholarships, mentoring programs and crucially, the development of an Australian Alternatives Validation Centre, with broader benefit to biomedical research. It is necessary to provide better treatments for more diseases, a level of healthcare congruent with the 21st century, improved abilities to understand the human species, and halt disease progression and end human and animal suffering. Therefore, Australia needs to urgently re-direct funding towards scientifically-valid, *in vitro* and *in silico* methods in alignment with the international movement.

Resources

To find out more on the non-animal models discussed here and international activity regarding human-relevant research, consider reviewing the following sources:

Alternatives Validation Centers associated with universities or scientific institutes

- CaCVAM - Canadian Centre for the Validation of Alternative Methods https://www.uwindsor.ca/ccaam/
- CAAT Europe - https://www.biologie.uni-konstanz.de/leist/caat-europe/
- Centre for Alternatives to Animal Testing (CAAT) John Hopkins (US) http://caat.jhsph.edu
- ECVAM - The European Centre for the Validation of Alternative Methods.
- ICVAAM (USA) - Interagency Coordinating Committee on the Validation of Alternative Methods https://ntp.niehs.nih.gov/whatwestudy/niceatm/iccvam/index.html
- JaCVAM - Japanese Centre for the Validation of Alternative Methods. https://www.jacvam.jp/en/index.html
- KoCVAM - the Korean Centre for the Validation of Alternative Methods http://www.nifds.go.kr/kocvamen/
- National Centre for Advancing Translational Sciences (NCATS) https://ncats.nih.gov/
- NKCA–The National Knowledge Centre on Alternatives to Animal Experiments (Netherlands). https://norecopa.no/inventory3rs/netherlands-knowledge-centre-on-alternatives-to-animal-use
- NC3RS – National Centre for the Replacement, Refinement & Reduction of Animals in Research (UK) https://www.nc3rs.org.uk
- Swiss 3R Competence Centre, https://www.swiss3rcc.org/en/about-us
- The German Centre for the Protection of Laboratory Animals https://www.bfr.bund.de/en/german_centre_for_the_protection_of_laboratory_animals.html and their associated unit:
- Unit: Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET) https://www.bfr.bund.de/en/unit__centre_for_documentation_and_evaluation_of_alternative_methods_to_animal_experiments__zebet_-53868.html

Alternatives development: key societies, programs, or databases

- ONTOX program https://ontox-project.eu/ and associated database https://aopwiki.org/aops
- European Organ-on-Chip Society (EUROoCS) https://www.eurooc.eu/
- List of all key alternative methodologies databases, Lush Prize, network toolkit: https://lushprize.org/background/lush-prize-1r-network/

Key advocacy groups with interest in promoting alternatives to animal use in science and medicine

- Avicenna Alliance – Association for Predictive Medicine https://avicenna-alliance.com/about-us/our-mission/
- Biomedical Research for the 21st Century (Biomed21) https://biomed21.org
- Fund for the Replacement of Animals in Medical Experiments (FRAME) http://www.frame.org.uk
- Humane Research Australia https://www.humaneresearch.org.au/
- Humane Society International https://www.hsi.org/
- New Zealand Anti-Vivisection Society https://www.nzavs.org.nz/
- Physicians Committee for Responsible Medicine PCRM (USA) https://www.pcrm.org/
- PETA Science Consortium International https://www.thepsci.eu/

Resources

To find out more on the non-animal models discussed here and international activity regarding human-relevant research, please consider reviewing the following sources:

Australian researchers, research institutes or companies identified as active in alternative methodologies (*in vitro* or *in silico*)

Australia Research Council, Centre for Personalised Therapeutics
In vitro: https://therapeutics-technologies.com.au/

MiniFab - chip technologies
https://schott-minifab.com/contact-minifab/minifab-australia

Royal Melbourne Institute of Technology
In silico: Jingliang Dong: https://orcid.org/0000-0002-2812-6188

University of Technology, Sydney
In vitro: Majid E. Warkiani : https://orcid.org/0000-0002-4184-1944

University of Sydney
In silico: Agisilous Koumatsis: https://www.sydney.edu.au/engineering/about/our-people/academic-staff/agisilaos-kourmatzis.html

Wal-yan Respiratory Research Centre, Perth Western Australia:
In vitro: Alexander Larcombe: https://orcid.org/0000-0003-4196-4482,
In vitro: Katherine Landwehr: https://orcid.org/0000-0002-1543-6304
In silico: Natalie Anderson: https://orcid.org/0000-0002-7532-8372
In vitro: Thomas Iosifidis: https://orcid.org/0000-0001-8462-5865
Wal-yan Centre Research Communications Specialist: Hayley Goddard: linkedin.com/in/hayleygoddardjournalist

International institutes and companies active in non-animal inhalation research

AlveoliX AG, Bern, Switzerland
ARTORG Center for Biomedical Engineering Research, University of Bern, Switzerland
Emulate, United States (US)
Epithelix, Switzerland
Flemish Institute for Technological Research (VITO NV), Mol, Belgium
Institute for In Vitro Sciences, Gaithersburg, US
Iuliu Hațieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania
Kirkstall, UK
Luxembourg Institute of Science and Technology (LIST), Luxembourg
MatTek Corporation, US
Medical University of Innsbruck, Innsbruck, Austria
TissUse GmbH, Germany
Vitrocell Systems, Germany
Wyss Institute for Biologically Inspired Engineering, Harvard University, US

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