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Unravelling the molecular mechanisms underlying chronic respiratory diseases for the development of novel therapeutics via *in vitro* experimental models

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Abstract

Chronic respiratory diseases have collectively become a major public health concern and have now taken form as one of the leading causes of mortality worldwide. Most chronic respiratory diseases primarily occur due to prolonged airway inflammation. In addition, critical environmental factors such as cigarette smoke, industrial pollutants, farm dust, and pollens may also exacerbate such diseases. Moreover, alterations in the genetic sequence of an individual, abnormalities in the chromosomes or immunosuppression resulting from bacterial, fungal, and viral infections may also play a key role in the pathogenesis of respiratory diseases. Over the years, multiple *in vitro* models have been employed as the basis of existing as well as emerging advancements in chronic respiratory disease research. These include cell lines, gene expression techniques, single cell RNA sequencing, cytometry, culture techniques, as well as serum/sputum biomarkers that can be used to elucidate the molecular mechanisms underlying these diseases, and to identify novel diagnostic and management options for these diseases. This review summarizes the current understanding of the pathogenesis of various chronic respiratory diseases derived through *in vitro* experimental models, where the knowledge obtained from these studies can greatly benefit researchers in the discovery and development of novel screening techniques and advanced therapeutic strategies that could be translated into clinical use in the future.

Keywords: Chronic respiratory diseases; *In vitro* models; Asthma; COPD; Cystic fibrosis; Lung cancer

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Abbreviations

ADAM, a disintegrin and metalloproteinase

ADAM33, ADAM metallopeptidase domain 33

ADAM8, ADAM metallopeptidase domain 8

AEC, airway epithelial cell

AECOPD, acute exacerbation COPD

ALI, air-liquid interface

A-NO2, acidified nitrite

ASM, airway smooth muscle

BAL, bronchoalveolar lavage

CCL, cysteine-cysteine motif chemokine ligand

CCR, CC chemokine receptor

CF, cystic fibrosis

CFBE, CF bronchial epithelial

CFTR, CF transmembrane conductance regulator

CFU, colony forming units

COPD, chronic obstructive pulmonary disease

COVID-19, coronavirus disease 2019

COX, cyclooxygenase

CXCL, C-X-C motif ligand

DC, dendritic cell

ECM, extracellular matrix

EGFR, epidermal growth factor receptor

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EMT, epithelial-mesenchymal transition

eNO, expired nitric oxide

ER, endoplasmic reticulum

GLRX, gluteradoxin

GM-CSF, granulocyte-macrophage colony-stimulating factor

GSTP, glutathione-S-transferase

HDAC, histone deacetylase

HMDM, human monocyte-derived macrophage

HMGB1, high mobility group protein B1

HQNO, 2-heptyl-4-hydroxyquinoline N-oxide

HRV, human rhinovirus

HSP70, 70 kilodalton heat shock protein

ICS, inhaled corticosteroid

lncRNA, long codling RNA

MEG3, maternally expressed gene 3

IFN, interferon

Ig, immunoglobulin

IL, interleukin

IPF, idiopathic pulmonary fibrosis

LAMA, long-acting muscarinic antagonist

LMWH, low molecular weight heparin

LPS, lipopolysaccharide

LTA, lipoteichoic acid

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MC, mast cell

miRNA, microRNA

MK, midkine

MMP, matrix metalloproteinase

MPO, myeloperoxidase

NADPH, nicotinamide adenine dinucleotide phosphate

NCFBE, non-cystic fibrosis bronchiectasis

NF-κB, nuclear factor kappa B

NK, natural killer cell

NO, nitric oxide

NOS, nitric oxide synthase

NOX, NADPH oxidase

NSCLC, non-small cell lung cancer

PAFr, platelet activating factor receptor

PBMC, peripheral blood mononuclear cell

PCR, polymerase chain reaction

PEx, pulmonary exacerbation

PlGF, placental growth factor

PM2.5, particulate matter 2.5

PMN, polymorphonuclear leucocyte

PZP, Pregnancy Zone Protein

RSV, respiratory syncytial virus

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

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SCLC, small cell lung cancer

shRNA, short hairpin RNA

STAT, signal transducer and activator of transcription

TGF, transforming growth factor

Th, T helper

TLR, Toll-like receptor

Treg, regulatory T cell

Z-AT, Z α1-antitrypsin

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1.0 Introduction

Chronic respiratory diseases primarily include chronic obstructive pulmonary disease (COPD), asthma, cystic fibrosis (CF), non-cystic fibrosis bronchiectasis (NCFBE), idiopathic pulmonary fibrosis (IPF) and lung cancer (Meenu Mehta, Daljeet S Dhanjal, et al., 2020). These chronic diseases can affect individuals of all age groups, and they are one of the notable causes of global morbidity and mortality. The significant economic and social burden brought upon by these diseases have prompted the need for the development of novel therapeutics that are safe and effective in combating chronic respiratory diseases (Y. Chan, Ng, Dua, & Chellappan, 2021; Y. Chan, Ng, Liew, et al., 2021). Recently, the coronavirus disease 2019 (COVID-19) pandemic that arose from the outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has claimed the lives of more than a million individuals globally ("WHO Coronavirus (COVID-19) Dashboard,"). As such, an in-depth understanding of the pathogenesis and the molecular mechanisms underlying chronic respiratory diseases is important as it can facilitate the discovery of novel therapeutic targets that could ultimately cure these diseases.

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Changes and alterations in genetic information and expression patterns play a vital role in some of the chronic respiratory diseases. For example, the expression of mRNAs is inhibited by microRNAs (miRNAs) through dysregulation of gene expression by a post-transcriptional mode (Meenu Mehta et al., 2021). Development of COPD and progression of lung cancer are reportedly related to epithelial-mesenchymal transition, modulation of autophagy, and lung ageing. Furthermore, extracellular vesicles are involved in the intercellular communication and sharing among miRNAs (Eapen, McAlinden, Myers, Lu, & Sohal, 2019).

Under anaerobic conditions, mutant strains of *Pseudomonas aeruginosa* have been reported to cause acute exacerbations of CF and COPD. These exacerbations are represented by sensitivity or resistance to acidified nitrite (A-NO_2-). In planktonic and biofilm cultures, the *mucA* allele is observed to be more sensitive to A-NO_2- than the truncated Δ*mucA* deletion mutant. The levels of wild-type transcription of protective NO_2- and nitric oxide reductase in the Δ*mucA* mutant are usually high, but within the A-NO_2-sensitive *mucA22* mutant, their levels are low (Panmanee et al., 2019). The human immune system is implicated in the pathological progression of chronic respiratory diseases involving innate immune cells such as dendritic cell, neutrophils, and adaptive immune cells such as plasma cells, B and T cells. The B cells produce immunoglobulin (Ig) E (IgE) which plays a key role in the pathophysiology of allergic diseases. B cells undergo differentiation with the help of T cell through cognate interactions with specific T cell subsets and results in immunoglobulin secretion. B cells also play a major role in chronic allergic lung diseases through antigen presentation (Lindell, Berlin, Schaller, & Lukacs, 2008).

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Dendritic cells (DCs), which are present in the airways along with the airway epithelial cells (AECs), initiate immune responses and maintain tolerance against harmful pathogens and antigens. At homeostasis and during infections, the immune function of the lung is affected by the interaction between DCs and AECs. Age influences the functions of both DCs and AECs. Age is also associated with the dysregulation of inflammatory and immune responses and hence, increases the risk of chronic respiratory diseases in the elderly (Agrawal, 2017). In chronic lung disease, the neutrophils and their products mediate airway inflammatory alterations (Chellappan et al., 2020). They also lead to the development of disease-associated pathological features such as emphysema

and mucus hypersecretion, which contributes to the involvement of neutrophils in the disease progression. Changes in neutrophil migration, degranulation, reactive oxygen species production, bacterial colonisation and recurrent infective exacerbations may impair the defense mechanism of neutrophils (Jasper, McIver, Sapey, & Walton, 2019).

Chronic respiratory diseases may also generally result from abnormal immune response to various environmental agents. Pathogenesis of chronic respiratory diseases and newer targeted therapies have significant connections with airway epithelial cells and innate immune cells. The airway epithelial cells and innate immune cells involve themselves in the progression of chronic respiratory diseases (Holtzman, Byers, Alexander-Brett, & Wang, 2014). An example of an environmental agent that causes chronic respiratory disease is the 'particulate matter 2.5' (PM2.5). Such particles may harm the human respiratory system, alter the epigenetic and microenvironmental properties leading to lung cancer. PM2.5-associated lung cancer may result in autophagy and apoptosis of tumor cells. PM2.5 induces the release of several pro-inflammatory cytokines which act as a mechanism to trigger and aggravate asthma and COPD. These activities confirm the role of PM2.5 in the pathogenesis of chronic respiratory diseases (R. Li, Zhou, & Zhang, 2018; Z. Wang, Zhao, Wang, Du, & Xie, 2019). It is established that the human immune system is associated with the development of chronic respiratory diseases and their pathological progression. As the immunity levels of the biological system become defective, the growth rate and pattern of microbes may change. The common microbes involved are bacteria, fungi, and viruses. In chronic respiratory disease and even during exacerbations, the immigration, elimination, and relative growth rates of microbes change significantly (Keshav Raj Paudel, Vivek Dharwal, et al., 2020). The respiratory dysbiosis causes abnormal host immune response which alters the

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growth conditions of microbes in patient airways, promoting further dysbiosis (Dickson, Martinez, & Huffnagle, 2014). In chronic respiratory diseases, especially lung fibrosis, *Aspergillus fumigatus* is more frequently present (Fukuda et al., 2018). Patients suffering from chronic respiratory diseases are susceptible to lung infections. Respiratory viruses can exacerbate the chronic respiratory diseases such as CF through various mechanisms. The dysregulation of host response to virus is observed in these chronic diseases. The host immune responses are important to develop new therapies for virus-induced exacerbations. Viral infections modify the epithelial barrier and microbial environment in airways and the epigenetic alteration such as modulation of miRNA (Hendricks & Bomberger, 2016; Tan et al., 2020).

Prevotella histicola, a gram-negative bacterium activates Toll-like receptor (TLR)-2, which induces antigen- presenting cells to produce T helper 17 (Th17) polarizing cytokines. *Prevotella* is reported to stimulate the epithelial cells to produce interleukin (IL)- 8, IL- 6 and cysteine-cysteine motif chemokine ligand (CCL)-20 which promote the mucosal Th17 immune responses and recruitment of neutrophils. The inflammation of mucosa results in a systemic dissemination of inflammatory mediators, bacteria and its products which may affect the systemic disease outcomes. The immune cells and stromal cells release inflammatory mediators resulting in increased inflammation exhibited by *Prevotella* (Larsen, 2017). Other than the human immune system, metabolic and biochemical processes also correlate with chronic respiratory diseases and their pathological progression. The quality of life, economic burden, management, and disease mortality are influenced by metabolic comorbidities such as diabetes mellitus, osteoporosis, and thyroid dysfunction. Metabolomic studies are carried out to identify novel pathogenetic pathways

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and potential therapeutic targets (Janssen-Heininger, Reynaert, van der Vliet, & Anathy, 2020; Papaioannou et al., 2018).

