European Commission

Advanced Non-animal Models in Biomedical Research

Respiratory Tract Diseases

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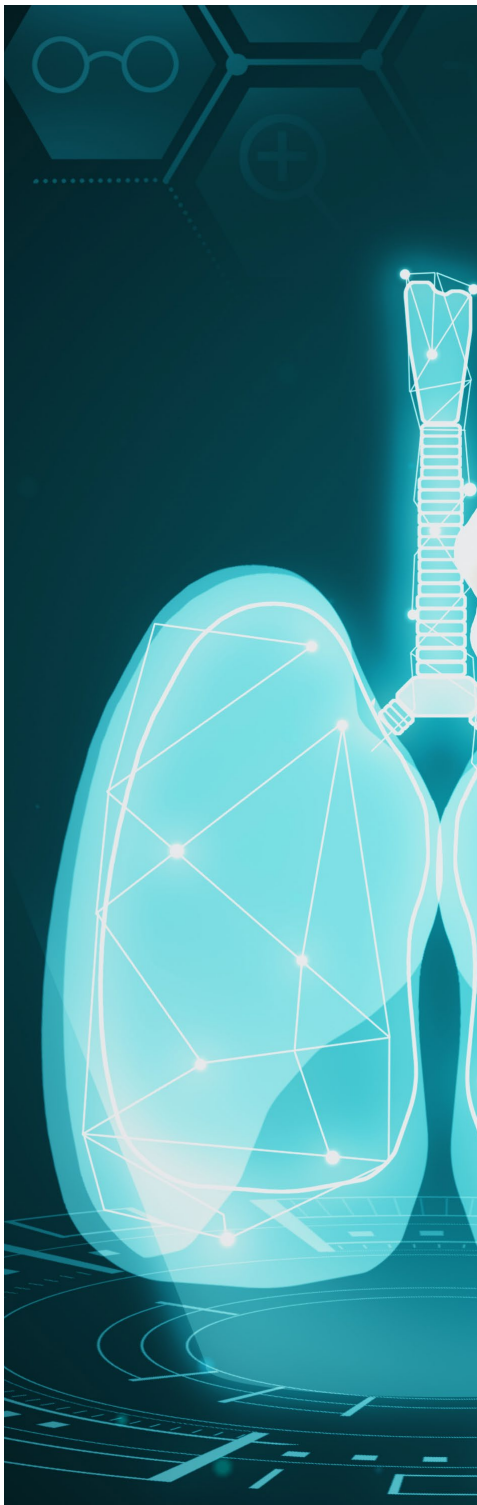

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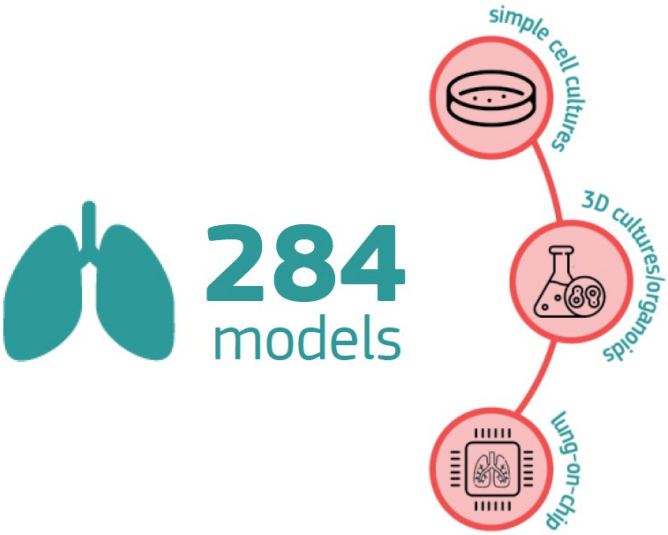
Abstract

The European Commission's Joint Research centre (JRC) has undertaken a study to review available and emerging non-animal models in the field of respiratory tract diseases.

In a detailed analysis of the scientific literature, over 21,000 abstracts (11,636 non-cancer and 9,421 cancer) were scanned for relevant non-animal models of respiratory disease. From this, a total of 284 publications were finally identified as being promising candidate models according to a set of inclusion/exclusion criteria. *In vitro* cell and tissue cultures, human *ex vivo*, *in silico* approaches were chiefly considered.



These models have been collated into a catalogue of biomedical disease models that will form a key knowledge source for researchers, educators and national ethics and funding authorities.

Simple cell culture models using immortalised cell lines are long-established, are inexpensive and quick, however they poorly reflect complex disease mechanism observed *in vivo*.

The emerging use of more physiologically-relevant models of disease, such a 3D human tissue cultures, spheroids, organoids, and microfluidic 'lung-on-a-chip' based systems shows immense promise for the development of *in vitro* model systems that can more accurately mimic human respiratory diseases.

> ***Bioengineering approaches hold the promise of more human-relevant disease models that can be used to elucidate mechanism of disease and aid in the development of new therapies***

This review shows that, while simple models are still prominent and have their uses, research focus has, in the past 5 years, being shifting towards increasingly sophisticated bioengineering approaches that recapitulate lung development, anatomy and physiologic functions *in vitro*. Such approaches hold the promise of more human-relevant disease models that can be used to elucidate mechanism of disease and aid in the development of new therapies.

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1 Introduction

Lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), chronic respiratory infections, pulmonary fibrosis, and lung cancer are major contemporary health problems. Over 235 million people are affected by asthma and around 65 million live with moderate-to-severe COPD (Forum of Respiratory Societies, 2013).

Existing models of respiratory disease rely heavily on *in vivo* endpoints including (histo) pathology, histology, mediator analysis and in diseases such as asthma, measures of airway (hyper)-responsiveness.

Despite increased prevalence of chronic airway diseases, the failure to translate promising drug candidates from animal models to humans has led to questions over the utility of *in vivo* studies in this area of disease research.

Failures in the current paradigm for drug development have resulted in soaring research and development costs and reduced numbers of new drug approvals. Over 90% of new drug programmes fail to progress to market and the rate of successful transition from phase II to phase III clinical trial transition is far lower than other phases (Thomas *et al.*, 2016) due largely to efficacy failures or unexplained toxicity.

This is particularly evident for drugs targeting respiratory disease, where the phase II to phase III transition rate, calculated at 29%, is lower than the overall average for all disease indications (Waring *et al.*, 2015). Thus, there is now an increased interest in using alternative approaches, in particular non-animal models, as predictors of respiratory diseases.

There is a financial, as well as a scientific rationale, underpinning this. Respiratory diseases represent a huge burden worldwide, both economically and from a health perspective. In fact, chronic respiratory diseases are one of the leading causes of morbidity and mortality globally. There are many unmet needs regarding effective medications for respiratory diseases, and the inability of animal models to accurately recapitulate the full spectrum of clinical features of these conditions (e.g., asthma; COPD and cystic fibrosis) has a further negative impact on drug discovery.

> *Chronic respiratory diseases are one of the leading causes of morbidity and mortality globally*

The future development of safe and efficacious new therapies for respiratory diseases is therefore beginning to shift to models based on human data and the mechanistic basis for disease rather than the continued use of animal models which poorly reflect the clinical situation.

The purpose of this study is to develop a current overview of available and emerging non-animal models in the field of respiratory tract diseases, including information on their applications, biological relevance, and development status. This review aims to cover all existing and in-development non-animal approaches to respiratory disease modelling, including *in vitro*, *in silico* and *ex vivo* approaches.

1.1 Overview of respiratory diseases

The US National Cancer Institute (NCI)[1] defines respiratory disease as:

> *"A type of disease that affects the lungs and other parts of the respiratory system. Respiratory diseases include asthma, chronic obstructive pulmonary disease (COPD), pulmonary fibrosis, pneumonia, and lung cancer. Also called lung disorder and pulmonary disease."*

For this project, cystic fibrosis (CF), a genetic disorder that affects mostly the lungs, but also the pancreas, liver, kidneys, and intestine, is included in this definition.

1.1.1 Asthma

Asthma is a non-communicable inflammatory disease of the lung airways. It is characterised by variable and recurring symptoms, reversible airflow obstruction, and easily triggered bronchospasms manifested as recurrent attacks of breathlessness, wheezing, coughing and chest tightness, which vary in severity and frequency between individuals (World Health Organization, 2017a).

Asthma causes are not completely understood, but are thought to be due to a combination of genetic predisposition and environmental exposure to substances and particles that may irritate the airways or cause allergic reaction. Other potential triggers include medications such as aspirin (Kowalski *et al.*, 2013) and beta blockers (Morales *et al.*, 2017).

Diagnosis of asthma is typically based on the pattern of symptoms, response to therapy over time, and spirometry. Asthma is classified according to the frequency of symptoms, forced expiratory volume in one second (FEV1), and peak expiratory flow rate (Khajotia, 2008). Asthma may be classified as atopic or non-atopic, where atopy refers to a predisposition toward developing a type 1 hypersensitivity reaction (Horak *et al.*, 2016).

There is currently no cure for asthma. Symptoms can be prevented or managed by avoiding triggers, such as allergens and irritants, and by the use of inhaled corticosteroids (ICS) in combination with inhaled long-acting B_2 agonist (LABA). Long-acting beta agonists (LABA) or antileukotriene agents may be used in addition to inhaled corticosteroids if asthma symptoms remain uncontrolled. Treatment of rapidly worsening symptoms is usually with an inhaled short-acting beta-2 agonist such as salbutamol and corticosteroids taken by mouth (Horak *et al.*, 2016).

The World Health Organization (WHO) estimates that 235 million people currently suffer from asthma. According to the latest WHO estimates, released in December 2016, there were 383,000 deaths due to asthma in 2015. Asthma presents a substantial burden to individuals and families and often restricts individuals' activities over their lifetime.

Although most asthma-related deaths occur in low- and lower-middle income countries, likely due to being under-diagnosed and under-treated, the disease occurs in all countries regardless of their level of development (World Health Organization, 2017a).

Animal models of asthma aim to mimic the pathophysiology of human disease (Aun *et al.*, 2017). Several animal species of animals have been used in experimental models of asthma, including *Drosophila*, rats, guinea pigs, cats, dogs, pigs, and primates. However, the most common species/strain studied is BALB/c mice, as they develop a good Th2-biased immunological response (Nials and Uddin, 2008). Animal models typically include

1 https://www.cancer.gov/publications/dictionaries/cancer-terms/def/respiratory-disease

two phases: sensitisation and challenge. Sensitisation is traditionally performed by intraperitoneal and subcutaneous routes, while intranasal instillation of allergens has been used to mimic human exposure to allergens by inhalation.

Few new drugs have made it to the market in the last 50 years. Most approaches cannot be translated to human disease, as asthma is a uniquely human condition. For example, mice do not develop asthma spontaneously, and so in order to create an animal model with symptoms of the disease, a protein such as ovalbumin is instilled into the airways to artificially induce the condition.

There are wide variations between animal species, protocols and allergens used. Recent approaches have tended to replace ovalbumin with the same aeroallergens that trigger clinical disease, for example house dust mites (Aun *et al.*, 2017).

Development of innovative approaches in the study of human asthma has been slow. Human tissue and tissue engineering approaches show promise but are limited by access to normal human tissue and also to diseased tissue (Edwards *et al.*, 2015).

The use of non-mammalian models (*Caenorhabditis elegans*, *Drosophila*, *Dictyostelium discoideum* and zebrafish) in respiratory disease research is almost non-existent. They have, however, been used to understand the molecular mechanisms of diseases in other organ systems, suggesting that they may be useful alternatives for asthma research.

Mathematical models can provide new insights to improve understanding of asthma, and a number of these are under development. *In silico* approaches have been used, mainly in deposition modelling of inhaled therapies (Munro *et al.*, 2018).

1.1.2 Chronic obstructive pulmonary disease (COPD)

Chronic obstructive pulmonary disease (COPD) is an umbrella term used to describe progressive lung diseases including emphysema and chronic bronchitis. The common terms 'chronic bronchitis' and 'emphysema' are no longer used, but are now included within the COPD diagnosis (World Health Organization, 2017b).

COPD is a chronic inflammatory lung disease that develops slowly and usually becomes apparent after 40 or 50 years of age. The most common symptoms of COPD are breathlessness (or a "need for air"), chronic cough, and sputum (mucus) production (World Health Organization, 2017b).

Chronic bronchitis is inflammation of the lining of the bronchial tubes, which carry air to and from the alveoli of the lungs. It is characterised by chronic cough and mucus (sputum) production.

Emphysema is a condition in which the alveoli at the end of the smallest air passages (bronchioles) of the lungs are destroyed as a result of damaging exposure to cigarette smoke and other irritating gases and particulate matter.

The leading cause of COPD is tobacco smoking. Long-term exposure to chemical irritants can also lead to COPD. Diagnosis usually involves imaging tests, blood tests, and lung function tests. There is no cure for COPD, but treatment can help ease symptoms, lower the chance of complications, and generally improve quality of life. Medications, supplemental oxygen therapy, and surgery are some forms of treatment (Balkissoon *et al.*, 2011).

WHO has estimated a prevalence of 251 million cases of COPD globally in 2016, with an estimated that 3.17 million deaths caused by the disease in 2015 (5% of all deaths globally in that year). More than 90% of COPD deaths

occur in low and middle income countries (World Health Organization, 2017b).

Animal models of COPD typically use mice, guinea pigs and rats. COPD is induced by exposure to cigarette smoke (CS), intra-tracheal lipopolysaccharide (LPS) and intranasal elastase. Parameters measured are mainly lung pathological data and lung inflammation (both inflammatory cells and inflammatory mediators) in most of the studies and tracheal responsiveness (TR) in only few studies. However, to date there is no standardised method or protocol for animal exposure to CS, and there is a wide diversity of methods by which a study on COPD in animals can be done. For example, the type of cigarettes used to generate smoke (commercial vs. research cigarettes, with or without a filter), the constituents of the CS used for exposures, delivery systems (whole body vs. nose-only), duration of exposure and most significantly, the dose of smoke delivered to the animals are important determinant factors that have yet to be standardised (Ghorani *et al.*, 2017).

Although CS has been shown to induce many features of COPD in animals, including pulmonary infiltration of macrophages and neutrophils, airway fibrosis and emphysema, these still do not accuratley describe the human situation.

For example, mice do not produce mucus in their bronchial tract and therefore cannot represent this aspect of COPD. Animal models do not reflect that variable pathology and different stages of COPD severity in humans, and thus are restricted to modellling a limited number of characteristic features of COPD in the clinic. Also, several studies have shown that different strains of mice show various levels of sensitivity to CS challenge (Mortaz and Adcock, 2012).

Non-animal approaches to modelling COPD are developing but are still limited. For example, cell lines are not considered as physiologically complex enough, whereas reproducibility of primary cells is variable between donors. 3D epithelial cultures are more physiologically relevant, but also suffer from reproducibility issues across different donors. *Ex vivo* lung tissues, for example precision cut lung slices (PCLS) are the most physiologically relevant of non-whole organ *ex vivo* models and allow study of cell types/functions (macrophages, airway contractility, etc.) that are not available in other models. However availability of high quality tissue is low, and variabilty across donors is still an issue (Adamson *et al.*, 2011; Benam *et al.*, 2015).

1.1.3 Pulmonary fibrosis

Pulmonary fibrosis is a respiratory disease in which scars are formed in the lung tissues, resulting in stiffness and difficulty in breathing, which can eventually lead to respiratory failure, heart failure, or other complications. Scar formation through the accumulation of excess fibrous connective tissue in the lung parenchyma (fibrosis) leads to thickening of the walls, and causes reduced oxygen supply in the blood. This manifests as a perpetual shortness of breath (Sgalla *et al.*, 2018).

Pulmonary fibrosis may be a secondary effect of other diseases, in particular interstitial lung diseases caused by autoimmune disorders, viral infections and bacterial infection (e.g., tuberculosis). Idiopathic pulmonary fibrosis (IPF) refers to a manifestation without any known cause. Current paradigms suggest that alveolar epithelial cell damage is a key initiating factor in IPF (Barratt *et al.*, 2018).

Spontaneous pulmonary fibrosis is known to occur in other animal species, including dogs, horses, donkeys, and cats. To date, preclinical investigations of fibrosis have typically used mouse models of bleomycin (BLM)-induced pulmonary fibrosis. However there are several limitations that prevent direct translation to human IPF. Preclinical efficacy of currently

approved anti-fibrotic agents (pirfenidone and nintedanib) was tested only in the BLM-induced model, with results based on histologic end points such as collagen deposition that are not clinically relevant. Also, reproducibility issues are evident due to widely variable experimental procedures between labs, leading to a lack of consistent results. Animal size needs to be balanced with the statistical power needed to generate robust data and that insufficient reporting of experimental animal data or unpublished negative therapeutic results severely hamper the validity of experimental studies (Tashiro *et al.*, 2017).

Current research is directed at identifying key biomarkers that may direct more clinically-relevant results (Barratt *et al.*, 2018).

Humanised *in vitro* models can enable the modelling fibrotic disease using human cells, and these are in theory more suitable than animal models in modelling human lung physiology, biology and immunity. Current *in vitro* models are limited as they only allow acute studies (1–2 days) and are primarily focused on TGF-β1 induced collagen production (Sundarakrishnan *et al.*, 2018).

Precision cut lung slices (PCLS) recapitulate aspects of the human lung better than other 3D tissue models. The American Thoracic Society (ATS) has recommended using these systems to corroborate results obtained from bleomycin animal studies (Jenkins *et al.*, 2017). Future work will involve comparative studies of anti-fibrotic drug effects in PCLTs versus bleomycin administered animal models, so as to understand species differences in drug responses.

Past studies have created lung-on-chip devices for studying pulmonary fibrosis (fibrosis-on-chip), but antifibrotic drug testing and mechanistic studies of fibrotic lung disease have not yet been fully developed. Lung spheroids and organoids are limited because they do not permit airway maturation. However, they also may be used to identify differences in drug responses between animal studies and organoid cultures (Sundarakrishnan *et al.*, 2018).

1.1.4 Cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive genetic disorder that primarily affects the lungs, but also the pancreas, liver, kidneys, and intestine. In the lung, CF results in a progressive bronchiectasis, leading to difficulty breathing and coughing up mucus as a result of frequent lung infections, and ultimately to respiratory failure, which is the leading cause of death in patients with CF (Goetz and Ren, 2019).

The cystic fibrosis transmembrane conductance regulator (CTFR) protein functions as a channel across the membrane of cells that produce mucus, sweat, saliva, tears, and digestive enzymes. The channel transports negatively charged chloride ions into and out of cells, regulating the movement of water in tissues, which is necessary for the production of thin, freely flowing mucus. Disease-causing mutations in the CFTR gene alter the production, structure, or stability of the chloride channel, resulting in the production of abnormally thick mucous which can subsequently blocks airways and glands, leading to the clinical symptoms.

The condition is diagnosed by a sweat test and genetic testing. There is no known cure. The average life expectancy is between 42 and 50 years in the developed world. Lung problems are responsible for death in 80% of people with cystic fibrosis (Ong and Ramsey, 2015).

Most current knowledge of CF pathophysiology has been derived from animal models, in particular gene-targeted mouse models. However, mice models, in contrast to pigs and ferrets, do not spontaneously develop lung disease as seen in humans. Pigs and ferrets exhibit similar lung function and architecture with humans and at birth, however they lack inflammation (Semaniakou *et al.*, 2019).

The main disadvantage of animal models of CF is the difficulty of keeping them alive long enough for study, as the animals typically die from intestinal obstruction soon after birth. Therefore, while animal studies may show lung pathology similar to that in CF patients, they are only based on experiments that have been conducted in a small number of animals, which are mostly newborn (Semaniakou *et al.*, 2019).

Conventional 2D cell culture models have been shown to be useful for preclinical research and testing, but they lack the structural complexity of intact organs (Cutting, 2015). The use of 3D airway and intestinal organoid models in CF research is increasing; however the most developed models currently are intestinal organoids that exhibit key physiological differences between the gut and the airway mucosal surface, including the observation that there is no mucociliary clearance in the gastrointestinal tract (Awatade *et al.*, 2018).

1.1.5 Lung cancer

Lung cancer, or lung carcinoma, is a malignant lung tumour characterised by uncontrolled cell growth in tissues of the lung, and which can metastasise to other organs and tissues. Most cancers that start in the lung (primary lung cancers) are carcinomas. Two main types are identified based on histology: small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). The most common symptoms are coughing (including coughing up blood), weight loss, shortness of breath, and chest pains.