Altered glutathione redox homeostasis and endoplasmic reticulum (ER) stress are also known to affect the development of fibrotic remodeling which is common in chronic respiratory diseases. Normalizing the redox homeostasis alleviates the ER stress and protein disulfide isomerase inhibits the mitochondrial oxidant production and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) enzymes. S-glutathionylation acts as a biochemical pathway enzymatically which is related to chronic lung diseases. Targeting S-glutathionylation impacts cell death, function of mitochondria, secretion of alarmins, immune responses, activation of epithelial and immune cells, and protease activities. The glutathione-S-transferase (GSTP) and gluteradoxin (GLRX) involve in catalyzing and reversing protein S-glutathionylation, which takes part in its covalent incorporation into proteins (Pryhuber, 2015). Some factors which are related to life forms, microbes and immunity are also involved in the progression of chronic respiratory diseases. The respiratory morbidity in premature infants is significant during infancy causing a negative impact on health, quality of life, and healthcare costs. In a prolonged lung disease state that has caused premature births, the susceptibility of infections and inflammation would increase. Pre-term births may alter host-pathogen interactions and immune responses and may affect chronic respiratory morbidity in such children (Restori, Srinivasa, Ward, & Fixman, 2018; Vallath, Hynds, Succony, Janes, & Giangreco, 2014). When it comes to disease management, the epidermal growth factor receptor (EGFR) signaling pathway may have a role in the management of chronic lung disease. The EGFR involves in lung development, homeostasis, repair, and disease ontogeny. Respiratory pathogens are also one of the possible causative factors for the development of chronic respiratory

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diseases. The recalcitrant biofilms that are formed render the antibiotic treatment ineffective. The common respiratory pathogens include *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Moraxella catarrhalis* to name a few. Community interactions on respiratory disease state impacts the progression of respiratory disease, morbidity, and mortality (Flick et al., 2020).

In this review, we present an updated compilation of important findings drawn from various *in vitro* studies with regards to chronic respiratory diseases in terms of their pathogenesis, diagnosis, and treatment strategies, paving the way for the extrapolation of these findings to *in vivo* studies, as well as eventual clinical translation and successful development of novel therapeutics that could improve public health and the overall well-being of the community.

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2.0 Overview of *In Vitro* Experimental Models in Respiratory Research

Over the recent years, preclinical research in respiratory therapeutics has witnessed substantial progress in the field of drug delivery where novel potent molecules have been delivered to the lungs (Artzy-Schnirman et al., 2021). Generally, drugs for respiratory diseases can be administered through various routes. However, not all of them are effective for instant symptomatic relief and disease treatment. For example, lung cancer therapeutics that requires systemic administration often results in low retention at the tumour site, leading to various systemic adverse reactions and reduced therapeutic efficacy. On the other hand, oral administration of high dose anti-inflammatory agents for treating asthma, COPD and cystic fibrosis often leads to liver and kidney toxicity. Therefore, a targeted delivery approach that directly deposits therapeutic moieties within

the lungs can facilitate the distribution of optimal drug concentration to the diseased site whilst minimizing systemic drug exposure and adverse drug reactions, which ultimately enhance therapeutic efficacy, treatment outcomes, and patients' quality of life (Y. Chan, Ng, Liew, et al., 2021; Y. Chan, Ng, Singh, et al., 2021; Cidem, Bradbury, Traini, & Ong, 2020). Nevertheless, the complex anatomical structure of the human lungs poses a huge challenge to researchers in the development of targeted drug delivery approach. There are also several biological barriers that could limit drug uptake and its subsequent efficacy (Y. Chan, Ng, Singh, et al., 2021; Cidem et al., 2020; Ruge, Kirch, & Lehr, 2013). As such, several *in vitro* experimental models have been developed, validated, and characterized to be employed as preclinical tools for the determination of ideal parameters of targeted therapies. Typically, these *in vitro* models mimic the biological and structural properties, as well as the heterogeneity of the human lung environment which can better replicate the exposure, deposition, and efficacies of therapeutic agents. Thus, *in vitro* models can increase researchers' understanding of how homeostasis is maintained in human lungs, and how diseases are initiated from the dysregulation of specific cellular processes, especially those within the deeper lung regions such as the alveolus (Cidem et al., 2020; Miller & Spence, 2017). Such *in vitro* models can also enable tightly controlled experiments that help researchers to study the direct roles of immune interactions, mechanical force, and the extracellular matrix (ECM) on progenitor differentiation and its response to injury (Miller & Spence, 2017).

Apart from that, *in vitro* experimental models can also assist in optimizing research in chronic respiratory diseases. Despite the sobering context of its rising prevalence, there have been remarkably fewer therapeutic agents approved as novel respiratory medicine in the past decade (Artzy-Schnirman et al., 2021). This can be attributed to the multiple hurdles faced with animal

experiments in which these *in vivo* models may not faithfully characterize human diseases, leaving doubts on the translational impact of *in vivo* findings. Namely, there have been various therapeutic agents that have presented promising performance in animal experiments but subsequently failed in clinical trials, thereby increasing the thrust for advocating a better predictive human model in respiratory research (Artzy-Schnirman et al., 2021; Upadhyay & Palmberg, 2018). Through the application of physiologically relevant *in vitro* experimental models, it offers a better scope to investigate detailed molecular mechanisms, cellular and subcellular functions, as well as cell-to-cell communication as compared with animal models. At the same time, *in vitro* models are more cost-effective, time efficient, easily scaled and replicated, and easier to perform as compared with animal models (Upadhyay & Palmberg, 2018). Hence, the leveraging of *in vitro* experimental models could act as an important preclinical step to bridge the gap between *in vivo* animal experiments and human clinical trials, thereby aiding the discovery of novel therapeutic targets whilst offering a scalable, powerful platform for the evaluation of novel therapeutics (Miller & Spence, 2017; Nossa, Costa, Cacopardo, & Ahluwalia, 2021). Ideally, a physiologically relevant *in vitro* experimental model replicates as many possible essential features with respect to its *in vivo* counterpart, in which cells must be cultured using defined protocols and must not lose their phenotypic characteristics (Nossa et al., 2021). Currently, there are several *in vitro* experimental models that are commonly employed to assist researchers in understanding the pathogenesis of chronic respiratory diseases, as well as the roles and mechanisms of various screening and therapeutic strategies for these diseases (Figure 1). An overview of several other key experimental models in respiratory research are presented in Table 1.

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Table 1: An overview of key experimental models in respiratory research

Context	*In vivo* models	*In vitro* models	References
Lung and airway mechanics	Assisted ventilation and non-assisted ventilation	Isolated airways, lung tissue culture (for airway dynamics) and cell stretching systems (mechanical cell stretching devices),	(Gerstmair, Fois, Innerbichler, Dietl, & Felder, 2009; Martin, Uhlig, & Ullrich, 1996; Nickles et al., 2014)
Oxygenation	Any *in vivo* model	No *in vitro* models	(Huh et al., 2010; Wolfgang M Kuebler et al., 2016; Uhlig et al., 2014)
Lung hemodynamics	Any *in vivo* model	Isolated perfused lung systems, shear stress models, isolated forms of blood vessels	(Kizub et al., 2013; Tabeling et al., 2015)

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Airway barrier function	Any *in vivo* model	Permeability and translocation assays, lung-on-a-chip model, isolated perfused lungs, ALI culture	(Huh et al., 2010; Wolfgang M Kuebler et al., 2016; Uhlig et al., 2014)
Lung tissue remodelling	Animal models of chronic lung disease (*via* cigarette smoke exposure; OVA or HDM models or knockout mice)	Smooth muscle cell proliferation, compensatory lung growth model, apoptosis, and chemotaxis assays; cell differentiation, ECM deposition and remodelling; cell signalling (juxtracrine, autocrine, paracrine, endocrine assays, ALI cultures	(Dabral et al., 2016; W. M. Kuebler, 2016)
Disease associated inflammation and innate immune responses	Models of infection, sterile inflammation, and autoimmunity	Adhesion and migration; cell signalling; phagocytosis and ALI culture	(Amatullah et al., 2017; Yin et al., 2016)
Therapeutic approaches	Any *in vivo* animal model	ALI culture, nanocarriers based drug delivery systems, gene expression analysis and editing	(Meenu Mehta, Saurabh Satija, et al., 2020; Keshav Raj

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	Paudel, Nisha Panth, et al., 2020; Raveendran, Rochani, Maekawa, & Kumar, 2017)

2.1 Air-Liquid Interface

Traditionally, submerged cell cultures of primary bronchial epithelial cells are commonly used as the gold standard to perform respiratory research. However, research under submerged cell culture conditions do not accurately address the complex interactions of human cells *in vivo* (Artzy-Schnirman et al., 2021; Nossa et al., 2021). A major drawback of the submerged culture model is that cells often lose their ciliated phenotype, therefore, do not accurately replicate human respiratory physiology. This has frequently led to misinterpretations and false conclusions that negatively impact drug development process in chronic respiratory diseases (Aydin et al., 2021).

To overcome this issue, researchers have developed new culturing techniques with physiological relevance to the human airway mucosa. An air-liquid interface (ALI) culture model is among the foremost important culture techniques in respiratory research in which mucus production and mucociliary differentiation can be achieved *in vitro*, and it is often utilized to recapitulate luminal airflow of the human respiratory system (Aydin et al., 2021; Cidem et al., 2020). As such, the use of ALI culture models may provide researchers with better information on *in vivo* processes in

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humans and enable the evaluation of molecular mechanisms that occur in humans, where some of which may differ or do not exist in animals due to physiological variations (Lacroix et al., 2018). Cells are generally seeded onto semi-permeable Transwell filters where both apical and basolateral chambers are submerged in culture medium. Upon reaching cell confluency, media is removed from the apical chamber, thereby exposing cultured cells to air, and enabling them to develop a pseudostratified morphology. Various immortalized respiratory cell lines have been shown to form tight junctions and/or possess the ability to produce mucus under ALI culture conditions. These include A549, Calu-3, NCI-H441, and 16HBE (Aydin et al., 2021; Cidem et al., 2020; Nossa et al., 2021). In short, these ALI culture models represent a unique *in vitro* platform that allows the replication of *in vivo* pulmonary drug delivery, in which drug particles can be subjected to differentiated cell layers of the model, thereby enabling downstream drug transport, efficacy, and cytotoxicity studies to be conducted. At the same time, these models also allow for a better understanding on the dynamic interplay between static and mobile cells that are present in typical diseased airways for evaluating the interaction of external insults with susceptible genes, as well as the interaction between airway cells during airway remodeling (Barnes et al., 2015; Cidem et al., 2020; Lacroix et al., 2018).