The majority (>85%) of cases of lung cancer are due to long-term tobacco smoking. Of the approximately 10–15% of cases that occur in people who have never smoked, a combination of genetic factors and exposure to radon gas, asbestos, second-hand smoke, or other forms of air pollution, or chronic infection, are implicated (Lemjabbar-Alaoui *et al.*, 2015).

Worldwide in 2012, lung cancer occurred in 1.8 million people and resulted in 1.6 million deaths, making it the most common cause of cancer-related death in men and second most common in women after breast cancer[2].

> *Worldwide lung cancer represents the most common cause of cancer-related death in men and second most common in women after breast cancer*

Modelling lung cancer in non-animal models presents many challenges. Whereas analyses such as tumour DNA sequencing can be performed in cell cultures *in vitro*, the outcome of mutations are best assessed in the context of an intact organism. Mouse models of cancer are widely used as they can reproduce many features of human tumours, and allow for re-engineering of combinations of complex lesions found in human tumours, providing a basis to model distinct tumour characteristics such as metastatic behaviour and response to therapy (Kwon and Berns, 2013).

The OncoCilAir™ human 3D *in vitro* lung cancer model has been available since 2015, offering the advantage of reproducibility that is implied with a commercial model, and which is of utmost importance to deal with emerging

2 https://www.wcrf.org/dietandcancer/cancer-trends/lung-cancer-statistics

issues with *in vitro* research beyond those that can be addressed with good cell culture practice (Pamies, 2018).

The use of patient-derived xenografts, where human tumour tissue is implanted into an animal in order to test different chemotherapy, has been discredited recently, as alterations in the tumours as they progress *in vivo* remove any resemblance to the patient's disease (Ben-David *et al.*, 2017).

1.1.6 Respiratory infections

Respiratory infections such as pneumonia and tuberculosis present a challenging field of study due to an intricate relationship between the pathogenicity of microbes and the host's defences.

Pneumonia is usually caused by infection with viruses or bacteria and less commonly by other microorganisms, certain medications and conditions such as autoimmune diseases. Risk factors include other lung diseases such as cystic fibrosis, COPD, and asthma, diabetes, heart failure, a history of smoking, a poor ability to cough such as following a stroke, or a weak immune system.

Tuberculosis (TB) is caused by the bacillus *Mycobacterium tuberculosis* and is the leading cause of infection-related death globally. TB affects over 10.4 million new individuals per year and is estimated to exist in a latent form in around 2 billion people worldwide (World Health Organization, 2018).

Human models of virus-induced asthma and COPD exacerbations have been developed but their use is limited to study viral infections, for safety and ethical reasons. Animal models of respiratory infection are more widely used. Species utilised include primates, bovids, sheep, rabbits, guinea pigs and mice (Saturni *et al.*, 2015).

Cattle have been widely used for modelling tuberculosis infections for vaccine development, as they are the natural host of *Mycobacterium tuberculosis bovis*, and they develop a disease very similar to humans (Rodriguez *et al.*, 2011). However, mice are the most common species used, principally for reasons of cost, ease of handling and opportunities for genetic manipulation (Bem *et al.*, 2011).

Studies on mouse models are not limited to common viral and bacterial infections, but also to complex infections like pneumonia. Animals can be manipulated in different ways to investigate various aspects of diseases. For example, immunological insights into pneumonia have been gained by photon-irradiation of mice subsequently challenged intra-tracheally with *Klebsiella pneumonia* (Keller *et al.*, 2003).

In vitro models are often the first step to study a respiratory infection mechanism, allowing evaluation the role of single elements in order to clarify the pathways leading to respiratory infections. The interrelationship between individual elements can then be studied in a complex setting as an animal/human model. A more complex setting is represented by the *ex vivo* models, which consist of human samples that are infected *in vitro*.

> *In vitro models are often the first step to study a respiratory infection mechanism in order to clarify the pathways involved*

In vitro models that recapitulate *in vivo* drug clearance profiles are increasingly important

in pharmacodynamic studies. And although *in vitro* pharmacodynamic models cannot model all variables seen *in vivo*, they can provide valuable information for the drug development process and the determination of optimal dosing regimens (White, 2001).

> *In vitro models that recapitulate in vivo drug clearance profiles are increasingly important in pharmacodynamic studies*

1.2 Non-animal models of respiratory disease

Many scientific discoveries and therapies have shown promise in the animal model of choice but subsequently failed to replicate the results when translated to human beings. This is due to the huge differences in associated risk factors, course of disease, pathogenesis, chronicity, related pathologies and comorbidities, symptoms, and genetic influences between the species (Akhtar, 2015).

Airways epithelial cells are differentiated and polarised *in vivo*, this aids normal function of the tissue and is vital to recreate effective models of diseases such as cystic fibrosis, where location and activity of an ion channel are of exquisite importance to pathophysiology. Culturing cells at the air liquid interface (ALI) has the added advantage of promoting polarisation and the role of this in disease pathology, and also in modelling infections, has been recognised (Hasan *et al.*, 2018). Whilst models of infection were not a major focus on this project, we were interested in models of important respiratory conditions, specifically, pneumonia and TB.

One of the major drawbacks of using animals to model infections is that the host-pathogen interactions are not recapitulated and differences in the immune response between animals and humans could create significant differences in disease progression and the ultimate resolution of infection. However, it is apparent that non-animal models are beginning to address thus and are becoming more complex, incorporating various different immune cells in order to increase complexity and physiological relevance (Iakobachvili and Peters, 2017).

This increase in complexity has led to more multidisciplinary approaches to create models, with a rise in the use of bioengineering approaches, to scale-up basic research models in order to create solutions for clinical problems such as lung transplantation. A growing number of tissue engineering approaches are exploring the potential to generate lung tissue *ex vivo* for transplantation (De Santis *et al.*, 2018). We did not extensively explore the issue of generating tissue for clinical purposes through this project, but note that regenerative medicine is potentially a very important endpoint for *in vitro* modelling, and particularly for lung disease, given the high incidence and severity of these conditions globally.

Cancer remains a leading cause of morbidity and mortality globally and lung cancer specifically accounts for more deaths than breast and prostate cancer combined[3]. The rise in tissue engineering has enabled the development of complex three dimensional, 'spheroid' tumour models that take account of the niche surrounding the tumour and can facilitate studies of cell signalling within and outside of the tumour mass (Rodrigues *et al.*, 2018).

3 https://www.lungcancereurope.eu/lung-cancer/

> *The rise in tissue engineering has enabled the development of complex three-dimensional, 'spheroid' tumour models*

1.2.1 In vitro 2D and 3D cultures

Cell cultures from primary and immortalised cell lines have traditionally been performed in two dimensions (2D), where the cells of a single type are grown submerged in nutrient medium on plastic or glass culture dishes. This has allowed for a consistent approach to the study of individual cell type features such as morphology, genetics, and physiologic response and enable high throughput applications, particularly in drug development.

However, despite these advantages, 2D cell culture has major drawbacks when compared with animal models of disease. Most obvious is that 2D systems cannot replicate the *in vivo* situation where tissues and organs are comprised of multiple cell types which function together as units through cellular communication and contact, as seen in the complexity of whole organs (Huh *et al.*, 2011). 2D cultures cannot reproduce the key features of the airways such as the formation of a differentiated, polarised epithelial barrier with different epithelial cell types. For example, cells in these system fail to undergo mucociliary differentiation (Marshall *et al.*, 2015).

The development of three-dimensional (3D) air-liquid interface (ALI) cultures has enabled the creation of more physiologically accurate models recapitulate the pseudostratified mucociliary phenotype observed *in vivo.*

The defining feature of ALI culture is that the basal surface of the cells is in contact with liquid culture medium, whereas the apical surface is exposed to air. Airways epithelial cells, for example human bronchial epithelial cells (HBECs), are plated onto specialised culture inserts such as Transwells® or Snapwells™, and grow on a porous filter that physically separates the lung epithelial tissue from the underlying media. Cells can thus be induced to differentiate to create a pseudostratified epithelial layer. Ciliated and mucous-producing cells become polarised and take on features of *in vivo* tissue, including functional apical cilia and/or mucous secretion from goblet cells (Bals *et al.*, 2004).

Air-liquid Interface (ALI) cultures of primary human epithelial tissue have been especially useful in understanding cellular mechanisms of cystic fibrosis (CF), allowing for the study of the cellular effects of CFTR mutations or defects in epithelial barrier function (Randell *et al.*, 2011). ALI cultures are also used to study the effects of toxins such as cigarette smoke, environmental particles, or infectious agents on bronchial epithelial cells (Schamberger *et al.*, 2015; Miller and Spence, 2017).

A number of commercial ALI systems have become available in recent years, for example the MucilAir™ human airway epithelia model[4]. These provide a well-characterised 3D model of several pathologies, thus allowing high data reproducibility.

3D cell culture also allows for the co-culture of different cell types along with a synthetic scaffolding and an extracellular matrix (ECM), such as hydrogels, which leads to self-

4 http://www.epithelix.com/products/mucilair

assembly of the tissue into various forms that partially reflect the structure of the lung.

> *Three-dimensional (3D) air-liquid interface (ALI) cultures can recapitulate the pseudostratified mucociliary phenotype observed in vivo*

Spheroids are simple 3D models generated by spontaneous cell aggregation, and do not require any external scaffold, instead relying on cellular contact and spontaneous ECM deposition to bind them together. Cell-only spheroid size is limited due to the absence of vasculature, which also makes long-term culture (beyond days) difficult (Barrila *et al.*, 2010).

Spheroids of different co-cultured cell types can develop physiologic cell-cell contacts and secrete mucins similar to *in vivo* tissues, allowing them to be used to study cell metabolism, necrosis, angiogenesis, and adhesion (Konar *et al.*, 2016). They are widely used for drug screening and also provide a platform for various electrophysiological and immunohistochemical studies (Mueller-Klieser, 1997).

Spheroids also develop similar characteristics to *in vivo* tumors and are widely used in cancer biology to study invasion, metastasis, antineoplastic drugs, radioresponsiveness, and hypoxic effects (Weiswald *et al.*, 2015).

Recent work has seen the development of *in vitro* models of the human lung using embryonic and induced pluripotent stem cells (hPSCs). This provides a unique tool for disease modelling and drug discovery, and show promise for the study of regenerative medicine (Dye *et al.*, 2016).

1.2.2 Ex vivo tissue culture

Ex vivo tissue explant cultures involve the growth of human organ explants on various substrates, which has the advantage of retaining that microarchitecture, functions and molecular interplay of the lung and airways.

A common method is the production of precision cut lung slices (PCLS), which offer a phenotypically-accurate model of lung behaviour. PCLS of defined thickness are sectioned from lung tissues using a vibratome, before being placed in fresh media. The slices can then be stimulated with drugs or pathogens for study of specific characteristics of normal and diseased lungs. PCLS-based lung models have been used to study the effects of allergens, stimulated bronchodilators, smooth muscle contractility, smoking-related toxicology, and the effects of infectious diseases (Sanderson, 2011).

Although explant cultures make it possible to study *in vivo* microenvironments *in vitro*, they are difficult to obtain and optimise. The majority of tissue explants are obtained from thoracic surgery, mostly involving lung carcinoma, but whole lungs rejected for transplantation are also used. Road traffic accidents are the major source of tissue available from asthmatics and these are rarely available. The variability of source organs can create experimental variance, and the handling of organs introduces possibilities for contamination (Sanderson, 2011; Zscheppang *et al.*, 2018).

1.2.3 Organoids

Recent advances in tissue engineering have resulted in the generation of multicellular tissue models, called organoids that can reflect the functional properties of human tissue and organs. An organoid is a minituarised 3D representation of an organ that accurately replicates the histological and functional aspects of *in vivo* tissue (Dye *et al.*, 2015).

Lung organoid culture involves the culture of epithelial stem and progenitor cells extracellular matrix (ECM) which is supplemented with growth factors and/or stromal cells. These organoids then self-organise into complex structures that retain clusters of epithelial cells from multiple lineages. The most common 3D environment used for the formation of organoids are hydrogels, (e.g., Matrigel), that contain mixtures of extracellular matrix components, including laminin and collagen (Nadkarni *et al.*, 2016).

Depending on the origin of the starting cells, organoids can grow into spheres called, for example, bronchospheres, bronchioalveolar spheres or alveolospheres. Lung organoids recapitulate features of the lung including heterogeneous cell composition, spatial organisation, and retention of a stem cell population, giving them the capacity to self-renew and differentiate.

Lung organoids were initially developed using combinations of induced pluripotent stem cells in 3D culture conditions. Subsequently, resident progenitor cells from adult tissues were cultured in supportive 3D systems, and these can also be induced to form organoids (Dye *et al.*, 2016).

Lung organoids represent a physiologically relevant culture system, particularly of the distal (alveolar) lung. Thus they make for highly promising models in basic and translational approaches such as disease modelling and drug screening.

> *Lung organoids represent relevant models in basic and translational approaches such as disease modelling and drug screening*

1.2.4 Lung-on-a-chip

Huh and colleagues (Huh *et al.*, 2010) first designed a multifunctional, microfluidic device to emulate the human alveolar-capillary interface, seeking to reproduce key structural, functional, and mechanical properties of the alveolar-capillary interface in an effort to create a highly relevant model of lung microphysiology.

For the formation of an alveolar-capillary interface, human alveolar epithelial cells and pulmonary microvascular endothelial cells are seeded in the device chambers and allowed to adhere to matrix-coated membrane surfaces. The microchannels are then perfused with culture media to support cell growth. The alveolar epithelial cells are exposed to air, which induces differentiation while being fed from the basal side, leading to the formation of a microengineered barrier tissue composed of the alveolar epithelium and capillary endothelium in close proximity.

The lung-on-a-chip model provides new opportunities to reconstitute organ-level physiological functions that arise from complex interplay between multiple tissue types in the living human lung.

For example, introduction of pro-inflammatory cytokines and bacteria into the upper alveolar compartment induced activation of the endothelial cells on the opposite side of the membrane and their increased expression of adhesion molecules.

The device can also be used in toxicology studies, for example to examine intracellular reactive oxygen species (ROS) in response to alveolar exposure to nanoparticles. Further, it has been shown to effectively model pulmonary edema in human lungs (Huh *et al.*, 2012a).

Further development has led to a "breathing lung-on-a-chip" microdevice which recreates physiological breathing movements by applying a vacuum to the side chambers and causing mechanical stretching of the poly(dimethylsiloxane) membrane forming the alveolar-capillary barrier (Huh, 2015).

> *"The lung-on-a-chip provides new opportunities to reconstitute organ-level physiological functions"*

The development of effective models for respiratory research is vital for better understanding of human disease processes and mechanisms, for revealing possible sensitivities and toxicities and for finding and addressing novel drug targets. Our literature search revealed many salient reviews on this topic, that whilst we could not use them for extracting methodological data about individual models, were still very important for revealing the state-of-the-art. For example, in terms of investigation of lung toxicity, it is widely recognised that the 'traditional' submerged cultures are unsuitable for modelling the impact of an inhaled chemical onto airways epithelium and so models need to employ the air-liquid interface (ALI) platform (Hiemstra *et al.*, 2018; Upadhyay and Palmberg, 2018). Thus, the models that we have selected for future analysis in our collection focus on this set-up.

In summary, the enhanced availability of human cell types, both primary and immortalised cell lines has enabled more labs to use human cells in their research.

1.2.5 *In silico and mathemathical approaches*

Computational modelling of the lung has traditionally focused on representing lung functions, both normally in the healthy state and in the case of diseases, and have mainly focused on the modelling of the deposition of inhaled therapeutics.

The small airways and gas exchange tissue of the lung are relatively inaccessible to measurement and precise dosing with inhaled therapeutics. This has limited the development of new therapies for common lung diseases.

In response to this need, computational models can aid in the development of an overall framework so that its behaviour can be predicted in ways that are beyond direct measurement techniques (Tawhai and Bates, 2015).

Various modelling approaches have been developed that range from continuum models of tissue, airway, or fluid mechanics, to agent-based models of pathogen-host interactions.

For example, bronchoconstriction involves complex interactions among the small airways

that result in regions of poor ventilation (ventilation defects, VDefs) in the lungs. Mathematical modeling of the mechanisms involved in bronchoconstriction is now possible, allowing for insights into the complex airway behaviour that leads to VDefs (Winkler *et al.*, 2015). Computational models are available to assess patient response to acute disease (Burrowes *et al.*, 2011), and these are being expanded towards the development of computational models of patient-specific chronic disease (Clark *et al.*, 2015).

In the area of pulmonary disease research, computational (*in silico*) methods have become more prominent in the investigation of drug deposition and absorption, including the development of physiologically-based pharmacokinetic (PBPK) modelling and quantitative structure activity relationships (QSAR) to aid in the screening of promising drug candidates.

Inhalation is the administration route of choice for the delivery of drugs to treat respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD). Through inhalation, a drug is directly delivered to the airways of the lung, which can result in high pulmonary drug concentrations and low systemic drug concentrations. Therefore, drug inhalation is typically associated with high pulmonary efficacy and minimal systemic side effects (Borghardt *et al.*, 2018).

Due to the complexity of the lung, multiple pharmacokinetic (PK) processes exist that are specific to the pulmonary environment and the inhalation route, making pulmonary PK generally distinct and much more complex than those of drugs administered via other routes (Weber and Hochhaus, 2013).

Edwards *et al.* (2016) have reported on the development of a novel QSAR model (IPRLu) that can accurately predict pulmonary absorption, and act as a potential replacement for isolated perfused respiring rat lung model. With report good consistency against *in vivo* data, it is suggested that the IPRLu model is a suitable tool for investigating lung absorption.

QSAR based approaches have gained momentum in the structure-based drug design and therapeutic management of infections such as *M. Tuberculosis,* involving a combined knowledge of accurate prediction of ligand processes with statistically validated models derived from the 3D-QSAR approach. Such models are particularly used to screen libraries of potential synthetic candidates to identify docking hits for potential drug molecules to address multidrug-resistant tuberculosis. (Ahamad *et al.*, 2017)

The diagnosis and classifications of obstructive lung diseases are primarily based on global lung measurements from pulmonary function tests (PFTs). However PFTs are poor predictors of patient outcomes for individuals. To address these patient-specific issues, medical imaging is increasingly being used in diagnosis, however the small airways are not visible with current imaging techniques. For this reason a combined imaging and modelling approach is needed to obtain information about the respiratory system (Burrowes *et al.*, 2014).

A 5 year European-wide project, AirPROM[5] (Airway Disease Predicting Outcomes through Patient Specific Computational Modelling) ran between 2011 and 2014 with the aim to develop computer and physical models of the whole airway system, in particular for the investigation of asthma and COPD.

The main focus of the modelling within AirPROM was predicting ventilation and the impact of pathophysiological changes on resultant ventilation and lung function, resulting in data and models for both the large and small airways. Software has been developed to enable

5 https://www.europeanlung.org/en/projects-and-research/projects/airprom/home

automatic extraction of the morphological properties of central airways and lobes from patient CT data (Burrowes e*t al.*, 2014).

High-resolution computational meshes of the central airways and lung surface were generated for use in 3D CFD (FRI) simulation studies. 1D airway models were generated down to the gas exchange level using the VFB algorithm. Functional models that predict ventilation and impedance within the 1D networks have been developed. Correlation of model predictions with imaging and measures at the mouth will be used for model validation.

The development of better and more accurate models will assist in better diagnosis of different types of asthma and COPD, allow better monitoring of the disease, provide tools for more targeted research into disease mechanisms, and ultimately assist in the development of personalised medicine and the matching of appropriate treatments to each patient.

The process of modelling requires several simplifying assumptions of the system being modeled to enable feasibility of solution; models will never replicate the complexity of reality. However, a model need only be as complicated as required to answer a specific question.

This will require modellers and clinicians to work ever more closely to achieve the shared goal of patient-specific clinically relevant multiscale models. One of the major hurdles standing in the way of computational modelling and its clinical use is lack of model validation.

A technique has been developed (Pathmanathan and Gray, 2013) to provide a methodical way of validation through quantifying the accuracy and uncertainty in a pulmonary disease model.

2 Methodology

2.1 Definition of models and methods

IIn this project, the term 'method' is used as an umbrella term to describe approaches, models techniques, assays, experimental or computational approaches.

This definition was discussed during the project and the definitions were refined as follows:

- A model (more accurately a 'tool') can be thought of as a 'knowledge object' which would be the pivotal point of a database.

- A method has a purpose and should be more descriptive way to practically enact a model. A method is what you do with your model.

- With the growing emphasis on human relevance, we should talk about the human relevance of a model, not a method.

- A model is used to address a pathology, therefore it is important to keep the disease in mind.