2.2 Organoids

As mentioned earlier, *in vitro* experimental models that precisely resemble *in vivo* physiology are crucial to accurately evaluate the delivery, efficacy, and toxicity of therapeutic agents. Therefore, *in vitro* experimental models have now advanced beyond ALI monoculture techniques for the inclusion of both physiological and mechanical parameters of breathing, role of ECM, and multiple

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cell types (Cidem et al., 2020). For this instance, organoids are progenitor cell-derived three-dimensional structures within an ECM that recapitulate fundamental, functional and structural aspects of human organs. In contrast to ALI monoculture models, respiratory organoids represent the three-dimensional airway microenvironment by promoting the growth and differentiation of various cell types to physiologically replicate the diverse structural branching within airways (Barkauskas et al., 2017; Cidem et al., 2020; Y. Li, Wu, Sun, Shen, & Chen, 2020). When embedded within a complex mixture of ECM proteins that include fibronectin, laminin, and collagen, physiological and architectural support can be provided, allowing for sustained cell-to-cell and cell-to-matrix signaling pathways which facilitate an accurate spatial organization of heterogenous cell population, thereby generating luminal growth within the organoid. As such, respiratory organoids have been used as experimental models for various chronic respiratory diseases, including but not limited to CF, viral infections, and lung cancer. In a nutshell, these experimental models can serve as an effective platform for the evaluation of physiological and disease-relevant intracellular interactions when investigating the delivery, efficacy, and toxicity of novel therapeutic agents in chronic respiratory diseases (Cidem et al., 2020; Y. Li et al., 2020).

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2.3 Lung-on-chip Model

Advancements in technology have allowed the engineering of organ-on-a-chip-based models to precisely replicate the mechanical and physiological parameters which modulate and influence organ function and homeostasis. In terms of chronic respiratory diseases, lung-on-chip models can efficiently mimic *in vivo* respiratory environment via replication of breathing mechanics, air pressure and airflow dynamics, cellular heterogeneity, as well as vascular flow rates. Generally,

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lung-on-chip models feature two distinct chambers, separating epithelial and endothelial monolayers that are cultured on the opposite sites of an ECM-coated permeable membrane, forming the apical and basal chambers, respectively (Artzy-Schnirman et al., 2021; Konar, Devarasetty, Yildiz, Atala, & Murphy, 2016). At the cellular level, it has been established that exposure to mechanical stressors such as airflow changes and fluid shear stress can induce cell proliferation, differentiation, and its function, which proves the importance of integrating mechanical stress within *in vitro* experimental models when attempting to emulate an *in vivo* environment. In contrast to traditional *in vitro* experimental models that are largely static when considering the collection and/or exchange of media, the application of microfluidic systems allows continuous perfusion to replicate fluid-induced shear stresses. As a result, a lung-on-chip model closely mimics the alveolar-capillary barrier, and it can be employed as a highly relevant model of lung microphysiology to demonstrate the *in vivo* interplay between cell-to-cell, cell-to-matrix, and cell-to-mechanical forces. The overall complexity of the chip can also be tailored depending on the study, producing varying flow or pressure on the membrane to result in mechanical stress to a certain extent (Artzy-Schnirman et al., 2021; Bovard, Iskandar, Luettich, Hoeng, & Peitsch, 2017; Cidem et al., 2020). Hence, these lung-on-chip models allow researchers to perform appropriate *in vitro* investigations for evaluating the behaviour of novel inhaled therapeutics, such as their bio-pharmacokinetic profiles, aerodynamic performance, as well as therapeutic efficacy and toxicity. At the same time, such models also enable researchers to better understand how various mechanical and physiological *in vivo* parameters impact drug intake, which could lead to the optimization of drug formulation and eventual development of effective novel therapeutics for chronic respiratory diseases (Cidem et al., 2020; Konar et al., 2016).

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3.0 Understanding the Aetiology and Pathogenesis of Chronic Respiratory Diseases Using *In Vitro* Experimental Models

Single-nucleotide polymorphisms have been reported in transforming growth factor (TGF)-β1 including C-509T and T869C. Polymorphisms at a disintegrin and metalloproteinase (ADAM) metallopeptidase domain 33 (ADAM33) V4, T2, S2, and T1 have also been observed. These have been detected using polymerase chain reaction-restriction fragment length polymorphism. The associations of these single-nucleotide polymorphisms with the risk and severity of asthma were also evaluated. It was found that the risk of developing asthma during childhood is modified by the genetic variants at V4 and C-509T, with no association with disease severity, whereas TGF-β1 haplotypes and asthma risk are not significantly associated. Hence, ADAM33 haplotypes are capable of providing useful information in predicting the risk of asthma (H. Li et al., 2014; Sharma, Tripathi, & Awasthi, 2011). The vascular endothelial growth factor (VEGF), through the activation of VEGF receptor (VEGFR)-2/extracellular signal-regulated protein kinase (ERK)-1/2 (VEGFR2/ERK1/2) signalling pathway enhances the expression of ADAM33 and their cell proliferation, which may take part in the pathogenesis of airway remodeling (Naveed et al., 2017; Pei et al., 2016). In asthmatics, the interactions of cultured airway smooth muscle and mast cell promote the severity of asthma by raising the activation of matrix metalloproteinase (MMP)-1, growth of airway smooth muscle, and airway responsiveness (Pei et al., 2016). In an *in vitro* study, type 2 helper T cells expressed the CC chemokine receptors (CCR) CCR3, CCR4, and CCR8. All the T cells expressed interleukin IL-4 and CCR4, while eosinophils expressed CCR3. CCR8 co-expressed with CCR4 in 28% of the T cells. Upon an allergen challenge, the CCR4-specific ligands macrophage-derived chemokine and thymus, and activation regulated chemokine are expressed

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where they are strongly upregulated on AECs. This receptor and ligand are shown to have regulated lymphocyte recruitment in the asthmatic bronchi. The immunofluorescent analyses of the endobronchial biopsies were carried out a day after the allergen challenge (Panina-Bordignon et al., 2001). The airway epithelial cell-derived factors such as tumour necrosis factor-related apoptosis-inducing ligand and MID1 regulate the initiation of Th2 responses and stimulate the development of Th2 cell through signal transducer and activator of transcription (STAT)-6-dependent pathways (Foster et al., 2017). The release of cytokines from stimulated peripheral blood mononuclear cells (PBMCs) in asthmatics is modulated by low-molecular-weight heparins (LMWHs) such as enoxaparin and dalteparin to determine the specific components that play a significant role (Shastri et al., 2015).

Eosinophils may be involved in the pathogenesis of asthma progression primarily through the activation by Th2 cytokines such as IL-5 or granulocyte-macrophage colony-stimulating factor (GM-CSF), viral infection-related proteins such as C-X-C motif ligand (CXCL)-10 and interaction with neutrophils (Nakagome & Nagata, 2018). The soluble IL-6 receptor is often found within neutrophils in the lungs, and Asp358Ala variant of interleukin-6 receptor gene may elicit pro-inflammation in lung cells (Farahi et al., 2017). The IL-4 induced B cell autophagy can exacerbate asthma experimentally through multiple mechanisms (Xia et al., 2018). In BEAS-2B human bronchial epithelial cell line, the deletion of Src homology region 2-containing protein tyrosine phosphatase 2 (Shp2) which is involved in asthmatic airway remodeling was found to impair the production of IL-25, thereby alleviating airway inflammation and allergic reaction (Z. Qiu et al., 2017). The identification of serious asthma genotype is done by IL-27 together with Type-2/CCL26 signature which may reveal combined effects of IL-27 and IL-13 on STAT signaling.

IL-27 involves in the enhancement of Th1 responses such as the chemokine CXCL9 and suppression of Th2 responses. This is consistent with the findings from an *in vitro* study utilizing human bronchial epithelial cells cultured in ALI, where the induction of Th2 responses by IL-27 was found to be enhanced by IL-13 due to the increased activation of STAT1 and decreased activation of STAT3 (Xie et al., 2015).

It has been reported that the concentrations of induced sputum and plasma that contain 70 kilodalton heat shock proteins (HSP70) were higher in asthmatic patients than in non-asthmatics. Spirometry was carried out before the induction of sputum and the levels of HSP70 in induced sputum and plasma were measured using an enzyme-linked immunosorbent assay (ELISA). The HSP70 levels were similar in patients with eosinophilic and non-eosinophilic asthma. There was positive correlation between plasma HSP70 levels and neutrophil count, while sputum HSP70 levels positively correlated with lymphocyte count. The induced sputum and plasma HSP70 may act as useful marker for assessing degree of obstruction in asthma (Hou, Zhao, Li, et al., 2011). The sputum and plasma high mobility group protein B1 (HMGB1) concentrations in patients with asthma and COPD were higher than in control subjects. Spirometry was performed before sputum was induced and the HMGB1 levels in induced sputum and plasma were measured using ELISA. The HMGB1 levels in COPD patients were higher than in asthmatics and were inversely related to the forced expiratory volume. The levels of HMGB1 in both diseases were positively related to percentage and count of neutrophils. Multivariate analysis revealed both diseases and their severity independently predicted the sputum HMGB1, but did not correlate with smoking, age, or use of inhaled corticosteroids. Hence, HMGB1 plays a crucial role as a biomarker and diagnostic tool in diagnosing asthma and COPD (Hou, Zhao, Liu, et al., 2011).

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Cell viability and tube formation of primary human lung endothelial cells were evaluated *in vitro* by exposing them to LF-15, T3 and T7 tumastatin-derived peptides. LF-15 and T7 tumastatin-derived peptides may be used as new therapeutic tools because of their smaller size and their anti-angiogenesis property. The LF-15 and T7 tumastatin-derived peptides reduced both the viability of endothelial cell and formation of tube (Grafton et al., 2014). In COPD and allergy-related asthma, the *in vitro* expression of placental growth factor (PlGF) was elevated implying its role in pathogenesis. The airway-microvascular remodeling was supported by stimulation of isolated human lung microvascular endothelial cells with PlGF (D. Wu et al., 2017). Exposure to environmental particulates during childhood increases an individual's risk of developing asthma. In *in vitro* conditions ambient particulates injured the epithelia through oxidative stress and both pro-inflammatory cytokines and Th2-promoting cytokines such as IL-33 were observed to cause this. However, when airway epithelial cells were treated *in vitro* using antioxidant compounds, the pro-inflammatory cytokine response to these particulates were inhibited (Herbert et al., 2013).

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Viral infections cause severe acute exacerbations in both asthma and COPD especially in those co-infected with bacteria under chronic conditions. Due to the exacerbation of disease, the patients require readmittance to the hospital. Rhinovirus A was the most frequently isolated virus when throat, nose swabs as well as sputum samples were obtained and examined by both bacterial culture and multiplex polymerase chain reaction (PCR) for respiratory viruses. The confounder was controlled multivariate analysis (Hewitt et al., 2016; Wark, Tooze, Powell, & Parsons, 2013). The number of respiratory episodes in the first few years could be correlated with asthma developed later, but not because of any particular virus (Bønnelykke, Vissing, Sevelsted, Johnston, &

Bisgaard, 2015). Human rhinovirus (HRV) which is associated with severe clinical conditions such as hypoxemia or hypercapnia is known as a major pathogen that can cause exacerbations of asthma (Saraya et al., 2014). The species of HRV-C is identified by partial and complete genome sequencing and its biologic features such as structure are significantly different to other HRV. These findings provide a new dimension for understanding the HRV biology. The treatments for allergies or viral infection decrease the risk of illnesses like allergies and virus-induced asthma. The data on inflammatory patterns observed in asthma and antiviral responses are linked by specific mechanism and can provide an opportunity to develop new therapeutic strategies (Gern, 2010). The increased IL- 10 mRNA and inducible expression of protein- 10 gene correlates with viral persistence. Essentially, the induced sputum is collected from patients to detect viral and inflammatory mediators. The non- infective human rhinovirus is the most common persistent virus, and the source of persistent infection is a viral RNA. The changed cytokine environment will lead to a host inflammatory response and the long- term outcomes in asthma will be caused by these effects (Wood et al., 2011). In asthmatics, the level of IL-15 in bronchoalveolar lavage (BAL) fluid is reduced. The virus load and airway hyper-responsiveness are inversely related to it during rhinovirus infection. There is impaired induction of IL-15 in BAL macrophages by rhinovirus which depends on replication, nuclear factor kappa B (NF-κB) and α/β interferon and are inversely correlated to lower respiratory symptom severity during experimental rhinovirus infection (Laza-Stanca et al., 2011).

In AECs, the control of respiratory syncytial virus (RSV) and influenza A virus and the response of interferon (IFN) to these viruses in asthmatics were observed to be similar to controls. The AECs activate the IFN-dependent system. The human bronchial epithelial cells from asthmatics

and controls were cultured and infected with either one of the two viruses. The viral levels together with IFN-β and IFN-λ and gene expression that is stimulated by IFN were also determined (Patel et al., 2014). The increased risk of COPD exacerbation is generally correlated with elevated inflammation in airways which can be caused by microbial infections and pollutants. The inflammatory responses result in mucus hypersecretion which in turn causes airway obstruction and related exacerbations. The rate of relapse in patients with chronic mucus hypersecretion is lower after initial treatment for acute exacerbation (Ko et al., 2016; Tesfaigzi, Meek, & Lareau, 2006). Both bacterial and viral infections are involved in COPD which lead to chronic infection, and exacerbation (Beasley et al., 2012; Buss & Hurst, 2015; Martinez, Erb-Downward, & Huffnagle, 2013). Furthermore, development of COPD requires macrophages and tryptase-expressing mast cells (MC). The recombinant MC tryptase induces pro-inflammatory responses from cultured macrophages (Beckett et al., 2013). In acute-exacerbation-COPD (AECOPD) patients, the early-endothelial progenitor cells are reduced and are dysfunctional, which may alter vascular endothelium (Liu, Liu, Huang, Lin, & Xie, 2017). In COPD, the cytotoxicity of natural killer cells (NKs) against lung epithelial cells is enhanced by lung DC-mediated priming through IL-15 trans-presentation on IL-15Rα. In *in vitro* isolated CD56+ it was reported that NKs eliminate CD326+ epithelial cells (D. K. Finch et al., 2018).

Wnt4, a protein coded gene, is reported to cause both infiltration of neutrophils and inflammation through activation of epithelial cell remodeling in which airway epithelial activation induces Wnt4 because of oxidative stress. The microarray analysis of bronchial biopsy samples from COPD patients reveal that Wnt4 is upregulated in COPD and correlates with IL8 gene expression (Durham et al., 2013). The neutrophil profiles of COPD patients were not influenced by tumour

necrosis factor (TNF)-α and GM-CSF-induced dominant signals as the systemic neutrophil responses in COPD patients are generally caused due to the interplay between multiple inflammatory signals. This was demonstrated in a study in which stimulated peripheral neutrophils with TNF-α, GM-CSF, or TNFα + GM-CSF resulted in the formation of regulated protein spots, which was consistent with the significantly regulated protein spots identified in peripheral neutrophils isolated from COPD patients as compared with age-matched healthy subjects (Langereis, Schweizer, Lammers, Koenderman, & Ulfman, 2011). The deficiency of ADAM metalloproteinase domain 8 (ADAM8) elevates lung inflammation induced by cigarette smoke, emphysema, and metaplasia of airway mucus cells. Therefore, approaches that increase or prolong expression of ADAM8 in the lung may have therapeutic efficacy in COPD (Polverino et al., 2018). In smokers, the activity of vessel-associated TGF-β1 is increased in the bronchial reticular basement membrane, especially in those with COPD. The immunostaining for TGF-β1 antibody is carried out on the biopsies of bronchia from smokers with normal lung function and smoking COPD subjects and the results were found to be comparable (Soltani et al., 2012).

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When exposed to a cell-polarizing condition involving TGF-β1, features like instability and plasticity were exhibited by CD4+CD25−Foxp3+ T cells allowing the conversion of such cells to Th17 cells. Essentially, TGF-β1 generates CD4+CD25−Foxp3+ T cells which help in the generation of Th17 cells and may result in chronic inflammation in COPD (J.-H. Wu et al., 2019). In COPD, aggregated intracellular mutant Z α_1-antitrypsin (Z-AT) invokes specific harmful cellular inflammatory phenotype. The intracellular Z-AT polymerization in epithelial cells is induced by oxidants leading to ER stress and excessive promotion of cytokine and cellular inflammation (Alam et al., 2014). The upregulation of epithelial platelet-activating factor receptor

(PAFr) expression is observed in smokers, especially in those with COPD. However, treatment with inhaled corticosteroids does not significantly influence the PAFr. The bronchial biopsies from COPD patients are collected to investigate the regulation of PAFr expression and effect of inhaled corticosteroids (ICS) on PAFr expression (Shukla et al., 2014). In small AECs from COPD patients, DNA damage foci are correlated with increase in telomere without detection of telomere shortening. In a study conducted on primary human small airway epithelial cells, it was reported that cigarette smoke accelerated the dysfunction of telomere through reactive oxygen species, thereby leading to decreased lung function that is commonly observed during aging and in COPD patients (Birch et al., 2015). The long noncoding RNA (lncRNA) maternally expressed gene 3 (MEG3)-short hairpin RNA (shRNA) provides protection to cigarette smoke extract-induced human pulmonary microvascular endothelial cells suggesting that lncRNA MEG3 could be a novel COPD therapeutic approach (Bi et al., 2020).

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Damaged lung cells release acetylated H3.3 leading to serious damage and resulting in the progression of disease promoted by the addition of H3.3 secretion. The cytotoxicity of H3.3 on lung cells is evidenced by the involvement in perturbating the homeostasis of calcium ions and toxicity of mitochondria. In human AECs, the toxicity of H3.3 can be reversed partially by antibodies to either the C or N terminus of H3 (Barrero et al., 2013). The alteration of gene expression associated with COPD is summarized by the overexpression of activating transcription factor 4 in AECs. The change of transcription in the bronchial airway epithelium represents the molecular events of disease activity at more distal sites (Steiling et al., 2013). In COPD, the airway remodeling and inflammation occurring through regulation of epithelial-mesenchymal transition (EMT), and cyclooxygenase (COX)-2 expression were also found to be caused by overexpression

of farnesoid X receptor in small airways (Chen et al., 2016). Upregulation of hsa-miR-664a-3p in COPD patients and downregulation of target gene FHL1 positively associate with FEV1/FVC%. Cigarette smoke extract regulates both hsa-miR-664a-3p and FHL1 (Zhong et al., 2019). The overexpression of Kv1.3-channels is observed to promote COPD progression in T-lymphocytes isolated from COPD patients (Kazama & Tamada, 2016). Both rs1048829G > T and rs6435156C > T variants in BMPR2 are correlated with increased susceptibility to COPD. The binding of T variants of rs6435156 and hsa-miR-20a may elevate COPD risk and may further cause the downregulation of BMPR2 expression in the lung (J. Wang et al., 2016). In the diaphragm of COPD patients, the levels of myocyte enhancer factor 2C protein are found to be higher than in controls, whereas the expression of muscle-specific microRNA is downregulated. However, the types and sizes of muscle fiber and methylation of DNA between patients and controls were similar. In COPD, the sustained inspiratory loads of the respiratory system can be overcome by epigenetic events which act as biological adaptive mechanisms (Puig-Vilanova et al., 2014). The impaired pulmonary function in COPD can be caused by the components of the metabolic syndrome especially hyperglycaemia (S. M. H. Chan, Selemidis, Bozinovski, & Vlahos, 2019). Prolonged exposure to biomass smoke such as smoke produced by wood and coal burning increase the risks of COPD development, especially in women and children (Capistrano, van Reyk, Chen, & Oliver, 2017). Ambient air pollution levels lead to COPD exacerbation as pollutant particle matters induce apoptosis of airway epithelial cells (Yan et al., 2019).