2.2 Selection and exclusion criteria (methods/ publications)

It was agreed that the exclusion criteria should not extend to drug effects demonstrating receptor expression. Studies that might enable the uptake of new *in vitro* methods were retained, so as to ensure a wider uptake of *in vitro* methods, particularly in the preclinical sector.

It was also questioned whether the exclusion criteria should extend of studies examining gene expression in patient populations. It was agreed to exclude these studies if they were not useful or applied for deeper understanding of disease mechanisms, therefore expert judgement should decide on their inclusion.

The agreed list of inclusion and exclusion criteria are presented in Annex - Table 1.

2.3 Format for extraction of models

It was agreed that the extracted data should be expressed in the form of selections from a series of drop-down boxes. This would facilitate easier insertion and management of the final inventory. It is assumed that interested researchers will use more than one data source when conducting their literature searches/proposals. The final list of field definitions is presented in Annex - Table 2.

2.4 Information sources

With regards to primary literature searches, standard multidisciplinary reference databases were defined for the retrieval of studies in the open literature, as well as a number of journals specialising in respiratory diseases, references from which should be adequately captured in the multidisciplinary databases in Annex - Table 3.

A number of relevant organisations were also identified as potential sources of information for the project, encompassing societies involved in research in respiratory diseases, commercial entities developing specific non-animal models for respiratory disease, and organisations providing specialised information on non-animal models in Annex - Table 4.

It was also proposed to contact various research groups, clinical-teaching hospitals and commercial laboratories known to be

active in the field, with request to provide information specific to their areas of research.

It is assumed that there is a significant volume of unpublished or yet-to-be published work going on in the field and it was therefore expected that contacting these relevant research groups would provide a better picture and improve the quality of the deliverables.

A standard letter was prepared and sent to researchers individually by email. Where necessary, further personal contacts were pursued, for example during attendance of the project experts at international conferences.

2.5 Search phrases used

The principal areas of research interest for respiratory disease modelling were defined as investigations of **asthma**, **chronic obstructive pulmonary disease (COPD)**, **cystic fibrosis**, non-specific **fibrosis** and **lung cancer**. These diseases were identified as primary keywords for the pilot searches.

In order to specify for non-animal models, the following modifying keywords were applied.

("*in vitro*" OR "*in silico*" OR "*ex vivo*" OR "3r" OR "non animal" OR "animal alternative*" OR organoid*) AND ("respirat*" OR "lung" OR airway* OR alveol*)).

A conservative (broad) search strategy was chosen in order to aim at retaining high sensitivity rather than optimising specificity, i.e. to minimise the risk of missing potentially relevant literature.

Using these search terms, a series of pilot searches were carried out in the Web of Science (WoS database), with searches limited to 2006–2018 publication years. The outputs of the pilot searches are shown in Annex - Table 5.

Because of the large number of references identified in the pilot searches it was agreed with the project experts to limit the abstract scan initially to non-cancer models to include COPD, asthma, and (cystic) fibrosis. A search for cancer models was subsequently conducted, and the results included here.

Searches were conducted in the databases listed in Annex - Table 6.

2.5.1 Abstract retrieval — Non-cancer references

In total 11,636 unique abstracts were retrieved for the non-cancer endpoint models.

Abstracts from each search were retrieved, collated to remove duplicates, and uploaded to EcoMole's custom reference manager. This is a web-based tool greatly facilitating screening and characterisation of literature. All necessary information (title, abstract, citation) is presented to the reviewer in a structured and well-arranged way, as illustrated Figure 1, which shows screenshots of a single record in the system.

The reference manager allows for various types of statistical analysis of the contained information, and can be easily sorted and filtered according to tags, reviewer, date, etc.

For organisational purposes, full-text references selected for further review were uploaded and appended to each corresponding record in the reference manager.

2.5.2 Abstract retrieval — Cancer references

9,421 cancer abstracts were assessed, covering the past 5 years. Each abstract was reviewed for relevance and were reviewed and tagged with reason for acceptance or rejection (Figure 2).

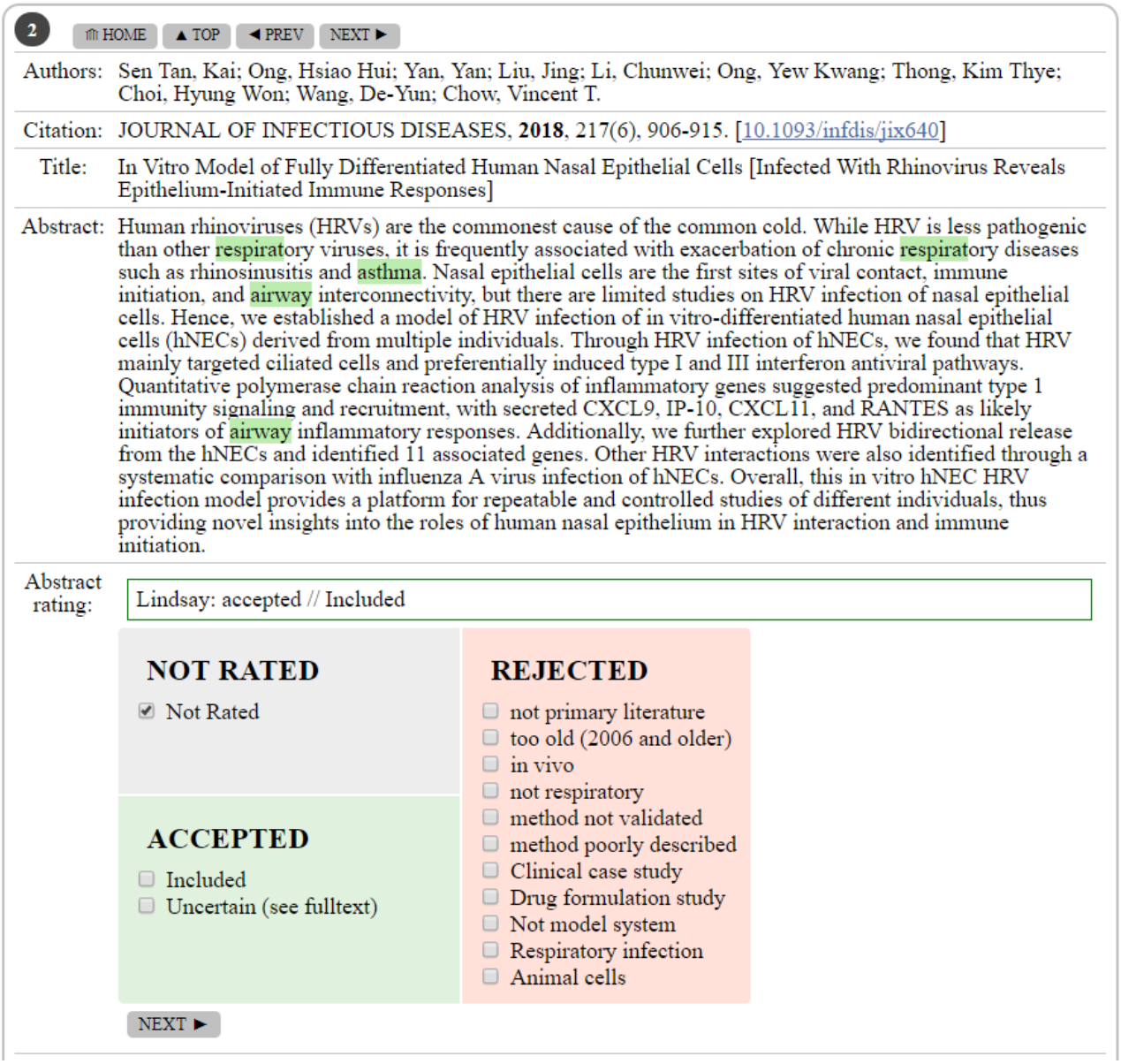


Figure 1: View of single abstract record in reference manager.

The main reasons for rejection of abstracts were that they were not related to respiratory disease, or they described studies *in vivo* or using animal cell/tissue, that they did not describe a model system, or were not from the primary literature. References to non-receptor mediated drug formulation studies, clinical studies and infection studies were also reasons to reject.

After abstract screening, the full-text articles of accepted references were retrieved from subscribed online journal libraries and, similar to attached directly to the relevant abstract record in the reference manager. Thus, all information was collected in an easy to access and well-organised database system.

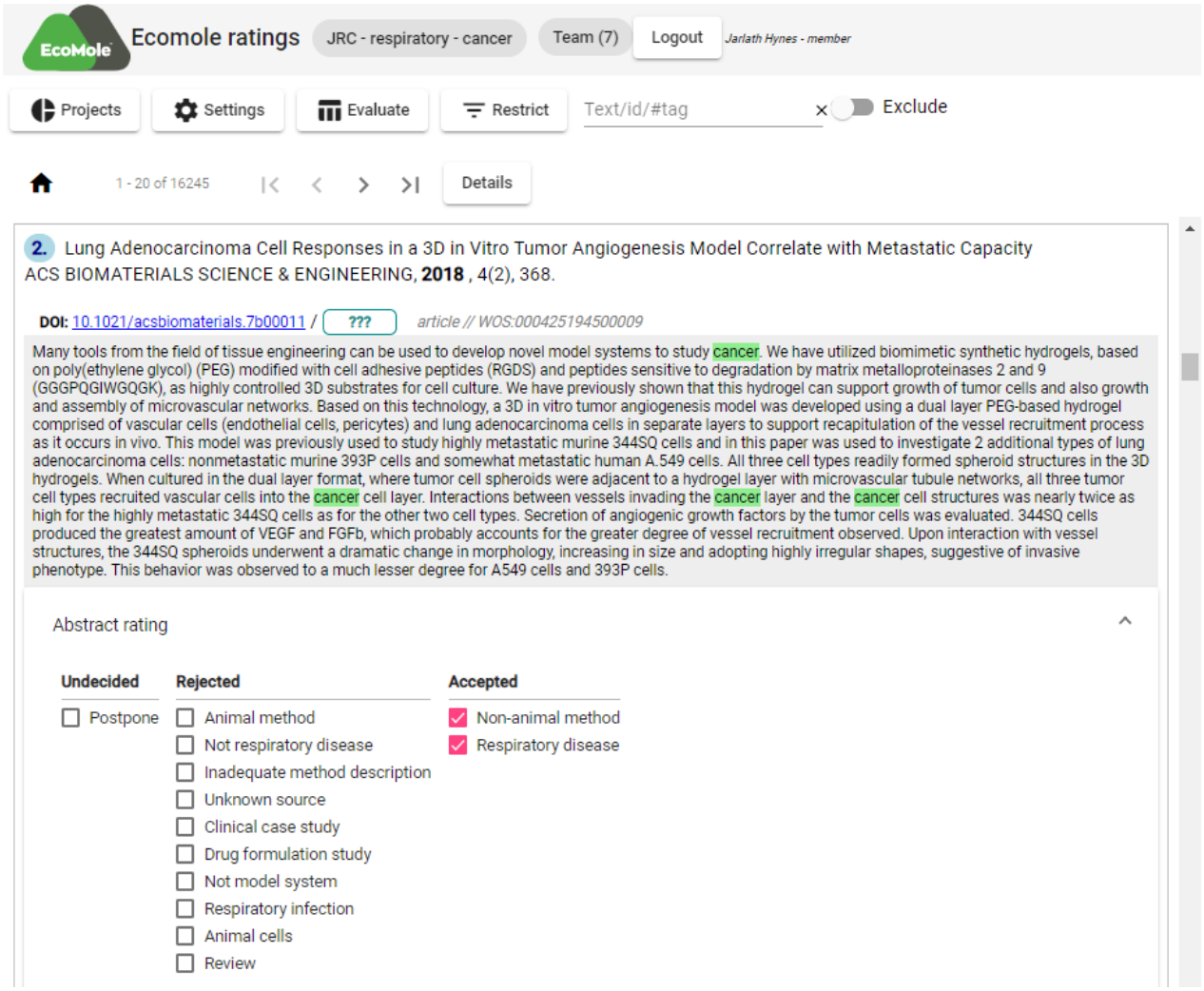


Figure 2: EcoMole reference manager record – abstract view.

3 Results

3.1 Categorised list of models

The full list of models with their biological endpoints studied and measurement techniques/technologies used can be found in the JRC catalogue[6].

When reviewing each full-text paper, a crucial determination was whether the model is directly relevant ('stand-alone') or is supportive of other models according to its context of use. Models that may be used in comparative studies for prediction and/or validation of non-animal models are also described.

When entering the descriptions into the central spreadsheet, the purpose of each method/model was detailed, as were the cell types used and the culture conditions, and the source of the cells.

Using this approach a comprehensive outline of each method/model can be provided, with direct reference back to the original source should more details be needed. Further, it allows for easy filtering/searching and parsing of the data to allow extended functionality of the resulting database. It was envisioned that researchers accessing these data online would be able to capture and compare alternative approaches that use more than one method/assay, and to allow for a meta-analysis to create a more 'holistic' approach.

All 11,636 non-cancer and 9,421 cancer abstracts captured by the search phrases were scanned for relevance using the EcoMole Reference Manager. References that were clearly relevant were marked and tagged (categorised) according to the system outlined above. Wherever there were doubts over the relevance of a reference, it was marked as 'uncertain' and sent for a full-text review.

A total of 464 non-cancer and 315 cancer references were selected from the initial abstract screening and sent for full-text review. References where relevant models were clearly identified from the abstract were marked as 'accepted'. Unless there was a clear reason to reject the reference based on the abstract text (e.g., where the reference is clearly an *in vivo* study) papers were marked as 'uncertain' as to their relevance, indicating that they may not directly fit the inclusion criteria, but nevertheless may contain some useful data.

Full-text papers were retrieved for all abstracts tagged as 'accepted' or 'uncertain', and these were uploaded to the Reference Manager forming the pool of data to be reviewed in the next phase. Each full text paper was attached as a link to the relevant record in the reference manager.

Screening of full text papers involves the sequential opening and review of the attached full-text papers by two reviewers working independently. If the paper contains potentially relevant non-animal model data the details of the model are extracted to a central spreadsheet containing the method/model descriptions using the agreed field definitions list described above (see Section 2.3).

3.2 Analyses of the retrieved data

After all relevant references were reviewed and data-extracted, the identified cancer and non-cancer models were pooled to a single spreadsheet for analysis and the preparation of graphics.

In total, 284 unique methods/models were extracted across cancer and non-cancer

6 https://europa.eu/!Tn39yB

references. The breakdown of models per disease area is presented in Figure 3.

Clearly, lung cancer models dominate, followed by general disease models, which were not focused on a specific disease area, but were more concerned with model development. Models for asthma are well represented in the research literature, followed by models concerned with COPD, cystic fibrosis and pulmonary fibrosis. Toxicity models and 'cigarettes' were differentiated, as the models involving exposure to cigarette smoke model general exacerbations. It is must be noted that infection-based models were also used in exacerbation studies, but in these cases directed at investigating a specific disease such as asthma, COPD and pulmonary fibrosis, and therefore were grouped under these disease areas.

Figure 4 shows the breakdown of models by disease feature. General exploratory models are most prominent and indicate model development activities, applicable across disease areas. These include, for example, the development of microfluidic/chip systems which have a wide applicability in respiratory disease research.

Annex - Table 7 provides an overview of disease features for extracted models mapped against the respective disease area.

For asthma, unsurprisingly, inflammation (n=12) and airway remodelling (n=11) dominate. Models for COPD are more evenly spread, but largely deal with those looking at inflammation, barrier integrity, mucous production and oxidative stress. Models of cystic fibrosis cover a range of features. For pulmonary fibrosis, the key feature investigated is IPF — idiopathic pulmonary fibrosis of unknown aetiology.

Lung cancer models primarily look at the tumour microenvironment and metastasis, but toxicity, cell/tissue viability and drug resistance

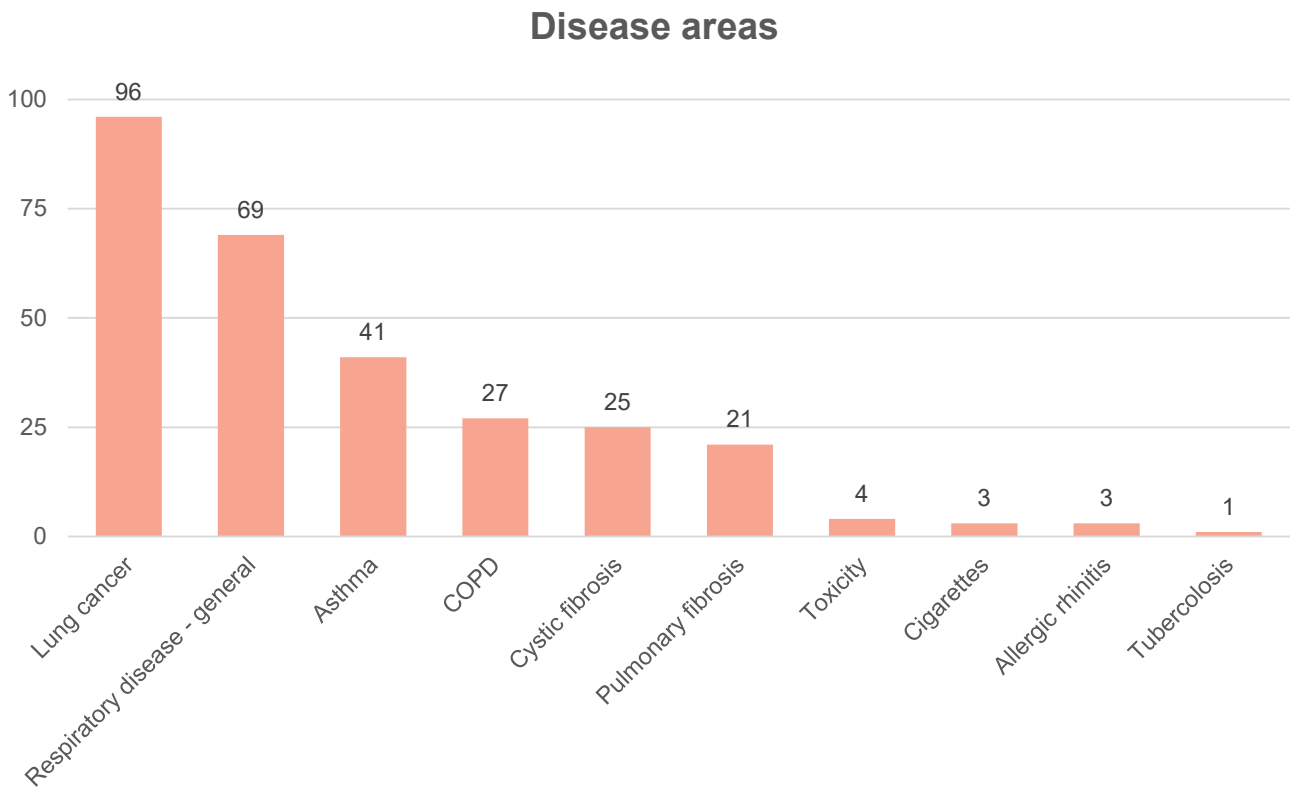


Figure 3: Number of unique non-animal models identified and extracted per disease area.

Figure 4: Number of unique models identified and extracted per disease feature.

studies related to drug development are also prominent.

Models looking at respiratory disease in general are focused in model development leading to wide application across disease areas, however infection models and general models of inflammation are also well represented.

Figure 5 and related Annex - Table 8 show the breakdown of models by category. Cell cultures dominate. 2D and 3D culture and co-cultures were aggregated as it was not always possible to differentiate these by method descriptions. Human-derived primary/stem cells and *ex vivo* human tissues are popular model types. Microfluidic systems / bioreactors are well represented, as are *in silico*/mathematical models. Organoid models are less common than expected, indicating that spheroids are currently more common in cancer research.