Chronic colonization in the pathogenesis of COPD and the immune responses of colonized COPD patients often result in continuation of harmful immune responses comparable to those in AECOPD (Leung et al., 2017). The chronic *H. influenzae* infection is established through the

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exploitation of structural and immunological abnormalities in the COPD lungs. In most of the studies, the sputum cultures are commonly used to determine the presence or absence of *H. influenzae*, while novel molecular diagnostic methods are used to define the accurate prevalence of *H. influenzae* infections (Finney, Ritchie, Pollard, Johnston, & Mallia, 2014). Non-typeable *H. influenzae* (NTHi) promotes COPD exacerbations, and this is supported by the isolation of non-typeable *H. influenzae* from the sputum of COPD patients in a study. Its colonization persistently in the lower airway requires the adaptation of phenotype and virulent mechanisms with altering environmental pressures in the airway such as host immuno-inflammatory response (Su, Jalalvand, Thegerström, & Riesbeck, 2018). *H. influenzae* isolated from COPD exacerbated patients induce airway inflammation. Interaction with AECs results in larger numbers of exacerbation strains the epithelial cells and increased secretions of IL-8. Both these activities occur due to increased NF-κB activation and protein kinase signalling pathways activated by the p38 mitogen (Chin et al., 2005). *H. influenzae* reduces the activity of histone deacetylase (HDAC) in a co-culture of macrophage-like cell line and epithelial cells. The glucocorticoids only partially inhibit the NF-κB-mediated inflammation regardless of COPD and is induced by *H. influenzae*. Targeting the activity of HDAC can overcome the problem (Cosío et al., 2015). During *H. influenza*e infection, IgA proteases variants are expressed. These proteases are highly specific proteases that cleave the hinge region of human IgA1. IgA proteases mediate invasion and trafficking in human respiratory epithelial cells and accommodate the persistence of *H. influenzae* (Murphy et al., 2015). The lysosomal-associated membrane protein 1 is cleaved and intracellular survival in respiratory epithelial cells is mediated by IgA proteases B1 and B2-expressing strains. The expression of IgA proteases B1 and B2 is important for the pathogenesis of infection and selected by intracellular persistence of non-typeable *H. influenzae* (Murphy et al., 2017). The regulation of non-typeable

H. influenzae virulence factor is carried out by using slipped-strand mispairing in simple sequence repeats to undergo adaption for survival in the human airways (Pettigrew et al., 2018). In steroid-treated COPD, mucosal-associated invariant T (MAIT) cells is deficient in the blood and bronchial tissue. Non-typeable *H. influenzae* is a target for pulmonary MAIT-cell immune responses which is inhibited by corticosteroids (Hinks et al., 2016). *Haemophilus parainfluenzae* is commonly isolated from the sputa of patients with COPD. Increased levels of *H. parainfluenzae*-specific IgG are observed in the patients' sera in comparison to control subjects. The response of both specific antibody and antigen specificity are observed and have been reported. The existence of a specific immune response reveals *H. parainfluenzae* as an important pathogen in COPD (Mitchell & Hill, 2000). Host and bacterial proteases degrade midkine (MK), an antibacterial protein and causes the impairment of airways defense in COPD. Staphylococcal metalloprotease aureolysin causes MK proteolysis leading to impairment of bactericidal activity. In COPD sputum, the degradation of recombinant MK occurs more rapidly, and proteolytic activity confirmed by zymography is greater (Linge et al., 2013). Post-rhinovirus infection increased the bacterial burden and significant outgrowth of *H. influenzae* from existing microbiota is observed in COPD patients. The rhinovirus infection in COPD modifies the respiratory microbiome and thus, may cause secondary bacterial infections (Molyneaux et al., 2013).

Oxidative burden, nitrosative stress, airway inflammation, and damaged HDAC2 are primarily reported to exacerbate virus-induced COPD. This was observed in a study where the HDAC2 activity was reported to be measured *in vitro* (Molyneaux et al., 2013). Lung whole tissue explants demonstrated an enhanced COPD response. The innate immune responses were amplified by multiple stimulations of TLRs (Pomerenke, Lea, Herrick, Lindsay, & Singh, 2016). In AECOPD

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patients, increased expression of RSV-TLR3 correlates with decreased lung function (D. Liu et al., 2018). The experimental rhinovirus infection acts as a COPD exacerbation model which acts as a representation of casual connection between rhinovirus infection and COPD exacerbations and can aid in investigating mechanisms for virus-induced exacerbations. The neutrophilic inflammation and impairment of IFN produced by the BAL cell can act as vital mechanisms in COPD exacerbations (Mallia et al., 2011). The upregulation of dipeptidyl peptidase 4 expression has been reported in the lungs of smokers and COPD patients and is found to be an important cause for individuals to be more susceptible to Middle East respiratory syndrome coronavirus infection (Seys et al., 2018).

Mutation of CF transmembrane conductance regulator (CFTR) gene causes an autosomal-recessive, monogenetic disorder also known as CF. CFTR, a transmembrane protein, transports ions across the surface of epithelial cells. Dysfunction of CFTR has its impact on many organs, especially lung. The patients with rare or difficult to treat alleles may need an individualized treatment method including test systems or biomarkers. The cells are collected from patients for testing the most promising treatment combination *in vitro*. The transformation of nasal epithelial cells and intestinal cells into organoids was carried out in a study. The derivation of stem cells from skin fibroblasts or blood cells occurs which continue to transform into AECs (Ratjen et al., 2015). Both COPD and CF correlated and resulted in neutrophilic inflammation in the airway, progressive obstruction in airflow and repeated exacerbations. The dysfunction of airway epithelium is proven to be involved in disease onsets and progression through the morphological and molecular changes of the epithelium, as airway epithelium is important in the maintenance of normal function of the airway. The host defenses, immune responses and repair are affected by

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such abnormalities and cause lung damage and failure in lung function (De Rose, Molloy, Gohy, Pilette, & Greene, 2018).

Cell recognition receptors such as the phosphatidylserine receptor, CD36, and αv integrins are known to remove the inflammatory cells that undergo apoptosis. They are associated with the resolution of inflammation. In CF airways, persistent inflammation causes the removal of impairment of apoptotic inflammatory cells by the alveolar macrophages. However, in *in vitro* conditions the removal process was inhibited by airway fluid of CF and bronchiectasis in a neutrophil elastase-dependent manner. The neutrophil elastase can act as inflammatory marker both in early and later disease states. The phagocytosis of apoptotic cells is disturbed by cleaving phosphatidylserine receptor by neutrophil elastase. The specific cleavage may cause airway clearance of apoptotic cells to defect which involves ongoing airway inflammation. The apoptotic cells are found in the sputa from CF, NCFBE and chronic bronchitis. However, the apoptotic cell number in sputa from CF and NCFBE is higher than those with chronic bronchitis. The sputum from patients with CF, NCFBE and chronic bronchitis is collected and cultured to determine bacterial and fungal pathogens. Human monocyte-derived macrophages (HMDMs) and polymorphonuclear leukocytes (PMNs) were isolated using Percoll gradient centrifugation. Jurkat cells or PLB985 cells and human PMNs were exposed to ultraviolet irradiation and cultured. All cells underwent apoptosis through nuclear condensation. HMDMs were co-cultured with apoptotic Jurkat cells or PLB985 cells suspended *ex-vivo* in the absence of human serum. HMDMs were suspended in HBSS containing 2% FBS and incubated with primary Ab on ice. After washing twice, they were incubated with secondary Ab on ice. The analysis of washed macrophages was

carried out on a FACScan cytometer using PCLysys software (Gramegna et al., 2017; Vandivier et al., 2002).

In respiratory tract epithelium, the dysregulation of nitric oxide synthase (NOS) isoforms has been reported with a reduction of expired nitric oxide (eNO). The intense airway infiltration of phagocytes which accompany later disease stages has high levels of the hemoprotein myeloperoxidase (MPO), activity of NOS, and reactive oxygen species production. At airway surfaces, the bioavailability of NO is decreased by MPO which plays a role as a phagocyte-derived NO oxidase. *In vitro* studies show high MPO levels in CF sputum leading to high rates of NO oxidation. Hence, in CF sputum, MPO activity negatively correlates with the eNO levels. The sputa collected from CF patients was incubated, processed, and treated with DNase. The eNO was measured by using chemiluminescence-based NO analyzer, while the heme peroxidase activity is identified by measuring the rate of H2O2-dependent oxidation of tetramethylbenzidine. The NO catabolism was measured using a NO- specific electrode and Macintosh-based PowerLab data acquisition software package was used to record data. The MPO protein was analyzed using ELISA and Western blot. The polyclonal antibody against human MPO was used to carry out the immunodepletion of MPO. The NO metabolites (NOx) in sputum were quantified using Antek 7020 Chemiluminescence NO detector (Chapman et al., 2010).

CF causes disease onset and mortality as a result of chronic respiratory infections. The colonization of bacteria occurs in the CF airways in childhood, and then bacteria pathogens display the commensal microbes over time. Hence, lung health of patients decreases as microbial diversity declines (Kiedrowski & Bomberger, 2018). The respiratory viral infection may lead to CF

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progression. The viruses that commonly involve in that progression include RSV, influenza, parainfluenza, adenovirus, and rhinovirus. The persistence of bacteria and CF pathogenesis in the respiratory tract is indirectly promoted by these specific viruses (Kiedrowski & Bomberger, 2018). Early viral infections are correlated to significant neutrophilic inflammation and bacterial pathogens. They play a vital role in the initiation of lower airway inflammation in infants with CF. Antibiotics are often used to treat bacterial infections. Nasopharyngeal swabs are used to collect samples, whereas multiplex PCR assays were used in the detection of respiratory viruses. The bronchoscopy with BAL and pulmonary function testing are generally carried out in such patients (Kiedrowski & Bomberger, 2018).

Enhanced biofilms are formed due to coinfection of RSV on human AECs which is confirmed by the presence of *S. aureus* isolates. Presence of *P. aeruginosa* enhances the biofilm formation. On CF AECs, the growth of *S. aureus* is evaluated to investigate the impact of virus co-infection on its growth. The growth of *S. aureus* increased within 24 h and biofilm-like clusters of bacteria on CF AECs are observed under microscopy. The secreted factor produced during virus infection contributes to the formation of *S. aureus* biofilms. This is proved by the formation of biofilms without host epithelium promoted by apical conditioned medium from RSV-infected cells. The characterization of *S. aureus*-RSV coinfection is carried out by dual host-pathogen RNA sequencing to determine dedications of *S. aureus* and RSV to the host response during co-infection. The increased availability of host nutrients and upregulation of *S. aureus* genes involved in growth, protein translation and transport, and amino acid metabolism during RSV coinfection through the dual host-pathogen RNA sequencing (Kiedrowski et al., 2018).

P. aeruginosa often co-infects with other microbes and increases the morbidity and mortality in CF [95]. *P. aeruginosa* from various regions has similar transcriptional profiles although genetically different. In the upper and lower airways of CF lung, *P. aeruginosa* is isolated from five different sampling areas. The *in vitro* transcriptional profiles were analyzed by RNA sequencing. The determination of genetic diversity within and across the different sub-compartments is carried out by using colony re-sequencing and deep population sequencing (Kordes et al., 2019). *P. aeruginosa* is cultured and two *in vitro* phenotypes of *P. aeruginosa* isolates are exhibited to distinguish stages of early and later infection through pyoverdine production and decreased protease production. The consecutive pulmonary exacerbation is predicted through mucoidy and decreased twitching among the phenotypes tested. The prognostic markers of transition to chronic infection and advanced lung disease are indicated by these phenotypes (Mayer-Hamblett et al., 2014).