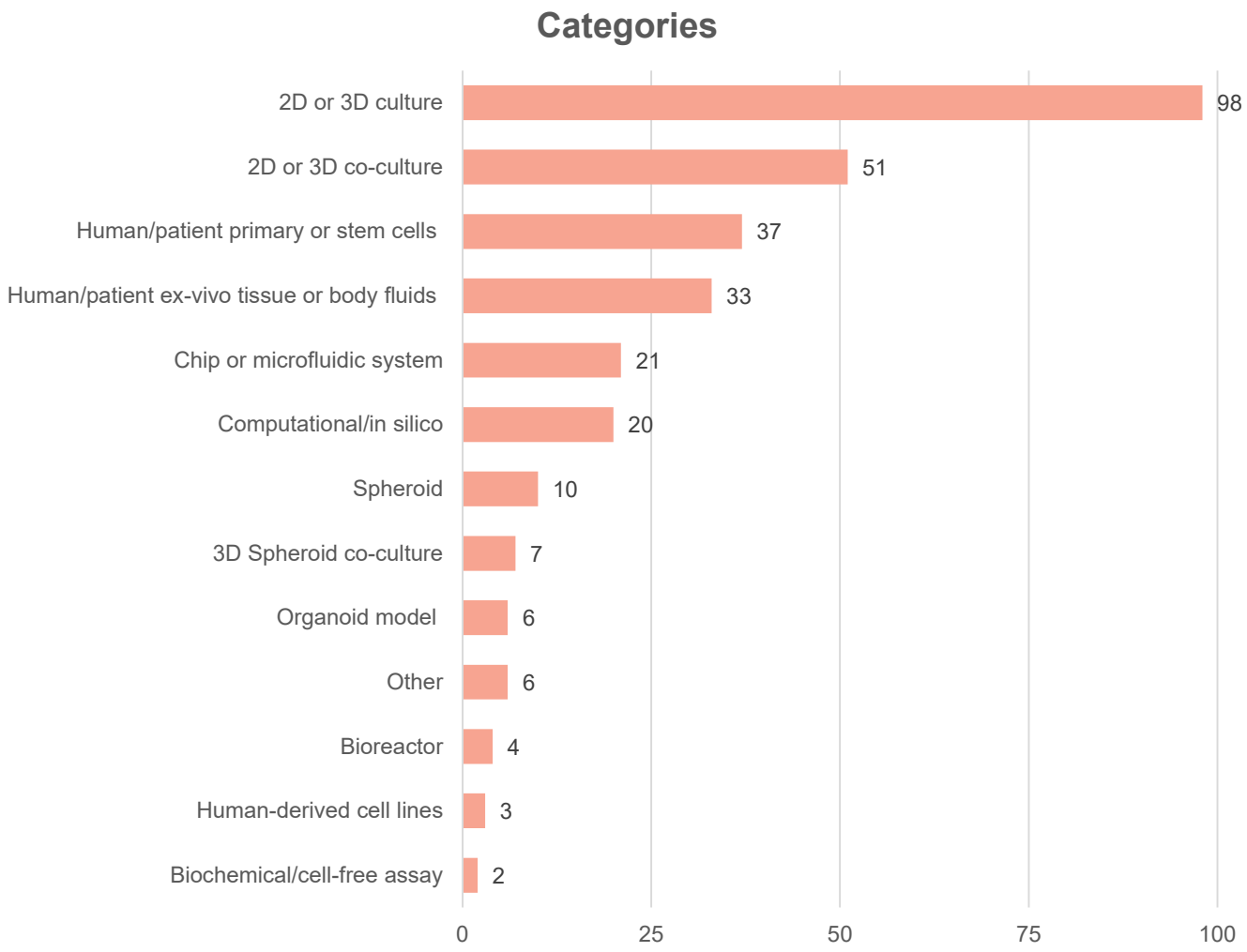


Figure 5: Number of unique models identified and extracted per category.

Figure 6 represents the main cell/tissue types reported. As there are >150 reported types, only those cell/tissue types appearing in 3 or more models were included in this graphic. The full list is provided in Annex - Table 9.

The large number of reported cell types, many of which a duplicate descriptions is, as discussed below, indicative of a lack of descriptive standardisation. Cell and tissue types are duplicated, but it was decided that these should be reported exactly as described in the corresponding literature references.

The A549 epithelia cell line dominates as this is a popular and easily accessible choice for investigating the efficacy and, mainly, toxicity cancer drug candidates. Human bronchial epithelial cells (HBEC) are commonly utilised in models investigating asthma and COPD. 'Other' cell types are notable in cancer models where their identity is not always apparent.

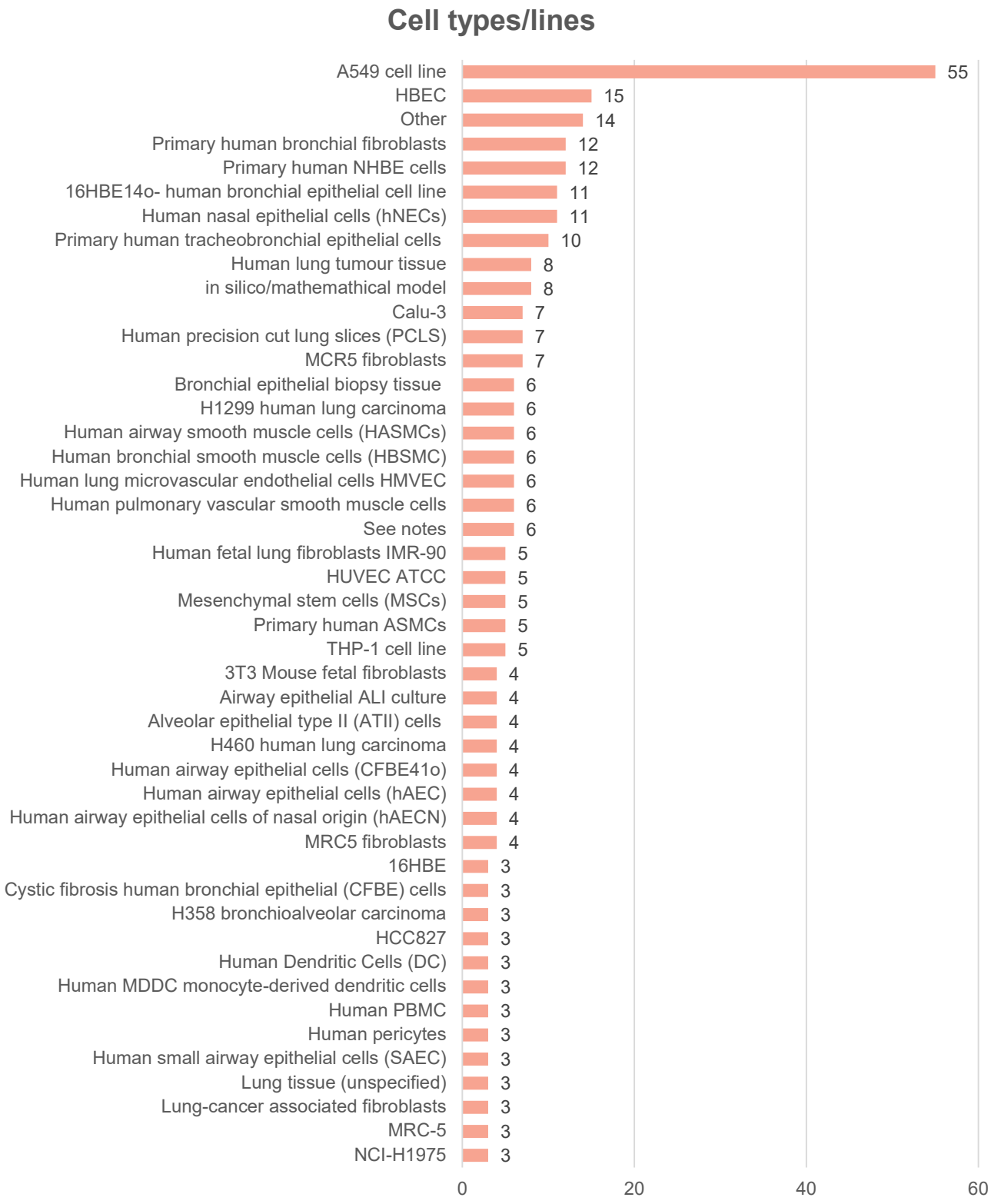


Figure 6: Number of methods per tissue/cell type.

Figure 7 reveals the most common applications of extracted models, with each model mapped against the respective disease areas shown in Annex - Table 10.

Experimental model development leads the list, not just in general respiratory disease research, but also in lung cancer. Drug development and disease therapy are the most common applications in lung cancer research. Asthma models, as well as those for COPD and pulmonary fibrosis are primarily directed at the elucidation of disease mechanisms.

Figure 8 and Annex - Table 11 reveal the biological endpoints investigated, in total and mapped against disease area. Protein / Gene / RNA expressions are the most common endpoints investigated across all models. Cytokine and inflammatory mediator release are characteristic endpoints observed in asthma studies, however again these endpoints are popular markers and confirmatory indicesand outputs in the development of general disease models. Viability, cell migration, gene expression and metabolism are key endpoints used in *in vitro* lung cancer studies.

Key models identified from each disease area are described in Section 4.

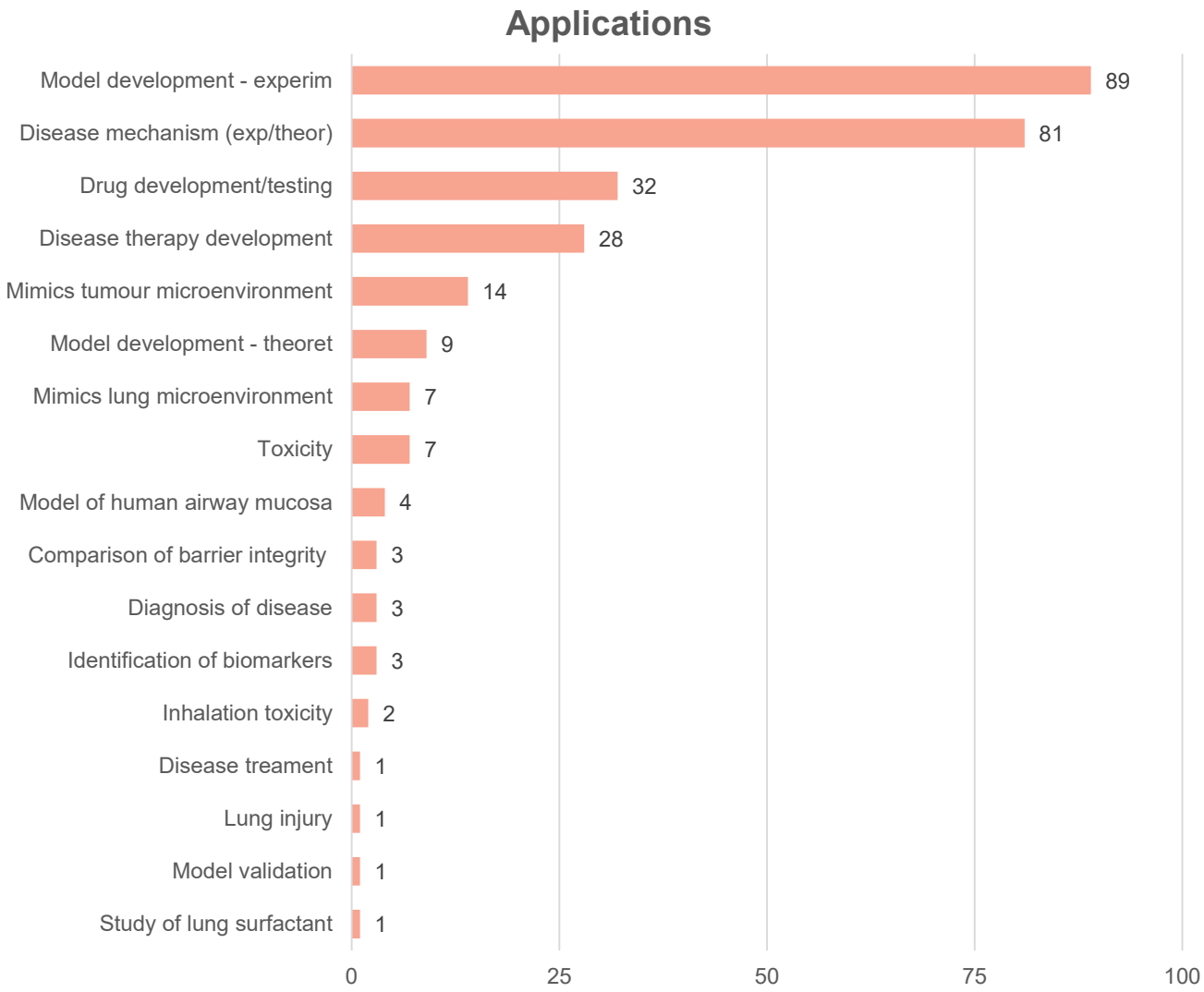


Figure 7: Number of unique models identified and extracted per application.

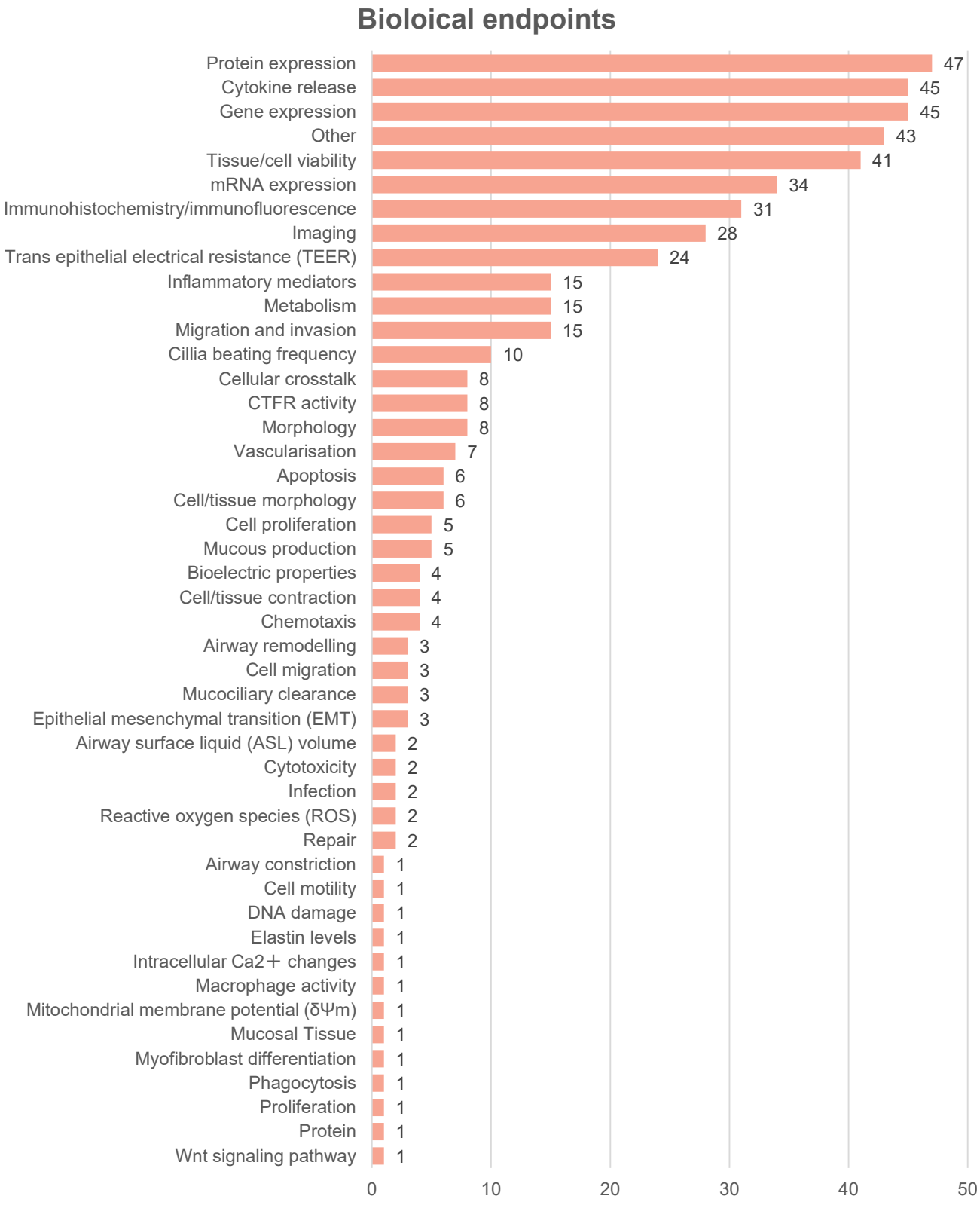
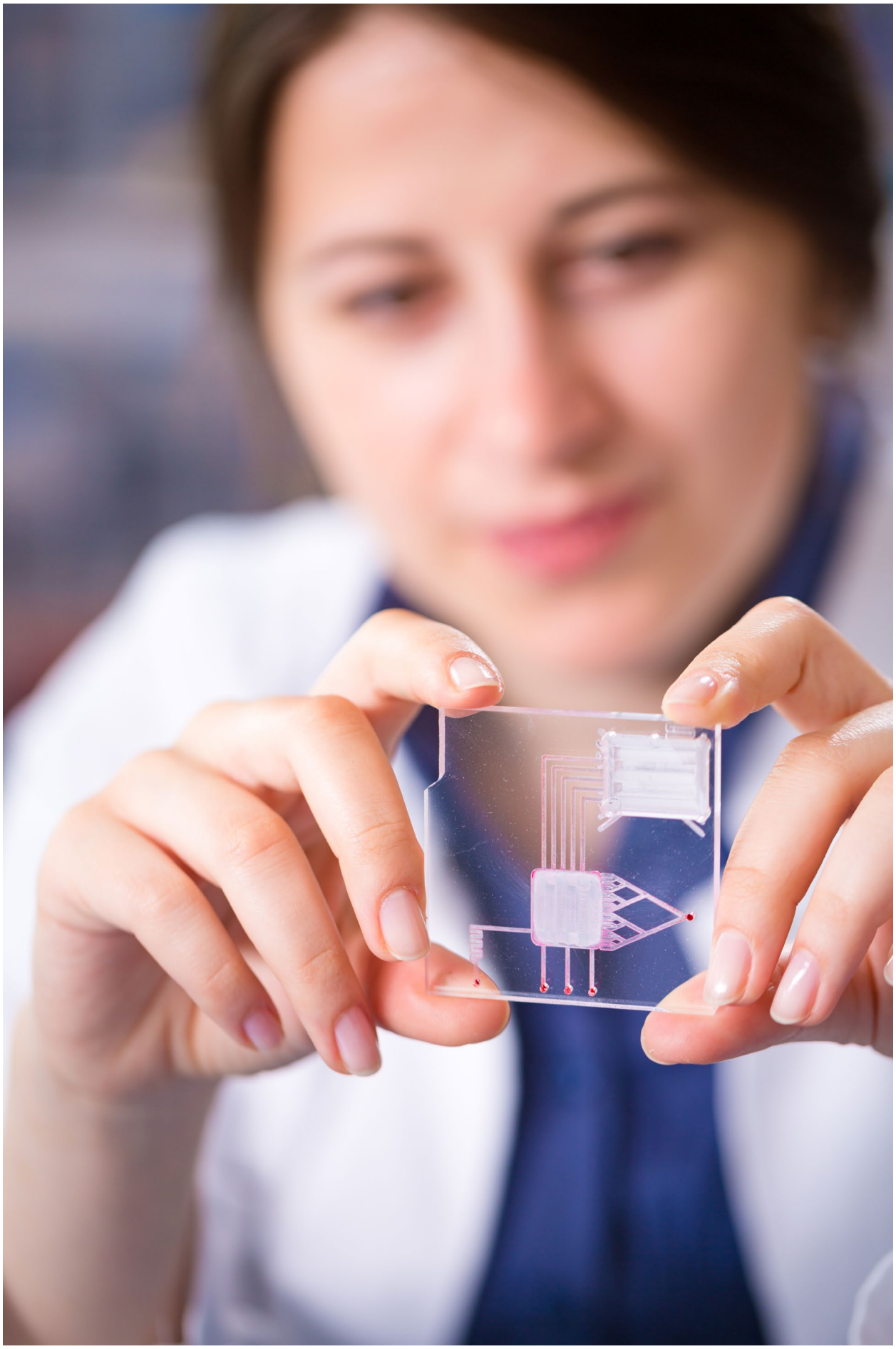


Figure 8: Number of unique models identified and extracted per biological endpoint.



4 Discussion

4.1 General observations on the extracted models

Despite the shortcomings of animal models for disease research, there has been little concerted effort to adopt non-animal approaches to investigate human diseases, in terms of elucidating the mechanism of disease and the development of new therapies. Consequently, there have been no moves to adopt standard methods. Research bodies are free to develop their own protocols and internal standards. Indeed, a major observation of the current project has been that a wide variety of approaches are used to investigate similar disease aspects.

A very obvious manifestation of this is that, of the 284 publications extracted in this current snapshot of non-animal biomedical models, 166 different cell and tissue types have been reported. Although the same cell and tissue types are used across multiple studies, there is no standard use of nomenclature. For example, Human Bronchial Epithelial Cells (HBECs) are variously decribed as HBEC, cHBEC, HBEC-4, saHBEC, dHBE, NHBE, Primary Human NHBE or the immortalised HBEC cell line 16HBE14o, which is also simply called 16HBE, depending on the publication.

The description of methods is also not always consistent, for example details of cells and tissue specifications are not routinely stated, particularly in cancer studies, and sometimes the source of cells is not provided. Nevertheless, the method descriptions in the identified publications are generally sufficient to allow to recreation of the methods if required.

In describing the various methods and underlying models, elucidation of the disease area, features, cell/tissue type, application and biological endpoints was typically straightforward. Many models investigate multiple endpoints, or use multiple cell/tissue types — often in combination as co-cultures or to cross reference and confirm findings.

Determining the potential of a model was a judgement call in the absence of clear potential utility in the field. Those models described as having 'high potential to become a disease model' were those that were well-developed and have a clear potential, for example microfluidic and lung-on-a-chip systems. Some indication of potential can be derived from the citation indices — the number of times a publication has been referenced by others — although older publications tend to bias this ranking. Nevertheless, it can be seen that the most cited recent references are those that describe lung-on-a-chip systems.

With regards to throughput, only 5 models were indicated as 'high throughput', and only when this was clearly stated by the authors. No 'high content' models were detected.

4.2 Main findings

In this study, over 21,000 abstracts were scanned for relevant non-animal model of respiratory disease. From this, a total of 284 models were identified as being promising candidates for application.

It is worth noting that, in this snapshot, models and methods were generally inseparable. The vast majority of publications describe a disease model and a specific method for utilising that model. In only a few cases was the same model subjected to different methods (Hill *et al.,* 2016; Munye *et al.,* 2017; Schleh *et al.,* 2012). This is largely a reflection of the infancy of this field, and the fact that non-animal models have been developed to investigate a very particular disease aspect. Such models — developed to focus on a very specific disease feature —

are generally not 'cross-cutting', i.e. they are not considered useful for the investigation of another disease area.

For example, in cystic fibrosis research, much importance is attached to the cystic fibrosis transmembrane conductanc regulator (CFTR). This is a protein that serves to maintain the balance of salt and water on many surfaces in the body, such as the surface of the lung. When the protein is dysfunctional — caused by a mutation in the CFTR gene — then chloride ions becomes 'trapped' behind the membrane of epithelial cells. The resulting dysregulation of epithelial fluid transport in the lung, pancreas and other organs means that water cannot hydrate the cellular surface, leading to the mucus covering the cells becoming thick and sticky, manifesting as the symptoms associated with cystic fibrosis. Therefore, cystic fibrosis models are usually direct at investigating the mechanisms behind CFTR dysfunction. Such models have limited or no application in other disease areas such as asthma or pulmonary fibrosis, where different disease mechanism are the focus of research.

Models looking at cancer are generally not informed by advances in non-cancer models. It was noteworthy that ALI cultures are used far less frequently than spheroids in cancer research, and that spheroids have not become widespread tools in non-cancer disease areas.

However, a large number of publications (n=69) describe general models, including those in the early stages of model development, with the aim of reproducibility and with potential application across multiple disease areas. For example, finding renewable sources of human epithelial cells, the recapitulation of lung development from human pluripotent stem cells (hPSCs), tissue and organoid models including novel microfluidic devices that mimic the lung microenvironment during homeostasis and disease states, and studies of respiratory absorption. Such models can find wide application in establishing *in vivo*-like conditions which can then be manipulated and studied for applications including drug discovery and development, and achieving a level of patient specificity that contributes to the development of personalised medicines.

4.2.1 Respiratory disease — general

In reviewing the full-text references, a number of models were identified that, while not describing a specific model of a disease, nevertheless represent important baseline, supportive models — including standardised/ commercial — that can be developed for wide utility for investigating respiratory disease *in vitro*.

Firth and colleagues (Firth *et al.*, 2014) describe a method to generate a renewable source of human epithelial cells, including multiciliated cells that can be used to study human respiratory diseases which have previously been difficult to study and model *in vitro*. Human induced pluripotent stem cells (iPSCs) and derived iPSCs provide an unlimited source of cells and also offer the opportunity for gene editing and clonal expansion of cells for disease modelling. There are several lung diseases with a known genetic origin, such as cystic fibrosis and primary ciliary dyskinesia (PCD) that could potentially be corrected by replacement of the defective gene with the correct one by gene-editing technology. It is hoped that, eventually, patient-specific iPSC cells can be used in this model, not only to provide a platform for understanding the cellular and molecular mechanisms of the disease, but to generate a gene-corrected transplantable cell type capable of engraftment into the lung. This paper has been cited 86 times since 2014.

Chen *et al.* (2017) describe the recapitulation of lung development from human pluripotent stem cells (hPSCs) in three dimensions (3D) that can allow deeper insight into human

development, as well as the development of innovative strategies for disease modelling, drug discovery and regenerative medicine. It involves the generation of lung bud organoids (LBOs) from hPSCs. These organoids contain mesoderm and pulmonary endoderm and develop into early alveolar structures after xenotransplantation, and in Matrigel 3D culture. It was shown that LBOs recapitulate lung development and may therefore provide a method to model lung diseases. Expression analysis and structural features indicate that the branching structures can reach the second trimester of human gestation. Infection *in vitro* with respiratory syncytial virus led to swelling and the shedding of infected cells into the organoid lumens. A similar outcome has been observed in human lungs. Introduction of mutations in HPS1, which causes an early-onset form pulmonary fibrosis, leads to accumulation of extracellular matrix and mesenchymal cells, suggesting the potential use of this model for the study of fibrotic lung disease *in vitro*.

McCauley *et al.* (2017) describe the directed differentiation of pluripotent stem cells (PSCs) into functional airway epithelial cells in response to cyclical modulation of developmental signaling pathways. The method was used to generate cystic fibrosis patient-specific iPSC-derived airway organoids showing a defect in forskolin-induced swelling that is rescued by gene editing to correct the disease mutation. These findings demonstrated the rapid generation of airway organoids by stage-dependent modulation of Wnt signalling, and provide proof-of-principle for the utility of these organoids in lung disease modelling.

Tseng *et al.* (2013) have detailed the assembly of a three-dimensional multitype bronchiole coculture model using magnetic levitation in conjunction with magnetic nanoparticles as a means of creating an organised three-dimensional (3D) coculture. The 3D coculture model is assembled from endothelial cells, smooth muscle cells (SMCs), fibroblasts, and epithelial cells (EpiCs). However, it was noted that this group does not appear to have taken the models any further than these initial, proof-of-concept studies and speculate that the development of methods such as 3D bioprinting may have overtaken magnetic levitation in terms of ease and accessibility.

Pageau *et al.* (2011) using a 3D culture model, discovered that elements of the stroma affected the phenotype of HBECs when cultured at an ALI. Concentration of type-I collagen matrix determined the thickness of the overlying epithelium, and incorporation of fibroblasts in the model altered the epithelial phenotype; such that fetal fibroblasts promoted the development of typical epithelium but lung cancer-derived fibroblasts promoted more invasive behaviour. The authors suggested that the effect could be mediated through transduction of biomechanical signals from the ECM, or through soluble factors secreted by the fibroblasts. This study showed the importance of the stroma in determining normal and pathological conditions, and provides further guidance to investigators pursuing 3D culture methods as a means of studying these events *in vitro*.

Wilkinson *et al.* (2016) developed a reproducible model system to integrate multiple human cell types, including iPSC-derived cells, to their correct anatomical location to form lung tissue that can be used to model lung diseases and perform high throughput drug screening for precision medicine. This approach uses bioreactor-assisted self-assembly to reproduce the anatomy of distal lung alveolar sacs by scaffolding mesenchymal cells into the interstitial spaces between closely packed, biocompatible hydrogel beads in order to generate pulmonary-like tissues ready for disease modelling. As this method is a bottom-up synthesis, it is possible to control hydrogel bead composition, size, stiffness, and functionalisation as well as number and type(s) of cells included. The method can be easily scaled in both size and number of organoids, potentially bridging the gap between disease

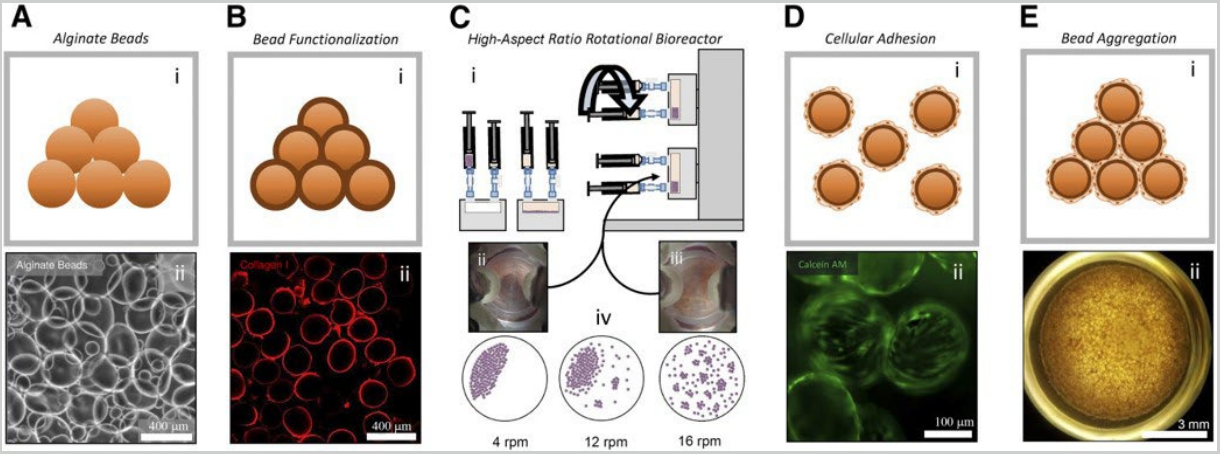
Personalised disease modelling through iPSC-derived mesenchymal cells grown into organoids

There are currently several methods to develop tissue-engineered organoids that replicate the organ's functionality. An important advantage is their potential for high throughput drug screening to identify targeted therapies

Wilkinson *et al.* (2016) generated human lung tissue containing self-assembled multiple lung cell types that allow cell-cell contact and recapitulation of the lung microenvironment.

The organoids formed through the agglomeration of cell-coated alginate beads either in a bioreactor or in a 96-well plate format and remained viable for 2 weeks without degradation.

This system has been found suitable with any cell types including iPSC-derived mesenchymal cells offering a great potential for use in personalised medicine or disease modelling and drug discovery.



Generation and characterisation of 3D pulmonary organoids.

modelling and the generation of transplant-ready tissues.

Morris *et al.* (2014) have described the development of a single scaffold comprising of two distinct topographies that recapitulate the different microenvironments that airway epithelial and fibroblast cells encounter in situ. This method shows how matrix development, when sympathetic to cellular requirements, can provide a relevant 3D *in vitro* platform to investigate important multicellular interactions that occur within the airway wall. The model cocultures MRC5 fibroblasts and CALU3 epithelial cells, with proven fibroblast penetration of the microfibre scaffold.

Punde *et al.* (2014) describe a two-layered microfluidic model of protein-induced lung inflammation, designed to emphasise the role of eosinophil cationic protein (ECP) in inflammation, and to demonstrate a dynamic migration tool which mimics the physiological flow conditions in the body. ECP plays a major role in attracting fibrocytes from blood vessels to airways. A 'lung-on-a-chip' microdevice, it mimics the lung microenvironment, providing a platform for perfusion culture, with potential for use in preclinical studies.

Reus *et al.* (2014) have demonstrated the feasibility of a commercial 3D human airway epithelial model (bronchial epithelial model) to study respiratory absorption, in particular to differentiate between low and high absorption of substances. This bronchial epithelial model was the commercially available MucilAir™ system and can differentiate between

substances with low and high absorption and could be used to predict respiratory transport for both safety and efficacy assessment.

Vicens-Zygmunt *et al.* (2015) describe a model that may be useful for investigating cell behaviour and phenotypic changes that occur during the processes of lung fibrosis and aging, as well as for testing different anti-fibrotic therapies *in vitro*. Human lung fibroblasts are able to grow into 3D collagenated and stiffened matrices under specific conditions. Increasing ECM cross-linking via non-enzymatic glycation at low ribose concentrations induces phenotypic and metabolic changes in the fibroblasts, resulting in a mechanical and oxidative modified environment which could resemble an aging/fibrotic ECM.

Huang *et al.* (2017) report on the establishment and characterisation of SmallAir™, a commercial *in vitro* human small airway model using primary small airways human cells. SmallAir™ epithelium is tight, with a transepithelial electric resistance (TEER) value close to that of upper airway epithelia, and is therefore ideal for studying permeation and drug delivery. This is a powerful tool for studying the physiology and function of small airways.

Ong *et al.* (2016) have demonstrated the feasibility of generating cultures of primary human nasal epithelia resembling *in vivo* epithelium physiology, with highly differentiated epithelial cells after 5 weeks in culture at the air-liquid interface. The development of functional cilia, mucus secretion by goblet cells, ability to induce inflammation and viable tight junctions represent a promising *in vitro* model for nasal drug delivery studies and provides a unique opportunity to study disease specific physiological differences of the airway epithelium. This model may also form the basis for the development of more complex systems of *in vitro* bacterial or viral cell infections and inflammation.

Bucchieri *et al.* (2017) report on the development of a robust bronchial mucosal model (the epithelial mesenchymal trophic unit or EMTU) where cellular outgrowth from bronchial tissue might enable development of a mucosal structure that recapitulates *in vivo* tissue architecture. Bronchial biopsies taken from subjects without lung disease were cultured in matrigel until a differentiated, multi-layered structure was formed. Structural changes observed following cigarette smoke extract (CSE) exposure suggest the model could be used for drug discovery and preclinical testing, especially those targeting airway remodelling.

4.2.2 Lung cancer

Scrutiny of the lung cancer database provided several important insights — the rejection of almost 95% (n=9,421) of all abstracts retrieved indicates the breadth of topic for cancer and difficulty in creating precise search terms that will only retrieve appropriate non-animal models with confidence of not missing anything.

Almost half of the rejected abstracts were categorised as 'not a model system', and of these, 15% were clinical studies that analysed patient samples for RNA or gene expression, monitored survival according to gene expression, or related protein expression to prognosis. 3,876 abstracts were rejected under the category 'not a model' and of these, 1,099 employed the A549 cell line. However, despite the relevance of this cell line, and its use in 30% of the accepted abstracts, there were many examples of where the use of A549 cells could not be classified as a model system. This included examples where A549 cells were used as one of a panel of cancer cell lines, in a test platform for a variety of potentially toxic agents, with no differentiation of culture techniques for the different cell types, or attempts to replicate the relevant microenvironments; or using A549 cells for assessment of different delivery vehicles like nanoparticles. It was felt

that the use of A549 under these conditions — cultured under similar conditions to other non-respiratory cell lines and used for a straightforward readout of toxicity, was not taking account of the physiological nature of the cells and could therefor not be classified as a model of lung disease.

There was evidence of the application of *in silico* approaches in the lung cancer field, with over 600 references referring to *in silico* or computational/mathemathical approaches. However, only 10 of these could be accepted for full text extraction. Reviewing the rejected abstracts indicated that the majority of these papers were using computational methods to examine docking between a possible drug and target, or to carry out drug screening studies that were not specific for lung cancer, and therefore were not included.

We noted that many rejections were due to the use of the patient-derived xenografts, where human tumour tissue is implanted into an animal in order to test different chemotherapy. This approach has been discredited recently (Ben-David *et al.*, 2017) as alterations in the tumours as they progress *in vivo* removes any resemblance to the patient's disease. However, our search returned almost 2,000 references with patient-derived xenograft in the title or abstract, of which we rejected all but 6.

Despite the continued animal focus in cancer research, it would appear that the field has benefited from previous work in the development of respiratory models in general, and that researchers are using these advances to create more sophisticated cancer models. Our analysis revealed eleven robust models with high potential, of which two were *in silico*/ computational models. Although we might have expected more good cancer models, it does seem that the advent of microfluidics, 3-dimensional culture and other approaches that are dominating the non-cancer field have transferred into cancer research, providing these models with an immediate advantage.

Our analysis revealed eleven robust models with high potential, of which two were in silico/ computational models

Robertson-Tessi *et al.* (2015) have presented a mathematical model of the impact of immune cells on tumour cell growth. The model derives from experimental and clinical data and incorporates several different types of T cell, antigen presenting cells as well as cytokines. It uses antigenicity and growth rate to characterise individual tumours and reveals the impact of the adaptive immune response on the control of tumour growth, offering insights into opportunities for personalised medicine.

Another model (Chen *et al.*, 2018) incorporated many salient features of cancer — including uncontrolled growth, hypoxia and treatment resistance to estimate the effective ability of various chemotherapy agents, with a view to providing a personalised therapy. This study used retrospective analysis of data from thirteen people with lung cancer and so it seems that a possible progression of this mechanistic approach would be to expand to offer more effective treatment options for people with cancer, based on their specific tumour characteristics.

The remaining methods were all *in vitro* and used a variety of cell types and categories, according to our classification. Four of these models considered tumour microenvironment and, interestingly given the method developments apparent in the respiratory disease database and in disease modelling as

whole, there was only one model that used the microfluidic or chip platform. It is also interesting to note that the use of spheroid cultures appears to be more common for cancer models, with 19 confirmed references compared to just 6 for non-cancer database. Application of spheroid cultures for cancer research have been known for over 30 years (Sutherland and Durand, 1984) and this platform is particularly relevant for cancer because these 3D models exhibit physiologically relevant cell-cell and cell-matrix interactions, gene expression and the structural complexity that reflect the tumour.

It is worth pointing out the commercial availability of a lung cancer model — the OncoCilAir™ from Epithelix — featured in only 5 abstract hits in the database, despite originating in 2015. The advantage of this would be the reproducibility that is implied with a commercial model and which is of utmost importance to deal with emerging issues with *in vitro* research beyond those that can be addressed with good cell culture practice (Pamies, 2018). We categorised one of the OncoCilAir™ papers (Benainous *et al.*, 2018) as reflective of a good model. This model combines different cell types, using human primary cells and exploits culture at the air-liquid interface to generate a physiologically accurate model of the airways, with incorporation of adenocarcinoma cells to enable tumour formation. Analysis revealed that the model recapitulates the invasive nature of tumour cells into surrounding tissue and that this potentially provides a useful and relevant platform for drug screening. We would have expected to see more broad use of this model, and can only speculate that the comparative costs of buying in ready-made cultures like this are preventing its use more widely.

It was felt that one of the 'good' *in vitro* models was particularly suitable for disease development and the remainder were more appropriate for drug discovery. This model used a human lung carcinoma cell line cultured with cytokine-induced killer cells derived from human peripheral blood monocytes (Chen *et al.*, 2018). Interestingly, this paper also included an *in vivo* element that showed that the use of cytokine-induced killer cells, which were effective at reducing tumour *in vitro*, also reduced tumour volume in mouse models.

Other important cancer models (Hassell *et al.*, 2017; Wu *et al.*, 2018; Wang *et al.*, 2018; Yang *et al.*, 2018; Alonso-Nocelo *et al.*, 2016; Solomon *et al.*, 2016; Yamazoe *et al.*, 2016) were all suited to drug screening and discovery through mimicking of tumour microenvironments. As expected, tumours are not a 'normal' tissue and thus capturing the hypoxic, proliferative, pro-angiogenic environment is of the greatest importance for modelling cancer. It would appear that, despite broad parallels with methodological approaches for both cancer and non-cancer models (air-liquid interface, co-culture, etc.), the significant differences in the cell types required to recapitulate the disease phenotype (T cells, antigen presenting cells, carcinoma cells) and the specifics needed to recapitulate the tumour microenvironment prevent any significant cross-over between the two.

Correia *et al.* (2017) described a model of bronchial dysplasia, the precursor lesion to squamous lung cancer. Earliest stages of bronchial epithelial carcinogenesis were modelled using using nonvirally immortalised human bronchial epithelial cells with a stable karyotype and an intact p53 signalling response/pathway. The model recapitulates human bronchial dysplasia *in vitro*. SOX2 deregulation drives dysplasia, and loss of TP53 is a co-operating genetic event that potentiates the dysplastic phenotype. Deregulated SOX2 alters critical genes implicated in hallmarks of cancer progression. Targeted inhibition of AKT prevents the initiation of the dysplastic phenotype.