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Diverse clinical sources have revealed the level of inducible phages in *P. aeruginosa* isolates to be higher than those of other Gram-negative bacteria. This shows a high amount in the reservoirs of mobile DNA that involve in the adaptation and evolution of bacteria in the lung. The phages can interact and translocate across epithelial surfaces and allow a further layer of unexplored complexity to chronic respiratory infections. In CF and NCFBE patients, the diverse communities of inducible phages that relate with disease progression is harboured by *P. aeruginosa*. High infectivity across its genotypes is showed by its temperate phages from more advanced disease. The PAO1 antimicrobial susceptibility will be modified by temperate phage infection at antibiotics' sub-inhibitory concentrations. Hence, may lead to antimicrobial resistance (Tariq et al., 2019). The association between reduced lung function and warmer temperature is mediated

respiratory pathogens in CF. *P aeruginosa*, a common pathogen in CF often lives in an environment with warmer temperature and its growth may lead to declined lung function and serious CF issues in patients. *P. aeruginosa,* mucoid *P. aeruginosa*, and methicillin-resistant *S. aureus* are cultured and the association between temperature and lung function is determined by clustered linear and assessed by logistic regression (Collaco, Raraigh, Appel, & Cutting, 2016).

The mucins that act as carbon reservoirs for the CF lung microbiota are degraded by oral-associated anaerobes when utilization of mucins by *P. aeruginosa* decreases. The amino acids, propionate and acetate are generated by the fermentative metabolism of mucin-degrading communities *in vitro* as they grow on mucins. Many similar metabolites are also found within expectorated sputum. The commensal microorganisms may contribute to degrading mucins which provide nutrients for pathogens. Mucin fermentation cultures contain anaerobic species, anaerobic consortium, and saliva-derived mucin-fermenting bacterial community are used to inoculate mucin in minimal medium in molten agar under anoxic conditions. The additional molten minimal medium agar without mucin is inoculated with *P. aeruginosa* after solidification of the mucin-fermenting fraction. Samples are poured over the mucin fermenting community fraction and allowed to solidify. The negative controls are shown by tubes without mucin-fermenters (Flynn, Niccum, Dunitz, & Hunter, 2016). The expression of specific virulence factors such as antioxidants and acquisition of adaptive mutations during chronic infection display that *P. aeruginosa* is resistant to antibiotics and innate immune effectors. BAL fluid and immune cells are directly collected from CF patients. The study revealed concentrations of proinflammatory markers increased in both supernatants of CF epithelial cell cultures that are free of cells and in *in vitro*

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infection-free CF tissue specimens. The concentrations of proinflammatory cytokines such as TNF-α and IL-8 increased within BAL fluid (Malhotra, Hayes, & Wozniak, 2019).

Improved clinical outcomes are not often observed by increased killing of *P. aeruginosa* during pulmonary exacerbation (PEx). The pathogens can be identified based on biomarker of PEx outcomes by understanding the polymicrobial nature of CF and the heterogeneity of *P. aeruginosa*. The sputum samples are generally collected from adults infected with *P. aeruginosa* who experience PEx. The sputum is analysed quantitatively during hospitalization and at the end of therapy and was compared to baseline and follow-up samples. Alterations in *P. aeruginosa* burden from baseline was evaluated for morphotypes, mucoid and non-mucoid isolates. The identification of PEx is failed as more than 90% of baseline pulmonary function did not recover (Lam, Somayaji, Surette, Rabin, & Parkins, 2015). Under anaerobic condition, *P. aeruginosa*-infected CF lung is invaded by Bcc bacteria which inhibits the biofilm-like growth of *P. aeruginosa.* It expands the macrophage included host bacterial niche from mucus. Two Bcc species, *Burkholderia cenocepacia* and *Burkholderia multivorans* are grown for *in vitro* experiments. In the CF lung, Bcc bacteria are formed in single cells or small clusters within phagocytes and mucus. However, biofilm-like masses are identified in *P. aeruginosa,* and their densities decrease when co-infected with Bcc bacteria. The fermentation is used by *B. cenocepacia* and *B. multivorans* to obtain energy but not anaerobic respiration as test medium receives alternative electron acceptors under anaerobic conditions. Mucinases are expressed by both Bcc species as they can also produce carbon sources from mucins for growth purpose. Both Bcc species grew anaerobically under *in vitro* conditions as *P. aeruginosa* was present (Schwab et al., 2014).

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In a separate study, *P. aeruginosa* and *S. aureus* were co-cultured *in vitro* on monolayers of epithelial cells in human bronchi homozygous for the mutation of ΔF508 CFTR to show the interaction mechanisms. The transition from aerobic respiration to fermentation in the expression profile of *S. aureus* is driven by *P. aeruginosa* depending on the production of both 2-heptyl-4-hydroxyquinoline N-oxide (HQNO) and siderophores by *P. aeruginosa*. The lactate provided by *S. aureus* acts as a carbon source to *P. aeruginosa* over medium-supplied glucose. Both the bacteria coexist but the viability of *S. aureus* declines in an HQNO- and siderophore-dependent manner as *P. aeruginosa* is overly co-cultured. The mechanism provided to kill *S. aureus* is mediated by *P. aeruginosa*. Factors required for the mechanism include genetic factor, environmental factor, nutritional factor and HQNO and siderophores. The killing of *P. aeruginosa* is decreased in *S. aureus* small-colony-variant (SCV) genetic mutant strains compared to their wild-type parent strains. In the presence of *P. aeruginosa*, SCVs aids in sustaining the presence of *S. aureus* (Filkins et al., 2015).

Swabs from the airway clearance devices brought by patients were taken before and after cleaning in a study. The collected samples were cultured for bacterial counts and the total colony-forming units (CFU) are determined. Such devices can get contaminated after use, but appropriate cleaning can reduce contamination. Although the cross-infection between CF patients and devices is unknown, but CF microbiome still can be altered by using contaminated devices. The alterations may also change the disease progression. CF microbiota cannot be identified by culture-dependent techniques. There is an interaction of immune system and microbiota such as competing with the invading pathogens. Both the immunity and disease progression are influenced by change and loss of microbiota diversity (Manor, Gur, Geffen, & Bentur, 2017). There is an association between

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the decreased respiratory function and declined bacterial diversity. *Aspergillus*, *Candida*, and *Scedosporium* genera are shown by the inter-kingdom network. The Phy-Lasso method is used to identify the association of respiratory microbe with CF pulmonary exacerbation and lung function. The CF pulmonary exacerbation is associated with *Aspergillus* and *Malassezia* while *Scedosporium* and *Pseudomonas* are associated with CF pulmonary exacerbation along with decreased lung function. The correlation network predicts the cross-domain interactions between *Aspergillus* and *Streptococcus in vitro* (Figure 2).

In an *in vitro* experiment, the strains of *Streptococcus oralis* and *Streptococcus mitis* were isolated from CF patients. At 37 °C and under aerobic conditions, co-cultures with *Aspergillus conidia* were carried out in brain-heart infusion medium with moderate shaking. *A. fumigatus* is grown alone or co-cultured with *S. oralis* or *S. mitis*. Their growth is measured every 24 hours for five subsequent days (Fig 2). The diluted aliquot of microbial brain-heart infusion-mixture is plated on chocolate agar and fungal CFUs are counted after incubating for 24 hours. The experiment was carried out in triplicate (Soret et al., 2020). The defective immune function and chronic airway damage or infection characterise bronchiectasis. The levels of IgG3, B cell lymphocytes and Th lymphocytes are lower than controls. They may have low oxidative burst in bronchiectasis. Full blood examination is carried out to analyse samples. The whole blood assay is used to assess the neutrophil oxidative burst and phagocytic function. The phorbol myristate acetate and N-formyl-Met-Leu-Phe act as the stimuli that are used generally to stimulate oxidative burst (King et al., 2006; Vandivier et al., 2002).

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There has been an association between Pregnancy Zone Protein (PZP) and airway infection, formation of neutrophil extracellular traps, as well as disease severity in bronchiectasis. PZP plays a role in the suppression of T-cell function during pregnancy for the prevention of fetal rejection. The identification of increased PZP is carried out by label-free liquid chromatography or mass spectrometry. The elevated PZP associates with airway infection with *P. aeruginosa*, while the sputum is correlated with the Bronchiectasis Severity Index, exacerbations frequency, and symptoms. There is also a direct relation between PZP in sputum and airway bacterial load. The formation of NETs with phorbol myristate acetate through induction by neutrophils releases high concentrations of PZP *in vitro*. The presence of PZP in neutrophil extracellular traps is confirmed by fluorescence microscopy, whereas the localization of PZP to the cytoplasm and nuclei of neutrophils is detected by fluorescence and electron microscopy (S. Finch et al., 2019). From the origin of adeno- and squamous cell carcinoma, the proliferation of non-small-cell lung cancer (NSCLC) cell lines such as A549 and H226 which express TLR2 mRNA are induced by the lipoteichoic acids (LTA) of *S. aureus*. The release of IL-8 in the NSCLC cell lines is also induced by LTA. The increased IL-8 mRNA expression and dose- and time-dependent IL-8 release accompany with the cell proliferation. The A549 cells undergo metabolism increasingly. The antibodies neutralized to target IL-8, inhibited the LTA-induced A549 cells proliferation.

In another *in vitro* study, A549 and H226 were cultured at 37 °C with 95% air and 5% CO_2 for experimental purpose and exposed to LTA. The LTA was prepared with high purity and its concentration for stimulation of A549 cells proliferation was lower than the concentration of lipopolysaccharide (LPS). The MTS assay is generally used to quantify the proliferation and metabolic activity of cells. The antibodies inhibit LPS- and TLR4-induced cellular responses.

However, it does not change the proliferation of LTA-induced NSCLC cell line. The LTA induces pro-proliferative A549 cells response and various kinetics are performed compared to LPS, with 24 hours as maximum and dropping afterwards (Hattar et al., 2017). MicroRNAs mediate stem cell pluripotency, differentiation, and malignancy. The miR-487b is repressed by cigarette smoke condensate which targets SUZ12, BMI1, WNT5A, MYC, and KRAS. The targets overexpress in primary lung cancers and are correlated with repressed miR-487b. Within the genomic locus of miR-487b, the methylation of DNA, occupancy of *de novo* nucleosome, and reduction of H2AZ and TCF1 levels occur together with repression of miR-487b. miR-487b is depressed and its CSC-mediated silencing is weakened by deoxy-azacytidine. Wnt signaling is removed by miR-487b expression. The CSC or miR-487b targets' overexpression mediate *in vitro* proliferation and invasion of lung cancer cells is also inhibited by miR-487b expression. ECM-coated semipermeable modified Boyden chambers are used for evaluation of cell invasion *in vitro*. miR-487b is able to suppress tumour and the epigenetic mechanisms silence it during tobacco-induced pulmonary carcinogenesis (Xi et al., 2013). EMT involves in small airway fibrosis and alteration in ECM. COPD is closely associated with lung cancer development and the development link with EMT as associated with angiogenesis. In bronchial epithelial cells, there are phosphodiesterase 4 inhibitors such as roflumilast N-oxide that regress EMT *in vitro* through the restoration of cyclic adenosine monophosphate levels in cells. In the small airway epithelium with COPD, an active EMT process is involved by increased Urokinase-type plasminogen activator receptor expression. In human small airway epithelial cells, the EMT that is induced by cigarette smoke is inhibited by targeted silencing of uPAR with the usage of shRNA (Sohal, Mahmood, & Walters, 2014).