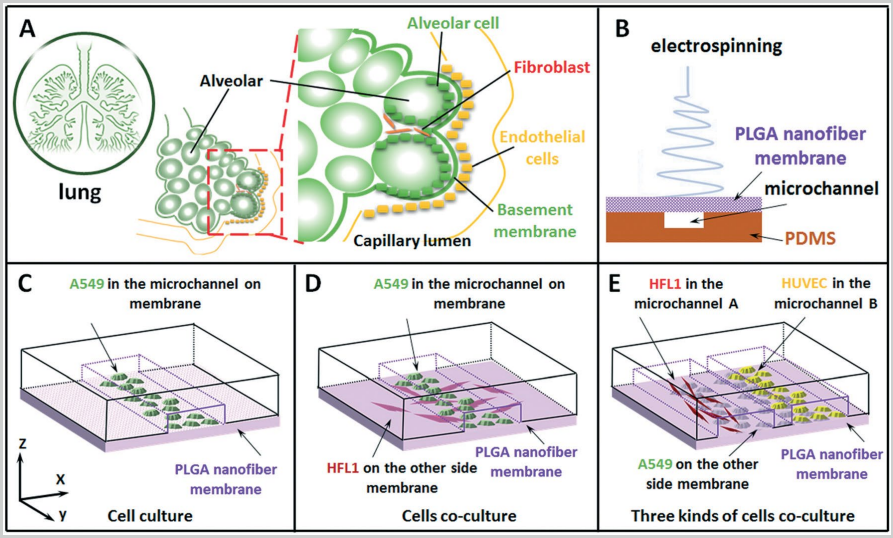
Yang *et al.* (2018) have developed an electrospinning nanofiber membrane-supported lung-on-a-chip microdevice for anti-cancer drug testing taking the alveolar microenvironment as a model. Gefitinib, an

Lung-on-chip to simulate tumour microenvironment

Microfluidic chip technology can be used to simulate the physiological and pathological conditions of human tissue and organs.

Yang *et al.* (2018) developed a simple lung tumor-on-a-chip with a an electrospinning nanofiber membrane as a substrate of the chip and the cell 3D culture scaffold. This model simulates the alveolar microenvironment and the tumour microenvironment alveolar biochemical factors, especially in the respiratory membrane.

On this chip, they grown 3D cell culture and co-culture of the human lung non-small cell lung cancer cell line A549 and human fetal lung fibroblast HFL1 cells, and tested the effects of the gefitinib, an EGFR-targeted anti-tumor drug



epidermal growth factor receptor (EGFR)-targeted anti-tumour drug, was tested in on-chip cell culture and co-culture of human non-small cell lung cancer cells (A549) and human fetal lung fibroblasts (HFL1). This simple, effective, and easy to operate system is expected to find important applications in personalised treatment of lung tumors and to play a potential role in other clinical treatments and tissue engineering.

Han and Hsu (2016) co-cultured mesenchymal stem cells (MSCs) and human non-small cell lung carcinoma cells (A549) on a thin biomaterial-based substratum (hyaluronan-grafted chitosan, CS-HA; similar to 2 μm), which self-organised into the 3D tumour co-spheroids with core-shell structure. Tumorigenicity observed in a zebra fish xenotransplantation model was consistent with that observed *in vitro*, suggesting that this 3D co-culture platform for cancer cells and MSCs may be a convenient tool for studying the cell-cell interaction in a tumour-like microenvironment and potentially for cancer drug testing.

4.2.3 Asthma

3,883 abstracts containing the keyword asthma were reviewed. Of these, 157 were selected for full-text review. The main reasons for rejection of asthma abstracts were that they described studies *in vivo* or using animal cell/tissue, that they did not describe a model system, or were not primary literature. After full-text review, 41 distinct references describing non-animal models of asthma remained.

The most important models identified include that of Chin *et al.* (2012) who describe the modelling of the mechanical properties of asthmatic airway smooth muscle using human ASM strips to study force induced by electric field stimulation (EFS) in situ. Mechanical properties of human ASM from asthmatic and nonasthmatic subjects were shown to be comparable allowing the modelling of bronchodilation in asthmatic subjects.

Freishtat *et al.* (2011) have developed a proposed model for airway epithelial-derived inflammation in asthma; an adapted *in vitro* epithelial injury model that allows for the study of epithelial repair processes in the lung. Normal and asthmatic primary differentiated human airway (i.e., bronchial) epithelia can be grown in 12-well plates on collagen-coated Transwell membrane inserts at an air-liquid interface. This allows for the measurement of inflammatory (i.e., IL-1b, IL-6, IL-10, and IL-13) and fibrogenic (i.e., transforming growth factor [TGF]-b1) cytokines in apical and basolateral secretions by flow cytometry. These cytokines have a prominent role in asthmatic inflammation and remodelling. The cultures were wounded by scraping and cytokine release and regeneration was assessed for asthmatic and non-asthmatic cultures. The asthmatic epithelial models were less able to regenerate and secreted more pro-inflammatory and fibrogenic mediators. This model demonstrated the role of the epithelium in the asthmatic phenotype and showed the positive effects of glucocorticoid treatment indicating possible utility for drug screening.

Hackett *et al.* (2011) showed that ALI cultures can be used to investigate and compare many of the phenotypic differences within the airway epithelium of asthmatic and non-asthmatic patients. In this model, asthmatic-derived ALI cultures respond with an increased expression of inflammatory mediators IL-6, IL-8, and GM-CSF to both environmental (infection with RSV, exposure to air pollution) and nonspecific mechanical damage, compared with nonasthmatic ALI cultures.

Hill *et al.* (2014) developed a theoretical biochemical/cell-free assay of mucus solids concentration as a potential biomarker for airways disease. Reference ranges for mucus solids concentration in healthy and disease states were derived from clinically-collected sputum samples, and mucus harvested from primary human bronchial epithelial (HBE) cell cultures. Analysis of the datasets provided a comprehensive assessment of barrier (diffusivity) and transport (viscoelasticity) functions of HBE mucus versus concentration. It was shown that clearance and barrier functions of mucus scale with mucus solids, providing a theoretical and practical basis for the use of mucus solids concentration as a clinical marker for phenotyping subjects with airway disease, and for the outcomes of clinical trials.

Gras *et al.* (2012) reported the development of an *ex vivo* model of severe asthma using reconstituted human bronchial epithelium. Human bronchial epithelial cells (HBEC) derived from endobronchial biopsy specimens of patients with mild and severe asthma maintained in culture in an air-liquid interface can reproduce a fully differentiated airway epithelium. This facilitates the practical study of features of airway remodelling, epithelial and subepithelial layers, as well as mucus production. Resulting differentiated epithelia could then be analysed for morphology and function based on ultrastructural analysis, IL-8 release, lipoxin A(4) generation, mucin production, and lipoxygenase gene expression.

Nesmith *et al.* (2014) have developed a model of human airway musculature on a chip, presenting an *in vitro* model of allergic asthmatic bronchoconstriction and bronchodilation. This system can mimic the structural architecture and functional hallmarks of contraction of BSM on a chip, which can be used as a practical model of responses to novel drug treatments.

4.2.4 Chronic obstructive pulmonary disease (COPD)

A total of 1,359 abstracts containing the keyword COPD were scanned for relevance, with 97 references marked for full-text review. After full-text review, 27 distinct references describing non-animal models of COPD remained. Rejection of abstracts was on the same basis as described above (*in vivo* studies, use of animal tissues/cells, etc.)

Key models identified include:

- Sul *et al.* (2018) developed computational fluid dynamics (CFD) model of airflows for steady expiration to investigate how terminal flows affect airflow patterns in respiratory airways healthy and diseased lung.
- Gohy *et al.* (2015) showed that epithelial-mesenchymal transition (EMT)-related de-differentiation of the epithelium occurs in COPD conducting airways and correlates with peribronchial fibrosis and with airflow limitation. These changes are recapitulated *in vitro*, during ALI-driven re-differentiation of the epithelium, at least in part as a consequence of TGF-β signalling. EMT therefore is an important component of airway disease in COPD and targeting it could reveal an attractive therapeutic strategy to restore epithelial barrier and integrity.

Wagner *et al.* (2014) demonstrated that cadaveric lungs from individuals with chronic lung diseases such as COPD can be appropriately decellularised and recellularised to retain characteristic histological architecture and ECM reflecting either normal or COPD, particularly emphysematous, origin. Inoculation of human bronchial epithelial cells, endothelial progenitor cells, bone marrow-derived mesenchymal stem cells, and lung fibroblasts via airway or vascular routes into small, excised segments of the decellularised lungs demonstrated that normal lung scaffolds robustly supported initial engraftment and growth of each cell type for up to one month. In contrast, despite initial binding, all cell types inoculated into decellularised emphysematous lungs did not survive beyond one week.

4.2.5 Pulmonary fibrosis

A total of 1,093 abstracts containing the keyword 'pulmonary fibrosis' were scanned for relevance, with 46 references marked for full-text review. After full-text review, 21 references describing non-animal models remained. A number of key models were identified.

He *et al.* (2017) have developed a microfluidic co-culture device as a human disease model of pulmonary fibrosis. The device can monitor the fibrotic process by looking at epithelial-interstitial cell communication. Epithelial cells, fibroblasts, and macrophages can be cultured in parallel channels to mimic the *in vivo* epithelial-interstitial tissue interface of the lung. Epithelial cells formed an intact monolayer lining the fibronectin-coated channel, representing the alveolar space and alveolar wall. Culture medium treated with BLM is introduced to induce injury and stimulate paracrine communication between the cell types. The epithelium sends paracrine signals to activate interstitial cells, resulting in the migration of fibroblast and inflammatory cells with a subsequent increase in collagen I deposition at the ECM. The study confirms that epithelial injury is a source of epithelial/fibroblastic cross-talk during BLM-induced fibrosis, indicating that this co-culture system recapitulates the critical pathological changes characteristic of pulmonary fibrosis.

Mikami *et al.* (2016) report on the development of the gel contraction assay as an *in vitro* model of contractility, which is a characteristic function of fibroblasts that contributes to wound repair and fibrosis. Cultured A549 human lung epithelial cells were induced to epithelial-mesenchymal transition (EMT) by TNF-α and TGF-β1. Type I collagen gels containing mesenchymal cells that underwent EMT were reduced in size, indicating contraction of those cells. It was thus suggested that the

gel contraction assay should be considered as an extended *in vitro* assay to evaluate contractility, as mesenchymal cells that underwent EMT are thought to contribute to the pathogenesis of fibrosis.

Rajangam *et al.* (2016) claimed to have developed the first biomimetic 3D *in vitro* fibrosis model. Three-dimensional cell masses (3DCMs) of human adipose-derived stem cells (hASCs) were shown to be transformed into activated myofibroblasts by growing them on a MBP-FGF2-immobilised substrate. The hypoxic microenvironment created in the interior of the 3D cell masses during the early stages of culture leads to activation and synthesis of TGF-β1. The gene expression of fibrosis-related molecules (TGF-β1, a-smooth muscle actin (aSMA), and collagen type I) were shown to be upregulated in the cell masses. Over time, overexpression of TGF-β1 led to differentiation of hASCs into activated myofibroblasts, which deposit excessive collagen type I and are characterised by aSMA expression. This model might therefore be used to study the common mechanism of fibrosis, which could then be targeted for the development of broad range antifibrotic compounds.

Roach *et al.* (2018) present an *ex vivo* human lung parenchymal model of fibrogenesis which recapitulates the pro-fibrotic events evident in idiopathic pulmonary fibrosis (IPF). 2mm^3 sections of human lung parenchyma were cultured for in medium containing TGFβ1. After 7 days a fibrosis-associated increased expression of ECM proteins (Collagens I and III), and fibroblast-specifc protein were demonstrated by immunohistochemical staining. The involvement of KCa3.1 ion channels — which play a key role in TGFβ1-dependent pro-fbrotic responses in human lung myofbroblasts — was demonstrated by the addition of senicapoc, a selective KCa3.1 blocker.

The authors contend that this model has potential to simplify and accelerate the evaluation of therapeutic targets for human lung fibrosis, and may also provide insights into the mechanism of drug action.

Surolia *et al.* (2017) explored the utility of 3D primary cell culture systems in creating an *ex vivo* model of the IPF lung. The authors generated 'pulmospheres' from lung biopsies from 20 IPF patients and 9 control subjects. These are 3D clusters of cells (spheroids) derived exclusively from primary lung tissue cultures *in vitro*, and including all cell types found in the human lung. Control pulmospheres were positive for surfactant protein C (a marker for epithelial type II cells), CD31 (endothelial cells), CD11b (macrophages), and α smooth muscle actin (α-SMA) (vascular smooth muscle cells and myofibroblasts). All these various types of cells were embedded in extracellular matrix (ECM) proteins — collagen type I, fibronectin-EDA, and collagen type IV. The IPF pulmospheres also demonstrated presence of α-SMA-positive, SPC-positive, CD31-positive, and CD11b-positive cells. Immunofluorescence staining demonstrated enhanced staining for ECM proteins, such as collagen type I, fibronectin-EDA, and collagen IV. These features of lung pulmospheres reflect the *in vivo* situation where cells are supported by surrounding ECM proteins. As these pulmospheres simulate the lung microenvironment, they may find utility as a predictive model to assess the efficacy of antifibrotic drugs in IPF patients.

4.2.6 Cystic fibrosis

A total of 1,651 abstracts were retrieved, with 72 references marked for full-text review, from which 25 models were extracted.

Sears *et al.* (2015) demonstrate that culturing well-differentiated HAE cells in a circular track is a simple and reproducible method to study mucociliary transport (MCT) *in vitro*. Normal mucociliary clearance (MCC) is essential for maintaining a healthy respiratory system, and

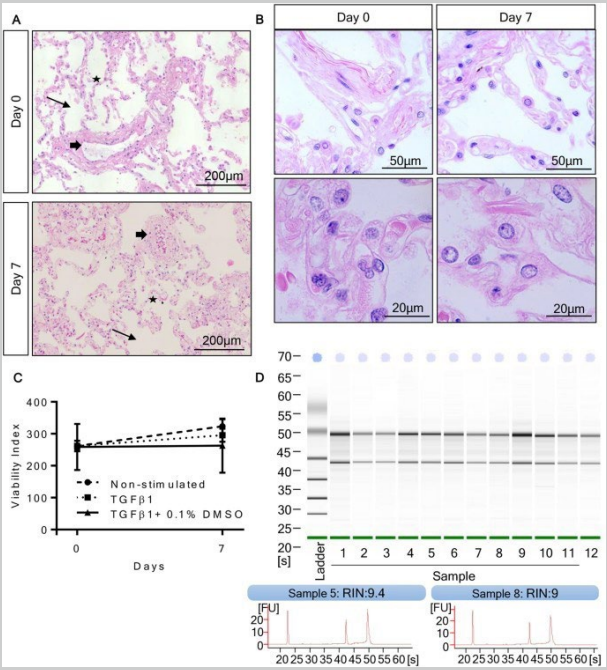
Ex vivo human lung parenchymal model of fibrogenesis

Pulmonary fibrosis is a severe respiratory disease in which scars are formed in the lung tissues, as a result of the accumulation of excess fibrous connective tissue.

Roach *et al.* (2018) have used *ex vivo* human tissue obtained from lung of patients undergoing lung resection for carcinoma to investigate their hypothesis that TGFβ1-induced pro-fibrotic structural responses are inhibited by a selective KCa3.1 blocker.

This model is based on *ex vivo* human lung parenchyma cultured for 7 days in serum-free medium containing TGFβ1 and essential nutrients.

By examining nuclear morphology, tissue necrosis, metabolic activity and RNA quality, the authors assessed the lung tissue viability and response.



Magnified H&E-stained images of tissue embedded on day 0 or after culture for 7 days in serum-free medium form the same donor shows preserved nuclear morphology with no sign of nuclear fragmentation.

impaired MCC is a feature of many airway diseases, including cystic fibrosis. Millicell cell culture inserts are modified to make MCT devices (MCTD). To create MCTDs, a plastic central cylinder (15-mm outer diameter) is glued with silicone sealant onto the membrane in the center of the insert, and coated with collagen. Cells from a significant fraction of donors are capable of coordinating their ciliary motility to produce mucus flow in a defined pattern, and, once established, this transport is stable for the lifetime of the culture. Using this device, it is possible to make repeated measures of the overall speed of transport, as well as many of the individual components of MCC, including cilia beat frequency (CBF), fluid heights, mucus concentration and composition, etc. The device is amenable to a wide range of *in vitro* manipulations, as illustrated by the studies presented above, and will be useful in the development and testing of therapies targeting MCC. The device will also be useful to investigate the mechanisms responsible for the coordination of ciliary motility and determine the effects of diseases including cystic fibrosis on MCT.

Pranke *et al.* (2017) assessed CFTR function *in vivo* via nasal potential difference (NPD) and in human nasal epithelial (HNE) cultures. HNE cells were sampled by nasal brushing of both nostrils after local visualisation of the nasal mucosa and HBE cells were isolated from bronchial explants. Freshly isolated HBE or HNE cells were then seeded on porous filters with culture medium and cultured in an air-liquid interface for 3–4 weeks. The level of CFTR correction in HNE cultures significantly correlated with the FEV1(Forced Expiratory Volume in the first second) change at 6 months in 8 patients treated with CFTR modulators. Importantly, this study correlated an *in vitro* readout (chloride channel activity) with *in vivo* data (lung function through FEV1 measurement) from the same patient to show the positive impact of the Vertex CFTR modifier

drugs. This study therefore provides the first evidence that correction of CFTR function by CFTR modulators in HNE cell cultures, measured by quantification of the CFTR-mediated Cl^- secretion, may be a valuable surrogate biomarker to predict clinical response and indicate respiratory improvement.

Darch *et al.* (2017) have developed an *in vitro* model based on chronic *Pseudomonas aeruginosa* infection of the CF lung. This model utilises a synthetic sputum medium that readily promotes the formation of *P. aeruginosa* aggregates with sizes similar to those observed in human CF lung tissue. They have used high-resolution imaging, to elucidate the life history of *P. aeruginosa* and the mechanisms that it employs to tolerate antimicrobials, specifically, bacteriophages. The initial goal of this study was to develop a model that recapitulates important aspects of *in vivo* bacterial growth and physiology and then use this model to provide insights into how microbes respond to antimicrobial therapies. The majority of people with CF will die because of lung infections, and around half of all people with CF are chronically infected with *P. aeruginosa*.

Ahmadi *et al.* (2017) adapted a fluorescence plate reader assay of apical CFTR-mediated chloride conductance to enable profiling of a selection of modulators on primary nasal epithelial cultures derived from patients bearing different CFTR mutations. This platform faithfully recapitulates patient-specific responses previously observed in the Ussing chamber assay. In proof of concept studies, they also validated the use of this platform in measuring drug responses in lung cultures differentiated from CF iPS cells. This medium throughput assay of CFTR activity has the potential to stratify CF patient-specific responses to approved drugs and investigational compounds *in vitro* in primary and iPS cell-derived airway cultures. The longer-term goal is to optimise the apical chloride conductance (ACC) assay so that large chemical libraries can be screened on patient-derived nasal cultures and iPS cell-derived epithelium.

Guimbellot *et al.* (2017) and Brewington *et al.* (2018) both describe the development of 'nasospheroids' derived from nasal epithelial cells (NEC) from CF and healthy patients. NECs are centrifuged in medium, forming pellets containing sheets of epithelia. Pellets are then seeded onto a collagen-coated dishes containing epithelial growth medium. Nasospheroids form spontaneously within 2–5 days and were shown to exhibit key features of mature respiratory epithelia including cilia, mucus secretion, and separate apical/basolateral membranes supporting luminal fluid secretion. CFTR activity can be measured by viewing changes in cross-sectional area using confocal microscopy.

Nasospheroids derived from healthy patients (active CFTR-mediated ion and fluid movement) show a reduction in cross-sectional area, whereas no such changes were observed in CF spheroids. Nasospheroids from F508del CF homozygotes that treated withlumacaftor and ivacaftor did show a significant reduction in cross-sectional area, indicating therapeutic efectiveness in restoring CFTR function (Guimbellot *et al.*, 2017). When stimulated with forskolin/IBMX — which rasie the levels of cyclic AMP, spheroids were seen to swell in the presence of functional CFTR, and shrink in its absence (Brewington *et al.*, 2018). Nasospheroids therefore show potential for the understanding of rare CFTR mutations. These spheroids are easily acquired, personalised and accurately represent the architecture and functions of airway tissues.

4.2.7 Other disease areas

After full-text review, 5 toxicity models, 3 related specifically to cigarette smoke exposure, 3 models of allergic rhinitis and one of tuberculosis were also included.

Park *et al.* (2016) demonstrated that, by means of minimally invasive nasal brushing, it is possible to harvest nasal cytology specimens in sufficient amounts for ALI culture of nasal epithelial cells in patients with allergic rhinitis (AR). ALI culture of ARNE cells showed distinguishing histologic morphologies and physiologic features compared to NHNE cells, and cultured ARNE cells preserved the pathologic conditions occurring in the nasal epithelium of AR patients. Such findings will expedite the clinical application of *in vitro* studies to predict the molecular mechanisms of AR and the potential role of the epithelium in allergic diseases.

Mathis *et al.* (2015) followed up on their earlier work comparing changes in gene expression in response cigarette smoke exposure to look at microRNA analysis of human organotypic bronchial epithelial cultures. They identified several cellular pathways that were affected by cigarette smoke exposure and that these could be both dose- and time-dependent. They propose that their model is useful in terms of adhering to the principles of Tox21[7] and so this approach may be more vaild for inhalation toxicity testing, but could be adapted for disease modelling.

4.3 Future developments

The last decade has seen significant progress in the develpment of new *in vitro* models of the human lung. *In vitro* (and *ex vivo*) model systems have enormous potential to improve our understanding of lung biology, such as investigating the effect of growth factors, oxygen availability, cell development and differentiation, and other factors that control cell fate and tissue morphology.

Nevertheless, there are many biological questions and technical challenges yet to be overcome.

As an example, the potential of human pluripotent stem cells (hPSC) has led to methods that promote differentiation of multiple lung epithelial cell types at the same time. However, methods for deriving lung tissue from hPSCs use different combinations of growth factors, leading to variations in the derived cell populations. The adoption of a more systematic approach should elucidate the mechanisms that control differentiation of specific cell lineages *in vitro*, allowing more controlled tissue engineering and translational applications of hPSC (Miller and Spence, 2017).

A notable disadvantage of current *in vitro*/*ex vivo* methods is that they cannot integrate the proximal (airway) and distal (alveolar) compartments of the lung into a single, reproducible model. The interaction between these distinct anatomical compartments and their unique cell types have important roles in lung development, homeostasis, and pathogenesis that cannot yet be captured outside of an organism.

The development of unifying models brings additional technical challenges, such as the need to grow different cells and tissues in different media. For example, epithelial tissues grow more readily in laminin-rich matrices (e.g., Matrigel), whereas endothelial and mesenchymal cells tend to favour collagen matrices (Miller and Spence, 2017). However these challenges are already being overcome, notably in the development of modular 'human-on-a-chip systems'. These already exist for toxicity testing (Abaci and Shuler, 2015; Prantil-Baun *et al.*, 2018) and could be adapted for disease modelling. The future should see an entire respiratory tract on a chip, incorporating nasal, upper airways and lower airways chips, each comprising different, relevant cell types.

Matrix stiffness affects cellular function and ongoing developments in the use of scaffolds

7 https://tox21.gov/

in cell culture — particularly hydrogels — offer the prospect of growing complex tissue / organoids in a well-defined physical environment, allowing precise regulation of cell fate and tissue homeostasis, and mimicking of the extracellular matrix (ECM) (Enemchukwu *et al.*, 2015). This should allow detailed study of the influence of ECM and other physical parameters such as stiffness and stretch, which are critically important factors in lung development, and which may exert influence on the progression to disease states (Miller and Spence, 2017).

Models looking at environmental or cigarette smoke exposure, as designed for inhalational toxicity studies, should attempt to adhere to Tox21 principles, but allowing for extension of resulting methods to look at disease modelling involving cigarette smoke induced lung damage and disease progression, and the impact of environmental pollution on, for example, diseases like asthma.

Undoubtably, if we are to describe the state-of-the-art in respiratory disease research, then the development of organoids and lung-on-a-chip technologies offer the best potential models for investigating disease mechanisms and potential therapies, including drug development. Nevertheless, their use as disease surrogates requires standardisation of all aspects of culture.

Lung organoid culture systems are ripe for development in disease modelling and treatment. They offer the ability to investigate the functions of various cells types in distinct regions of lung tissue, while also allowing the monitoring of their behaviour in different micro-environments. In particular, the generation of patient-derived organoids from biopsies will provide powerful research tools for a wide range of translational and medical approaches, such as drug efficacy and toxicity studies.

The development of lung-on-a-chip microdevices represents a major advance from traditional cell culture and offer the prospect of integrating microfluidics and microfabrication technologies with cultured living cells to reconstitute the most relevant architecture and functions of living human organs. It is thus encouraging to see that such devices are well represented by 22 seperate methods published since 2015.

> *“Organoids and lung-on-a-chip technologies offer the best potential models for investigating disease mechanisms and potential therapies”*

These devices are innovating to the extent that there now exists a “breathing lung-on-a-chip” microdevice which recreates physiological breathing movements by applying a vacuum to the side chambers and causing mechanical stretching of the poly(dimethylsiloxane) membrane forming the alveolar-capillary barrier (Huh, 2015).

Future developments in this area require the nurturing of a multidisciplinary approach, requiring cooperation between disease-focused biologists, bioengineers and mathematical modellers, so as to develop an all-encompassing understanding of how the structural environment of the lung relates to normal and diseases states.

5 Conclusions

This review of non-animal models and methods for respiratory disease research shows that we are on the cusp of an expansion of sophisticated methods to recapitulate in vivo human lung conditions, allowing us to target specific endpoints for disease research and to greatly enhance the development of therapeutics for major lung disease, including cancer, asthma, chronic obsructive pulmonary disease (COPD) and pulmonary and cystic fibrosis.

An important output of this study is a detailed catalogue (database) of models and methods that has been made publicly available via JRC Data catalogue. Every effort is needed to disseminate this inventory as widely as possible to maximise its utility and impact.

Biomedical researchers looking to find non-animal models in a particular disease area, national committees (ethics, funding), and teaching institutions (universities). can all benefit from the results of this study.

Other key stakeholder groups include Member State National Committees under Article 49 of Directive 2010/63/EU on the protection of animals used for scientific purposes and the European Network of Research Ethics Committees (EUREC).

Each stakeholder group will likely approach this knowledge source differently, for example funding committees may want to have a simple overview of existing non-animal models for a specific disease type. Researchers would require more detailed information on, for example, cell types utilised and specific details of the methods used.

A catalogue of non-animal biomedical models can offer added benefit here by allowing greater search capacity for accessing all models using a specific cell type. In this way the 'noise' of larger, general, database can be removed, reducing the time spent sifting through irrelevant references. As it stands, the use of citation indices extracted here could be used as an approximate marker of model acceptance by other researchers.

By promoting the catalogue as a uniform platform useful for researchers, evaluators and policymakers, it could be the focus of concerted efforts to satisfy the Three Rs in biomedical research.

Crucially, there is a need for more research funding to promote and develop these methods. National bodies and competent authorities have a role here — by cross-checking research applications against this database they will have a powerful tool to help assess whether proposals for animal-based research are truly compliant with the Directive when providing funding. In addition it contributes to the growing wish of the public at large to reduce and eventually eliminate the need for animal models of biomedical research.

In summary, a number of key areas for resolution have been identified in order to promote the uptake, development and standardisation of non-animal models for respiratory disease research:

1 Standard operating procedures (SOP) for model generation and testing will be essential and should be encouraged.

2 Define where models could be used — if models are better suited to address specific research questions (eg different diseases, specific pathologies, certain outputs) then model developers should indicate how their model is suitable for which aspect.

3 Lung-on-chip models will continue to grow in use as these can provide the ability to mimic breathing and allow realistic exposure to allergens and noxious particles such as cigarette smoke and infectious agents.

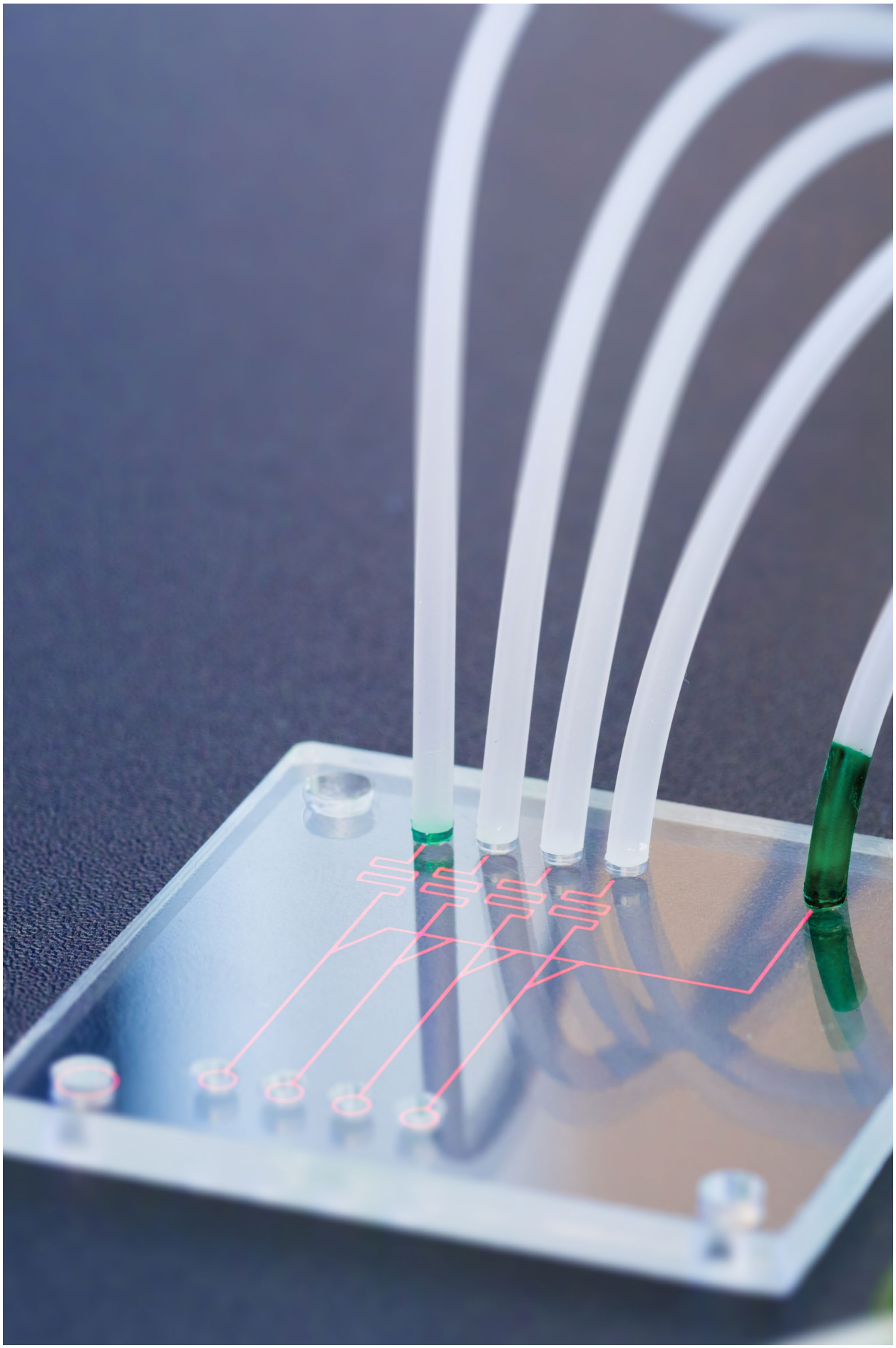
4 Lung-on-a-chip models also enable real-time analysis of readily accessible biomarkers. However, these models need to overcome use of plastic substrates as this is not representative of human tissue.

5 Promote more effective cross-talk between the fields of oncology and respiratory diseases — this will be essential for further development of organoid models.

6 Organoid models are already proving invaluable for the analysis of cell-cell and cell-matrix interactions in lung cancer models and these could be equally valid for the wider respiratory disease field.

7 Organoid models have proven useful in cystic fibrosis research — where increased swelling is used to represent enhanced mucus production. Functional readouts from organoid models could also be applied for investigation of other inflammatory and infectious lung conditions.

8 Precision cut lung slice (PCLS) models are probably the best model in terms of representation of the multiple different cell types, tissue architecture and physiological response of human lung tissue. However, their use is restricted due to tissue access and this situation is unlikely to improve in the future, particularly for conditions such as asthma and cystic fibrosis.



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7 Annex

Table 1: Non.animal respiratory disease models - Objective criteria for retaining or excluding models from consideration.

Inclusion criteria	Exclusion criteria
Review question: Define the suitability of a non-animal model for respiratory disease research	
Type of method	
The method does not require the use of live vertebrate animals, nor does it require the destruction of life each time it is performed (e.g specifically for the purpose of tissue extraction from multiple living entities). This includes: • *in vitro* methods (including cell cultures, organoids); • *ex vivo* methods using human tissues; • *in silico* methods (e.g., modelling of air flow patterns in lung). Studies that examine the effects of stimulation/drug exposure on receptor expression (excluded at first).	The method depends on the use of live vertebrate animals for its performance. *Ex vivo* methods using tissues derived from vertebrate animals. Human genome studies focused entirely on patient groups. Drug effects on cell cultures, where the culture model is poorly described, or appears to use submerged monocultures, and where the overall focus of the study is to look at DRUG effects, not disease mechanisms or model development. Mechanistic studies that are not developing models but are looking at/manipulating individual factors in a disease and are not comparing to a healthy system (i.e., not modelling disease). Studies that employ immune cells (T cells, B cells, eosinophils, neutrophils) in isolation (i.e., without a model). These studies are not considering their wider role, just looking at features/responses/profiles of the immune cells. (Note: In order to promote wider uptake of *in vitro* methods, particularly in the preclinical sector, JRC advised to retain all studies that might enable the uptake of new *in vitro* methods. Therefore such studies are brought under inclusion criteria). Studies that appear to examine the effects of stimulation with a drug/ stimulus/ pathogen/ receptor agonist without considering the baseline response (i.e., no “normal” model). Isolated studies using a particular cell type *ex vivo* — i.e., studies that extract blood/ respiratory/ immune cells and determine gene or protein expression in these cells. Analysis of one component of a particular cell type (e.g., gene or protein expression). Analysis of sputum or bronchoalveolar lavage (BAL) fluid from patient groups. Any studies looking at novel formulations of drugs, modifying formulations of existing drugs, or attempting to improve the inhaled characteristics or delivery devices for drugs.

Inclusion criteria	Exclusion criteria
Review question: Define the suitability of a non-animal model for respiratory disease research	
Area of applicability	
The method deals with modelling of diseases of the respiratory system. In some cases, respiratory endpoints may be secondary, in which cases it should be clearly indicated how this contributes to further understanding of respiratory disease research.	The method clearly does not address issues related to respiratory disease.
Purpose and rationale for the method	
The method addresses a scientific need, is based on sound scientific principles and is relevant for addressing the biological aspects and basis for respiratory disease. The method has physiological relevance and is intended to be developed for wider use.	There are no clear indications as to the purpose, rational or biological plausibility of the method for respiratory disease research.
Method description	
The method itself is clearly described, in terms of the design of the test system, the biological endpoints under investigation, and the exposure regime where relevant. The value of each target endpoint is clearly explained.	The method is poorly or inadequately described, or does not indicate in adequate details important aspects such as exposure regime.
Research entity Involved	
The method must come from a recognised research entity (Commercial, Educational, etc) with verifiable track record of research, and clearly identified so as to be contact for further information on the method, if required.	The source of the method is unclear and the research institute is unavailable for contact, or unwilling to answer questions about the status of the method.
Publication date	
All studies published in the last 10 years (since 1 Jan 2008).	Studies published prior to 2008. However, after identification of a first list of methods to be documented, specific searches shall be performed on the individual methods exceeding the given time-limit and extending the identified sources as appropriate and necessary for delivering an exhaustive and adequate description of the respective method. Based on the large number of cancer abstracts retrieved, most of which were found to be not relevant in an initial sampling, it was agreed to restrict the search for cancer models to the past 5 years.
Language	
The search strategy will be tailored to searching in English language, however, no languages are excluded.	-

Inclusion criteria	Exclusion criteria
Review question: Define the suitability of a non-animal model for respiratory disease research	
Publication type	
Primary peer-reviewed studies (articles) and grey literature (e.g., conference proceedings, dissertations and theses, etc.).	Secondary and tertiary studies (reviews, factbooks, etc.). Where reviews are found, the primary sources will be referenced for inclusion. Non-peer-reviewed sources (e.g., patents, communication letters, etc.)

Full text papers were retrieved for all accepted and 'uncertain' abstracts and individually reviewed for their relevance. Where a paper was found to describe a non-animal method and fit within the inclusion criteria, the details were extracted to a central MS Excel workbook. References that did not meet the inclusion criteria were included in a separate worksheet, with justification for their exclusion, referencing the agreed criteria.

Table 2: Format of data extraction.

Field	Field type	Drop-down options	Notes
Model number	Number	NA	This number refers to a model of a respiratory disease which is described in a paper. The same paper can describe more than a model.
Disease Area	Drop-down list	For example: Pulmonary disease - General Asthma COPD Lung cancer Pulmonary fibrosis Cysic fibrosis	Upon abstract screening, papers are broadly categorised by disease area.
Disease Feature	Drop-down list	For example: Elastin Viability Exploratory/ no specific feature Inflammation Protein aggregation Surfactant levels Tissue/cell viability Vasoconstriction	Specific endpoints of the disease with options for "Exploratory/ no specific feature" and "na".
Category	Drop-down list	For example: 2D or 3D co-culture Biochemical/cell-free assay Chip or microfluidic system Computational/*in silico* Human/patient *ex vivo* tissue or body fluids Human/patient primary or stem cells Human-derived cell lines Organoid model Other	Papers were categorised during the screening phase of abstracts, using the some model types decided upfront.
Type	Drop-down list	For example: Human pulmonary vascular smooth muscle cells Human precision cut lung slices (PCLS) Human samples from lung disease biobank (Germany) Human nasal epithelial cells (hNECs) RPMI2650 cell line A549 cell line HFL-1 cell line	More specifications, if applicable, on the model used.

Field	Field type	Drop-down options	Notes
Application	Drop-down list	Project-specific, for example: Comparison of barrier integrity Diagnosis of disease Disease mechanism (exp/ theor) Disease therapy developm Drug developm/ testing Identification of biomarkers Mimics lung microenvironment Model development - experim Model development - theoret Model validation Study of lung surfactant	Main scientific aim or application of the model.
Biological endpoints	Drop-down list	For example: Apoptosis Chemotaxis Cytokine Release Gene expression Imaging Immunohistochemistry Infection Inflammatory mediators Metabolism mRNA expression Phagocytosis Protein Repair Tissue/cell Viability	List of potential biological endpoints, used in a model system to describe either the characteristics of a model or changes in the model system related to disease feature.
Throughput	Drop-down list	High (automated) Medium/low (lab scale) Not relevant (e.g., *in silico*)	If applicable, the models is described in tems of throughput.
Potential	Drop-down list	For example: Biomarker Discovery Disease Progression Drug Development Drug Discovery Mechanistic Studies Pathogenesis Toxic insult/response Unknown	Potential for future development. This is subjective assessement.
Notes	Free text	NA	Additional information.

Field	Field type	Drop-down options	Notes
Reference	Free text	NA	Title of the paper which describes the model of respiratory disease.
DOI	Free Text	NA	DOI and link of the paper which describes the model of respiratory disease.
Author	Free Text	NA	Author(s) of the paper which describes the model of respiratory disease.
Year	Free Text	Automatically extracted from DOI	Year of Publication
Citation Index: Scopus and WoS	Drop-down	NA	Scopus and Web of Science citation indexes for the paper which describes the model of respiratory disease. It provides an indication of model maturity and use. It is automatically extraction from DOI reference.

Table 3: Information sources that were considered in searches for scientific literature relating to the review questions.

Multidisciplinary citation databases and indexing services	
Web of Science	*http://webofknowledge.com/WOS*
Scopus	*http://www.scopus.com/*
Google Scholar	*http://scholar.google.com/*
Biomedical sciences citation databases	
PubMed	*http://www.ncbi.nlm.nih.gov/pubmed*
Specific respiratory journals	
European Respiratory Journal	*http://erj.ersjournals.com*
American Journal of Respiratory Cell and Molecular Biology	*https://www.atsjournals.org/journal/ajrcmb*
American Journal of Critical Care Medicine	*https://www.atsjournals.org/loi/ajrccm*
Chronic Respiratory Diseases	*http://journals.sagepub.com/home/crd*
The Lancet Respiratory Medicine	*https://www.thelancet.com/journals/lanres/onlineFirst*
Thorax	*http://thorax.bmj.com*
Respiratory Research	*https://respiratory-research.biomedcentral.com/articles*
Journal of Cystic Fibrosis	*https://www.cysticfibrosisjournal.com*
International Journal of COPD	*https://www.dovepress.com/international-journal-of-chronic-obstructive-pulmonary-disease-journal*

Table 4: Organisations relevant to respiratory disease modelling and non-animal methods.