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4.0 Current Advances in the Development of Diagnostics and Treatment Strategies Using *In Vitro* Experimental Models

4.1 Asthma

The mechanisms of virus-induced asthma and exacerbation of asthma may be represented *in vitro* in human cells (Hansbro, Horvat, Wark, & Hansbro, 2008). An *in vitro* model can be used to mimic human pathophysiology and help in disease diagnosis (Saturni, Contoli, Spanevello, & Papi, 2015). In children with asthma, asthma attacks may be caused by the characteristics of host-microbiota interactions and the endotypes of asthma may be defined (Lejeune et al., 2020).

MiR-29c can be a biomarker and is used as a novel therapeutic approach for the monitoring of asthma. Exacerbated asthmatics have high B7-H3 and low miR-29c expression. The regulation of Th cell differentiation is carried out by the expression of miR-29c on macrophages by targeting B7-H3. The significance of miR-29c/B7-H3 axis is shown through the regulation of differentiation in Th2/Th17 cell. In co-enculturation of CD4 T cells, the expression of ROR-γt and GATA-3 is increased by transfection of anti-miR-29c into macrophages (X. Zhang et al., 2018). Various immune responses such as allergic, autoimmune, and graft-versus-host responses are regulated by regulatory T cells (Tregs). Several studies have shown that there is more efficiency in antigen specific Tregs transfusion which is significant in asthma prevention and control compared to polyclonal Tregs transfusion and is able to act on the site of lesion precisely. The antigen-modified Tregs which is expanded *in vitro* is used in most of the studies while only one uses gene-edited Tregs transfusion (Zhao & Wang, 2018).

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In *in vitro* conditions under the provision of strong bronchodilation, shortening of inflammatory responses is induced by allergens in the airways. The regulation in the proliferation of airway smooth muscle (ASM) cell, and alleviation of airway remodeling features are carried out by the agonists of bitter taste receptors (TAS2Rs). The properties of fibrotic airway remodeling decline due to TAS2R agonism. Functional tachyphylaxis is not caused by TAS2R therapy (Nayak, Shah, Michael, & Deshpande, 2019). The depolarization of *in vitro* human vagus is inhibited by dopamine receptor ligands and may help to treat cough and mucus secretion in asthma. The sensory nerve-mediated responses may be inhibited by dopamine receptor agonists. The drug, opioids are not very effective, but their side effect is low. Hence, this may act as a greater therapeutic window (Birrell et al., 2002). The effects of budesonide and formoterol are evaluated *in vitro* on the expression IL-17A, RORγ(t) and FOXP3 in cultured T lymphocytes in asthmatics and both of them may be used for therapeutic control. RORγ(t) and IL-17A are decreased, FOXP3 is increased by the combination treatment of Budesonide and Formoterol. The IL-17A mediated IL-8 secretion is declined by the combined treatment. The immunity mediated by Th17 may take part in the airway disease with allergic asthma in which Th17 cells and IL-17A are involved in the development of allergic diseases (Albano et al., 2013).

In asthma patients, the secretion of inflammatory cytokines from PBMCs is inhibited by enoxaparin and dalteparin on inflammatory markers because of the presence of components with various structures in the two LMWHs arising from various depolymerization approaches. PBMCs are isolated from collected blood, pre-treated with or without the presence of LMWHs in various concentrations and then stimulated by phyto-haemagglutinin for the secretion of IL-4, IL-5, IL-13 and TNF-α. The LMWHs were tested to determine the molecular weight, anticoagulant potency,

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and effect on cytokine secretion(Shastri et al., 2015). Seonpyejeongcheon-tang (SJT) can be used to treat asthma. The SJT microparticles (SJT-MPs) provided useful aerodynamic characteristics. No *in vitro* cytotoxicity against RAW 264.7 macrophages is observed with SJT microparticles at 0.01 to 3 mg/mL. For the inhibition of asthma-related adverse changes, the SJT-MPs is more effective compared to oral dexamethasone or SJT extract applied at a higher dose. The SJT-MPs protect against lung injury through the suppression of pro-inflammatory cytokines production (Yang et al., 2016).

Several studies state that vitamin D deficiency can lead to asthma. In patients resistant to steroids, the therapeutic responses to glucocorticoids are increased by vitamin D3. The inhibition of inflammation in the airways is carried out by vitamin D to maintain respiratory health through an additional pathway. The benefits of vitamin D effect in asthma are evidenced by the signal pathway of vitamin D receptor *in vitro* (Huang et al., 2013). *In vitro* study shows that after exposure to TNF-α, the higher levels of IL-10 are released by neonatal nasal airway epithelial which is exposed to a higher level of the maternal vitamin D (Hejazi, Modarresi-Ghazani, & Entezari-Maleki, 2016). The inhibition of VEGF-induced ASM cell proliferation is triggered by 1,25(OH)2D3 *in vitro* through the downregulation of ADAM33 and suppression of both VEGFR2 and ERK1/2 activation. The expression of ADAM33 in ASM cells correlates to asthma (S.-H. Kim et al., 2017).

In a study, selenium and several methyl donor dietary nutrients involved in the pathway of one-carbon metabolism correlated with increased life quality measures of asthmatics. Intake of that nutrients including folate correlated positively with LINE-1 methylation, the high levels of which correlates with greater dPFV, while nutrients intake correlates inversely with IFNγ CpG-186. The

food frequency questionnaire is used to evaluate dietary intake. The pyrosequencing assay is used to measure levels of LINE-1 methylation and methylation levels of IFNγ CpG-186 and -54 from buccal cell DNA (Montrose et al., 2017).

4.2 Chronic Obstructive Pulmonary Disease (COPD)

Airway and systemic inflammation measured by fibrinogen are greater in stable COPD patients with potentially pathogenic microorganisms detected by using quantitative PCR. The airway has a total pathogen-load threshold without systemic inflammation due to *H. influenzae* (R. Singh et al., 2014). The electronic nose (e-nose) aids in identifying the exacerbations of COPD, especially if it correlates with airway bacterial infection or pneumonia (Shafiek et al., 2015). The absolute values which are measured by using the impulse oscillometer and tremoflo devices may not be compared directly as that variation in the calibration procedures may cause differences (Lundblad, Miletic, Piitulainen, & Wollmer, 2019). Increased activity of indoleamine 2,3-dioxygenase (IDO) and tryptophan hydroxylase (TPH) acts as an important characteristic in COPD. The levels of TPH activity and serotonin are higher in smokers with COPD compared to non-smokers. The tryptophan metabolites are mediated by both IDO and TPH and correlate with gender (Naz et al., 2019).

The genes that correlate with COPD are identified experimentally by transcriptomic analysis and the differentiation of smokers and COPD patients from non-smokers is carried out accurately by 15 genes through a machine-learning approach. The risk continuum across smoking and COPD pathogenesis can be quantified by potential risk factor (PRF) index. The expression levels of 15 genes are generally changed among *in vitro* tissues exposed to factors such as oxidative stress,

cigarette smoke, inflammation, and DNA damage. The effectiveness in estimating the risk of tobacco products is shown by the dose-dependent rise of PRF which is demonstrated by *in vitro* tissues exposed to cigarette smoke (Matsumura & Ito, 2020).

The inhaled cigarette smoke often causes irreversible limited airflow in most COPD cases, but the genetic predisposition also leads to COPD. *In vitro* modelling is combined with whole-exome sequencing for the identification of COPD genes. The genes are identified based on the analysis of rare variant and exotic variants in various individual genes between susceptible and resistant smokers through the computational establishment of their function. TACC2 and MYO1E are identified as cigarette smoke-induced cytotoxicity and they elevate the COPD susceptibility through analysis by using both genetic and functional methods (Bruse et al., 2016). By using next-generation sequencing and bioinformatics, the cell-cell contact, leukocytes activation, T lymphocytes activation, and cellular homeostasis have been established to correlate with the miR6511a-5p–NT5E interaction which plays an important role in COPD. The interaction of miR641–PCDH7, miR3173-3p–SDK1, miR6511a-5p–NT5E and miR4435–TNS1 is observed in COPD bronchial epithelium (Chang, Tsai, Jian, Sheu, & Kuo, 2018). The phenotyping of AECOPD can be conducted by rapid molecular diagnostics on sputum. The primary diagnosis of AECOPD in sputum is carried out through molecular array. The patients can be grouped into positive or negative pathogen group based on the bacterial or viral pathogen and detected by molecular array. The best prediction is provided by bacteria-positive group, whereas the negative pathogen groups will give the opposite (Alotaibi et al., 2019).

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Recondensation of chromatin occurs during NETosis and double-helix DNA in 'beads-on-a-string' conformation is generated. The analysis of NET structure requires *in vitro*-induced NETs from human neutrophils. The marked presence of NETs and NETotic neutrophils is a characteristic feature of COPD sputa (Obermayer et al., 2014). Statins are shown to influence neutrophil functions positively which correlate with COPD pathogenesis and improve lung outcomes in the *in vitro* experiments (Walton et al., 2016). The expression of Club Cell Protein 16 (CC16) in airway inversely resembles airflow obstructive severity in COPD patients. The levels of CC16 increase when recombinant CC16 is used in cell culture. The CC16 overexpress due to mediation of plasmid and adenovirus in epithelial cells results in the reduction of inflammation and cell injury. The richest protein in bronchoalveolar lavage fluid is CC16. COPD is correlated with deficiency of CC16 as CC16 can undergo anti-inflammation(Laucho-Contreras et al., 2016). Stem cell therapies can be used to treat COPD and degenerative lung diseases. Various signals may be delivered by stem cells to host cells such as the mechanism of regeneration against alveolar destruction in the COPD lung. In elastase- and cigarette-induced COPD, the alveolus can be regenerated by mesenchymal stem cells as stem cells affect the regenerative gene. The mesenchymal stem cells also reduce the inflammation in the airway (Kokturk, Yıldırım, Gülhan, & Oh, 2018).