Specialised sources of information on respiratory disease research (publications, conference proceedings)	
American Thoracic Society – ATS	*http://www.atsjournals.org*
North American Cystic Fibrosis Foundation – NACFF	*https://www.cff.org*
European Respiratory Society – ERS	*https://www.ersnet.org/#publications*
Drug Discovery to the Lung – DDL	*https://aerosol-soc.com*
Respiratory Drug Discovery – RDD	*https://www.rddonline.com*
Commercial entities known to be developing respiratory disease models	
Epithelix	*http://www.epithelix.com*
Mattek	*https://www.mattek.com*
Emulate – lung-on-a-chip	*https://www.emulatebio.com/lung-chip*
Mimetas – organ-on-a-chip	*https://mimetas.com*
Specialised sources of information on non-animal methods	
UK National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs)	*https://www.nc3rs.org.uk*
Norwegian Inventory of Alternatives (NORINA)	*https://norecopa.no/NORINA*
Fund for the Replacement of Animals in Medical Experiments (FRAME)	*http://www.frame.org.uk*
Center for Alternatives to Animal Testing (CAAT)	*http://caat.jhsph.edu*
European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM)	*https://ec.europa.eu/jrc/en/eurl/ecvam* *https://ecvam-dbalm.jrc.ec.europa.eu*
OECD Test guidelines and guidance on non-animal, mechanistic methods	*http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm* *http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm*

Table 5: Respiratory disease non-animal models — pilot search outputs.

Hits	Specific search term
15,764	TS=((("in vitro" OR "in silico" OR "ex vivo" OR "3r" OR "non animal" OR "animal alternative*" OR organoid*) AND ("respirat*" OR "lung" OR airway* OR alveol*)) AND ("**lung cancer**")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, ESCI, CCR-EXPANDED, IC Timespan=2006-2018*
17,247	TS=((("in vitro" OR "in silico" OR "ex vivo" OR "3r" OR "non animal" OR "animal alternative*" OR organoid*) AND ("respirat*" OR "lung" OR airway* OR alveol*)) AND ("**lung cancer**" OR "**lung carcino***")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, ESCI, CCR-EXPANDED, IC Timespan=2006-2018*

Hits	Specific search term
24,980	TS=(((“in vitro” OR “in silico” OR “ex vivo” OR “3r” OR “non animal” OR “animal alternative*” OR organoid*) AND (“respirat*” OR “lung” OR airway* OR alveol*)) AND (**cancer*** OR **carcino*** OR **neoplas***)) *Indexes=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, ESCI, CCR-EXPANDED, IC Timespan=2006-2018*
4,069	TS=(((“in vitro” OR “in silico” OR “ex vivo” OR “3r” OR “non animal” OR “animal alternative*” OR organoid*) AND (“respirat*” OR “lung” OR airway* OR alveol*)) AND (“**fibrosis**” OR “cf”)) *Indexes=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, ESCI, CCR-EXPANDED, IC Timespan=2006-2018*
1,367	TS=(((“in vitro” OR “in silico” OR “ex vivo” OR “3r” OR “non animal” OR “animal alternative*” OR organoid*) AND (“respirat*” OR “lung” OR airway* OR alveol*)) AND (“**copd**” OR “**chronic obstructive pulmonary disease**”)) *Indexes=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, ESCI, CCR-EXPANDED, IC Timespan=2006-2018*
3,786	TS=(((“in vitro” OR “in silico” OR “ex vivo” OR “3r” OR “non animal” OR “animal alternative*” OR organoid*) AND (“respirat*” OR “lung” OR airway* OR alveol*)) AND “**asthma**”) *Indexes=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, ESCI, CCR-EXPANDED, IC Timespan=2006-2018*

Table 6: Information sources used in literature searches.

Specialised chemistry-oriented databases	
Chemical Abstracts (SciFinder)	http://www.cas.org/products/scifinder
Multidisciplinary citation databases and indexing services	
Web of Science	http://webofknowledge.com/WOS
Scopus	http://www.scopus.com/
Biomedical sciences citation databases	
PubMed	http://www.ncbi.nlm.nih.gov/pubmed

Table 7: Number of models per disease area mapped against disease feature.

Disease feature	Disease area										
	Allergic rhinitis	Asthma	Cigarettes	COPD	Cystic fibrosis	Lung cancer	Pulomonary fibrosis	Respiratory disease - general	Toxicity	Tuercolosis	Total
Exploratory/no specific feature		1	1		5	12	1	26			**46**
Inflammation	1	12		4	1	1	2	6		1	**28**
Tissue/cell viability	1	1	1	2	3	11	2	3	3		**27**
Tumour microenvironment						23					**23**
Airway remodelling		11						2			**13**
Gene expression				2	2	7	1	1			**13**
Toxicity			1			11		1			**13**
Barrier function/integrity	1	2		3	1			4	1		**12**
Infection		1			3			8			**12**
Protein expression		1		1	2	5	1	2			**12**
Idiopathic pulmonary fibrosis (IPF)							10				**10**
Mucous production		3		3	2			2			**10**
Metastasis						9					**9**
Angiogenesis		2				3	1	2			**8**
Drug resistance (chemotherapy)						7					**7**
Epithelial/fibroplastic cross-talk		1		1			1	3			**6**
Healing/repair				2	1		1	2			**6**
Epithelial mesenchymal transition (EMT)						4	1				**5**
Structural changes		1		1	1	1		1			**5**
Oxidative stress		1		3							**4**
Bronchoconstriction/ bronchodilation		2						1			**3**
Elastin viability				1	1			1			**3**
Primary ciliary dyskinesia (PCD)					2			1			**3**
Small airway smooth muscle		2		1							**3**
Bronchial dysplasia						1		1			**2**
CTFR Activity					2						**2**
EGF/EGFR expression						2					**2**
Mucosal clearance					2						**2**

	Disease area										
Disease feature	Allergic rhinitis	Asthma	Cigarettes	COPD	Cystic fibrosis	Lung cancer	Pulomonary fibrosis	Respiratory disease - general	Toxicity	Tuercolosis	Total
Protein aggregation		1						1			**2**
Airflow patterns				1							**1**
Chloride channel function					1						**1**
Emphysema				1							**1**
Goblet cell metaplasia						1					**1**
Hypoxia								1			**1**
Senescence				1							**1**
Sensitisation								1			**1**
Surfactant levels				1							**1**
Vasocontriction				1							**1**
Total per disease area	**3**	**42**	**3**	**29**	**30**	**97**	**21**	**70**	**4**	**1**	**300**

Table 8: Model categories mapped against disease area.

Model category/type	Disease area										
	Allergic rhinitis	Asthma	Cigarettes	COPD	Cystic fibrosis	Lung cancer	Pulomonary fibrosis	Respiratory disease - general	Toxicity	Tuercolosis	Total
2D or 3D culture	2	14	2	4	12	26	7	30	2		**99**
2D or 3D co-culture		2		4	2	27	2	12	1	1	**51**
Human/patient primary or stem cells		13		8	4		4	7	1		**37**
Human/patient *ex vivo* tissue or body fluids	1	7		10	2	5	4	4			**33**
Chip or microfluidic system		1			1	12	3	4			**21**
Computational/*in silico*		4		1	2	9	1	3			**20**
Spheroid					2	6	1	1			**10**
3D spheroid co-culture						6		1			**7**
Organoid model						1		5			**6**
Other		1				5					**6**
Bioreactor						1		3			**4**
Biochemical/cell-free assay		1		1	1						**3**
Human-derived cell lines			1					2			**3**
Total per disease area	**3**	**43**	**3**	**28**	**26**	**98**	**22**	**72**	**4**	**1**	**300**

Table 9: Cell types mapped against disease area.

	Disease area										
Cell type/cell line	Allergic rhinitis	Asthma	Cigarettes	COPD	Cystic fibrosis	Lung cancer	Pulomonary fibrosis	Respiratory disease - general	Toxicity	Tuercolosis	Total
A549 cell line				3		41	5	6			**55**
HBEC		5		4	2			5			**16**
Other						14					**14**
Primary human bronchial fibroblasts		4		2			4	2			**12**
Primary human NHBE cells			1	1	3	6		1			**12**
Human nasal epithelial cells (hNECs)	3				3			5			**11**
16HBE14o- human bronchial epithelial cell line		1		1	1	1		7			**11**
Primary human tracheobronchial epithelial cells		4			2			3	1		**10**
Human lung tumour tissue						8					**8**
In silico/mathemathical model		2		1	1	1		3			**8**
Calu-3						1		5	1		**7**
Human precision cut lung slices (PCLS)		1		3			1	2			**7**
MCR5 fibroblasts		1		1				5			**7**
Bronchial epithelial biopsy tissue		4		1				1			**6**
H1299 human lung carcinoma						6					**6**
Human airway smooth muscle cells (HASMCs)		4		1				1			**6**
Human bronchial smooth muscle cells (HBSMC)		4						2			**6**
Human lung microvascular endothelial cells HMVEC		2				2		1	1		**6**
Human pulmonary vascular smooth muscle cells		3		1			1	1			**6**
See notes		1			1		2	2			**6**
Human fetal lung fibroblasts IMR-90								5			**5**
HUVEC ATCC						5					**5**
Mesenchymal stem cells (MSCs)						4	1				**5**
Primary human ASMCs		2		1				2			**5**

Cell type/cell line	Disease area										
	Allergic rhinitis	Asthma	Cigarettes	COPD	Cystic fibrosis	Lung cancer	Pulomonary fibrosis	Respiratory disease - general	Toxicity	Tuercolosis	Total
THP-1 cell line						4	1				5
3T3 mouse fetal fibroblasts						4					4
Airway epithelial ALI culture		1	1	1	1						4
Alveolar epithelial type II (ATII) cells				1			1	2			4
H460 human lung carcinoma						4					4
Human airway epithelial cells (CFBE41o)					4						4
Human airway epithelial cells (hAEC)		1		1	1			1			4
Human airway epithelial cells of nasal origin (hAECN)					2			1	1		4
MRC5 fibroblasts						3	1				4
16HBE						3					3
Cystic fibrosis human bronchial epithelial (CFBE) cells					3						3
H358 bronchioalveolar carcinoma						3					3
HCC827						3					3
Human dendritic cells (DC)								3			3
Human MDDC monocyte-derived dendritic cells						3					3
Human PBMC						2				1	3
Human Pericytes							2	1			3
Human small airway epithelial Cells (SAEC)							1	2			3
Lung tissue (unspecified)		2		1							3
Lung-cancer associated fibroblasts						3					3
MRC-5		1					1	1			3
NCI-H1975						3					3
95-D human lung carcinoma						2					2
BEAS-2B						1		1			2
Cytokine-induced killer cells						2					2
H1650						2					2

	Disease area										
Cell type/cell line	Allergic rhinitis	Asthma	Cigarettes	COPD	Cystic fibrosis	Lung cancer	Pulomonary fibrosis	Respiratory disease - general	Toxicity	Tuercolosis	Total
H292 human lung tumour						2					2
HEK293T cells					2						2
Human lung parenchyma							2				2
Human MDM monocyte-derived macrophages						1				1	2
Human nasal turbinate slices	1							1			2
Human NSCLC						2					2
Human primary dermal fibroblasts						2					2
Human primary neutrophils		1		1							2
Human pulmonary artery endothelial cells (HPAEC)						1	1				2
Human pulmonary artery tissue				1			1				2
Human pulmonary epithelial cell lines NCI-H441					1			1			2
HUVEC isolated from human umbilical cords								2			2
Induced pluripotent stem cell-derived mesenchymal stem cells (iPSC-MSCs)				1				1			2
Lewis lung cancer LLC						2					2
Macrophages						2					2
NCI-H460						2					2
Normal human lung fibroblasts						2					2
Precision cut lung slices						2					2
Primary human airway epithelial (PHAE) cells				1				1			2
Small airway epithelial cells (SAEC)								2			2
SPCA-1 human lung adenocarcinoma						2					2
Wi-38 human fibroblasts						1	1				2
1205Lu metastatic melanoma						1					1
1HAEo2 cells		1									1
3T3J2 swiss mouse fibroblast cell line					1						1

Cell type/cell line	Disease area										
	Allergic rhinitis	Asthma	Cigarettes	COPD	Cystic fibrosis	Lung cancer	Pulomonary fibrosis	Respiratory disease - general	Toxicity	Tuercolosis	Total
3T3-L1 pre-adipose cells						1					**1**
Alveolar macrophages					1						1
Anip973						1					1
BAL Eosinophils		1									1
BEAS2B						1					1
BJ fibroblasts						1					1
Calu-6						1					1
Cancer-associated fibroblasts (LuCAFs)								1			1
CCL-153						1					1
CCL-210 fibroblasts						1					1
CF cell line (CuFi-1)								1			1
CFBE41o-					1						1
Colo66						1					1
Cystic fibrosis bronchial epithelial cell line (CF8Flp)					1						1
Decellularised human lungs				1							1
Endobronchial biopsy specimens (EBBs)				1							1
EpiAirway (primary BEC)			1								1
Erythroleukaemia cells						1					1
H292 human muco-epidermoid bronchiolar carcinoma cell line (ATCC)					1						1
H446						1					1
H520						1					1
H69						1					1
HBEC-4						1					1
HepRG (liver cells)						1					1
HFL-1 cell line						1					1
Human adipose-derived stem cells (hASCs)							1				1
Human airway epithelium (NuLi-1)								1			1
Human bronchial smooth muscular thin films (bMTF)		1									**1**
Human CL-1548 cells			1								**1**
Human ECFC-EC endothelial cells						1					**1**
Human fetal airway smooth muscle (ASM) cells		1									**1**

Cell type/cell line	Disease area										
	Allergic rhinitis	Asthma	Cigarettes	COPD	Cystic fibrosis	Lung cancer	Pulomonary fibrosis	Respiratory disease - general	Toxicity	Tuercolosis	Total
Human lung adenocarcinoma (HCC)						1					1
Human lung fibroblasts								1			1
Human microvascular endothelial cells								1			1
Human neonatal dermal fibroblasts						1					1
Human parenchymal lung tissue								1			1
Human pluripotent stem cells (hPSCs)								1			1
Human primary pulmonary artery smooth muscle cells (PASMC)				1							1
Human Reconstituted Nasal Epithelium (hRNE)			1								1
Human small cell lung cancer WA-hT						1					1
Immortalised human fetal hepatocytes						1					1
Induced pluripotent stem cells (iPSCs)								1			1
Jurkat E6.1 T cells						1					1
Mai9 human malignant pleural effusion-derived						1					1
MeT-5A human pleural mesothelial cell line (PMC)							1				1
Mouse 344SQ cells						1					1
Mouse lung adenocarcinoma Lacun. 3						1					1
Mouse lung epithelial cells						1					1
Mouse stromal fibroblasts WA-mFib cells						1					1
Murine 344SQ						1					1
Murine 393P						1					1
Myeloid-derived suppressor cells										1	1
Nasal ciliary cell sheets (human-derived)								1			1

Cell type/cell line	Disease area										
	Allergic rhinitis	Asthma	Cigarettes	COPD	Cystic fibrosis	Lung cancer	Pulomonary fibrosis	Respiratory disease - general	Toxicity	Tuercolosis	Total
Nasopharyngeal cancer cell line								1			1
NCI-H157						1					1
NCI-H1993						1					1
NCI-H292 cells								1			1
NCI-H522						1					1
NCI-H596						1					1
NHBE cell line				1							1
NIH 3T3 murine fibroblast cells		1									1
OncoCil Air™ cultures						1					1
Patient serum						1					1
Perivascular cells						1					1
Primary bronchial epithelial cells (PBEC)		1									1
Primary HAEC at ALI						1					1
Primary human BEC		1									1
Primary mouse lung fibroblasts							1				1
Rat heart-lung block						1					1
RPMI2650 cell line - nasal mucosa						1					1
SHP77						1					1
Synthetic sputum					1						1
VXN2 - primary lung tumour cell line						1					1
WM852 metastatic melanoma						1					1
Xenograft tumour tissue						1					1
Total per disease area	**4**	**51**	**5**	**32**	**32**	**198**	**30**	**89**	**4**	**3**	**448**

Table 10: Application of the models mapped against disease area.

	Disease area										
Application of the model	Allergic rhinitis	Asthma	Cigarettes	COPD	Cystic fibrosis	Lung cancer	Pulomonary fibrosis	Respiratory disease - general	Toxicity	Tuercolosis	Total
Model development/experim	1	4	1	2	12	30	2	38			**90**
Disease mechanism (exp/theor)	2	28		12	4	13	11	12		1	**83**
Drug development/testing				1	3	21	1	6			**32**
Disease therapy development		3		5	2	11	4	3			**28**
Mimics tumour microenvironment						14					**14**
Model development – theoret		2		1	1	4		1			**9**
Mimics lung microenvironment				1		1	2	3			**7**
Toxicity			2					1	4		**7**
Model of human airway mucosa		1		1	1			2			**5**
Comparison of barrier integrity				1				2			**3**
Diagnosis of disease		2		1							**3**
Identification of biomakers		1			1		1				**3**
Inhalation toxicity						2					**2**
Disease treatment					1						**1**
Lung injury								1			**1**
Model validation				1							**1**
Study of lung surfactant				1							**1**
Total per disease area	**3**	**41**	**3**	**27**	**25**	**96**	**21**	**69**	**4**	**1**	**290**

Table 11: Biological endpoints mapped against disease area.

Biological endpoints	Disease area										
	Allergic rhinitis	Asthma	Cigarettes	COPD	Cystic fibrosis	Lung cancer	Pulomonary fibrosis	Respiratory disease - general	Toxicity	Tuercolosis	Total
Protein expression		13	1	13	3		4	13			**48**
Gene expression	1	8		10	4	10	3	10			**46**
Cytokine release	1	11	1	5	1	4	5	14	3		**45**
Other		6		5	3	6	6	17			**43**
Tissue/cell viability		3	2	1	1	17	3	12	2		**41**
mRNA expression	2	5		5	3	1	5	13			**34**
Immunohistochemistry/ immunofluorescence		6		8		6	5	6			**31**
Imaging	2	5		4	2	1		14			**28**
Trans epithelial electrical resistance (TEER)	3	2	1	2	4			11	1		**24**
Inflammatory mediators		6	1	3	1	1		2		1	**15**
Metabolism		1			1	13					**15**
Migration and invasion						15					**15**
Cillia beating frequency					5			5			**10**
Cellular crosstalk						8					**8**
CTFR Activity					6			2			**8**
Morphology		1				7					**8**
Vascularisation		1				4	1		1		**7**
Apoptosis		1		2			3				**6**
Cell/tissue morphology		2					1	2	1		**6**
Cell proliferation		2		1	1		1				**5**
Mucous production	1			1	2			1			**5**
Bioelectric Properties					3			1			**4**
Cell/tissue contraction		1		1				2			**4**
Chemotaxis		2		1		1					**4**
Airway remodelling		1		1				1			**3**
Cell migration	1							2			**3**
Epithelial mesenchymal transition (EMT)						1	2				**3**
Mucociliary clearance					1			2			**3**

Biological endpoints	Disease area										
	Allergic rhinitis	Asthma	Cigarettes	COPD	Cystic fibrosis	Lung cancer	Pulomonary fibrosis	Respiratory disease - general	Toxicity	Tuercolosis	Total
Airway surface liquid (ASL) volume					1			1			**2**
(Cyto)toxicity				1				1			**2**
Infection		1				1					**2**
Reactive oxygen species (ROS)				1				1			**2**
Repair				1			1				**2**
Airway constriction		1									**1**
Cell motility		1									**1**
DNA damage		1									**1**
Elastin levels				1							**1**
Intracellular Ca2+ changes								1			**1**
Macrophage activity							1				**1**
Mitochondrial membrane potential (δΨm)				1							**1**
Mucosal tissue			1								**1**
Myofibroblast differentiation							1				**1**
Phagocytosis					1						**1**
Proliferation						1					**1**
Protein						1					**1**
Wnt signaling pathway								1			**1**
Total per disease area	**11**	**81**	**11**	**68**	**44**	**93**	**42**	**136**	**8**	**1**	**495**

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