Mesenchymal stromal cells which have robust immunosuppression can be collected from various tissues and could be efficiently expanded. The benefit in pulmonary function tests, quality of life reports, decline in systemic markers of inflammation can be enhanced by using mesenchymal stromal cells combined with another primary treatment (Antunes, Lapa E Silva, & Rocco, 2017). Inflammation of neutrophils from COPD patients *in vitro* is inhibited by corticosteroids and the

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inhibition is enhanced by long-acting muscarinic antagonists (LAMAs). The patients' neutrophils are obtained and the anti-inflammatory effects of LAMAs with corticosteroids and their additive effect are compared. In COPD patients, activation of corticosteroid-resistant neutrophil is reduced by aclidinium bromide. Increased anti-inflammatory effect was observed when combined with fluticasone propionate (Milara et al., 2016). The anti-inflammatory effect and immuno-modulation can occur in new macrolides used to treat COPD. However, it does not have antibiotic effects. The exacerbation frequency of COPD reduces with long-term use of macrolides. For the immuno-modulation, macrolides affect the adaptive immune response. Erythromycin causes *in vitro* downregulation of CD40 on dendritic cells. In combination with corticosteroids, it elevates the anti-inflammation of steroids for COPD treatment (S. Qiu & Zhong, 2017).

Carbocisteine demonstrates anti-inflammatory and anti-oxidative effects in human model *in vitro*. The recruitment of inflammatory cell to the airways reduces and the injury of endothelia and sensitivity of associated cough is attenuated by carbocisteine. It correlates with cough sensitivity and plays a role as free-radical scavenger. There are larger effects of mucolytic and antioxidant agents in patients without inhaled corticosteroid standard management (Hooper & Calvert, 2008). Multidrug resistance-associated protein 1 (MRP1) protects against toxic compounds and oxidative stress. The model MRP1 substrate, carboxyfluorescein is measured using functional flow cytometry. In bronchial epithelial cells, the activity of MRP1 is modulated by various drugs such as budesonide, formoterol, ipratropium bromide, and N-acetylcysteine (NAC). The *in vitro* effects of these drugs are analysed using immortalized human bronchial epithelial cells. Budesonide causes a concentration-dependent reduction in transport of carboxyfluorescein in MRP1, while the impact of formoterol is low. The transport of carboxyfluorescein increases as formoterol is added

to budesonide in its highest concentration. NAC also demonstrates a concentration-dependent increase in transport of carboxyfluorescein. Ipratropium bromide increases the transport of carboxyfluorescein at higher concentration but reduces the transport at low concentration (van der Deen et al., 2008). The interference of N-acetylcysteine (NAC) in the COPD pathogenesis and its ability to lower the oxidants level suggest that NAC can be beneficial in COPD therapy. The effectiveness of anti-muscarinic bronchodilators commonly used in COPD patients can be increased by NAC. The oral administration of NAC can be carried out efficiently, used easily and well-tolerated. The administration of NAC can also be carried out through other routes (Sanguinetti, 2016).

The combination of long- acting β- agonist and LAMA is the best therapy to decrease COPD exacerbations. The combined therapies are more effective compared to monotherapies for better symptom and quality- of- life scores. Based on the statistics of rank, LAMA that contains inhalers may be better for the prevention of COPD exacerbations compared to those without LAMA. The inhalers which contain corticosteroid (ICS) are correlated with an elevated pneumonia risk (Oba, Keeney, Ghatehorde, & Dias, 2018). PUR0200 therapy caused bronchodilation in COPD patients and its magnitude is similar to that of tiotropium HH. Its clinical effects on lung function enables PUR0200 with lower dose of tiotropium to be used compared with tiotropium HH. *In vitro* properties of aerodynamic particle size distribution of PUR0200 and tiotropium HH are shown. Through the comparison with the tiotropium HH, the raised fine- particle fraction of PUR0200 is indicated by testing using the next- generation impactor. The proportion of drug available for lung deposition will be raised by the raised PUR0200 fine- particle fraction (D. Singh, Ravi, Kane, Schmalbach, & Hava, 2018).

In COPD patients, personalized medicine is needed and various factors such as genetic, environmental, biological, and clinical factors are integrated into its experimental and computational models to interpret the multilevel COPD complexity (Franssen et al., 2019). It is normal that sometimes there is no response to treatment pharmacologically or non-pharmacologically. Hence, the disease managements with higher specificity and integrity are needed with a combination of the basic principles of predictive, preventive, personalized, and participatory medicine. The balanced immune response, maintained microbiome, reduced air pollutants and smoke, full inflammatory resolution, healthy diet with good antioxidant load, and physical activity can contribute to reducing COPD risk and exacerbation (Bagdonas, Raudoniute, Bruzauskaite, & Aldonyte, 2015).

4.3 Cystic Fibrosis

In a study, pediatric CF nasal epithelial cells were cultured at the ALI. Full differentiation into ciliated, mucus-producing, and basal cells was reported. This may be correlated with *in vivo* characteristics of the human respiratory epithelium satisfactorily. The cell samples were collected by nasal cytology brush sampling and the properties of cultures such as cellular content, ultrastructure, morphology, and functionality were characterized (Schögler et al., 2017). The CF respiratory microbiota analyses without culture such as Illumina MiSeq platform and PCR have proposed novel advanced CF care strategies to determine multiple bacterial communities, including genera not typically identified by usual laboratory cultures. The difficulty in investigating the CF infections' complexity was also emphasized in the study. Sophisticated

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models and analytic methods should be developed to link between CF respiratory microbiota and clinical consequence and finally express this information for the improvement of patient care (Caverly & LiPuma, 2018).

Potential biomarkers of infectious diseases, especially those diseases influencing the respiratory tract is represented by volatile molecules in exhalation. The CF bronchial epithelial (CFBE) cells with and without *P. aeruginosa* infection can be differentiated through the identification of volatile metabolic properties by Random Forest (RF) (Figure 3). However, CFBE with and without RSV infection cannot be differentiated by RF. The probability of identifying RSV-infected cells between uninfected CFBE and *P. aeruginosa*-infected CFBE show that infection of RSV causes the sharing of properties between the volatile metabolic profile and both of these groups. The *P. aeruginosa*-produced volatile metabolites without growing in CFBE is measured to explain the biological origins of the volatile metabolites accurately. The production of volatile metabolic signature is more likely occurs from the interaction of *P. aeruginosa* with the CFBE cells and gives clinical effectiveness in diagnosing and controlling CF (Castellani, Di Gioia, di Toma, & Conese, 2018).

Cellular models developed for the human CF airway epithelium are used to understand lung inflammation and mucus production. Two-dimensional and three-dimensional cultures are referenced for better generalization of the native airway epithelium. Fibroblasts, airway epithelial cells and immune cells such as macrophages and dendritic cells are co-cultured for involvement in the modelling of disease, discovery of drug, and identification of new therapeutic targets (Castellani et al., 2018). The finite supply of the airway epithelial cell source is overcome by two-

dimensional cultures, which are the backbone of *in vitro* cellular models and improved expansion protocols. The three-dimensional airway and intestinal organoid models are established and the challenges and latent improvements in each system are evaluated. The preclinical pharmacotherapy screening for identification of responsive patients is carried out through *in vitro* CFTR functional assays in patient-derived organoids. The organoids are useful as they take part in the explanation of disease mechanisms, design of new treatments and development of personalized management for CF patients (Awatade et al., 2018).

The bacteriophage-based therapy plays an important role in the treatment of respiratory infections in CF which is shown by *in vitro* data. The drug can be administered through the inhalation route. The sequencing of bacterial genome identifies putative target genes that associate with the activity of bacteriophage as the molecular biology develops. In the first randomized controlled study, the efficacy of bacteriophages to treat *P. aeruginosa* infections in CF patients is evaluated. The environmental strains of *P. aeruginosa* may be less susceptible to bacteriophages compared to clinical strains. The bacterial resistance to bacteriophages may result in altered or loss of receptors and exopolysaccharides overproduction such as alginates. In CF, there are more lysogenic prophages that occupy commonly within bacterial genomes compared to lysogenic form. However, it can convert to the lytic phenotype which involves in the regulation of bacterial densities. Besides, the horizontal gene transfer is correlated to temperate bacteriophages (Hraiech, Brégeon, & Rolain, 2015).

An *in vitro* model of a biofilm that is grown in trypticase soy broth supplemented with glucose and NaCl (TGN) or in artificial sputum medium evaluated the antibiotic activity in CF patients. In

terms of viability, the metabolic activity, biomass production, the activity of antibiotics in artificial sputum medium were observed to be less efficient than in TGN which is shown by the full concentration-response curves of antibiotics collected after one-day biofilms incubation. The usage of artificial sputum medium may support to define the effective concentration of drug or to assess novel therapies against biofilms in CF patients. In both media, rifampin shows the highest effectiveness towards biomass compared to other antibiotics (Diaz Iglesias, Wilms, Vanbever, & Van Bambeke, 2019).

4.4 Lung Cancer

A novel *in vitro* model of NSCLC, OncoCilAir™ comprising of a reconstituted human airway epithelium, lung fibroblasts and lung adenocarcinoma cell lines have been developed. This model employs two-photon laser induced autofluorescence combined with spectrally resolved imaging of human lung cancer cells. The autofluorescence provided by OncoCilAir™ is similar to the observations in lung tissues of patients. The spectral and intensity heterogeneity of autofluorescence is found at the edges of tumours which denotes the influence of tumour to its microenvironment (Kilin, Mas, Constant, Wolf, & Bonacina, 2017).

Volatile organic compounds released from cancer cells can act as odour signature and can perform as promising non-invasive screening of lung cancer employing gas array sensor devices. An *in vitro* study utilized e-nose technology with various statistical approaches to identify the best classifier. Lung cancer cells (A549 and Calu-3) were investigated using breast cancer cells (MCF-7) and non-cancerous lung cells (WI38VA13) as control. The study provided a list of possible

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volatile organic compounds that can be used as specific biomarkers for lung cancer and a linear discriminant analysis-based one versus all-support vector machine classifier that can produce high performance differentiation of lung cancer from breast cancer cells and normal lung cells (Thriumani et al., 2018).

In small cell lung cancer (SCLC) patients, the medium survival time is usually less than 2 years. In addition, biopsy sample for investigation especially from the extensive stage patients is scarce. Thus, repeat sampling at advanced stage of the disease can be rarely performed. In order to resolve this limitation, the circulating tumour cell-derived explants of SCLC have been developed as they imitate the donor tumour pathology and response of chemotherapy. The short-term cell-derived explants tumour cell cultures are beneficial in identifying the clinical biomarker, establishing mechanisms of resistance that leads to recalcitrant tumour and screening of compound (Lallo et al., 2019). lncRNA HOTAIR is a marker of cell cycle dysregulation in lung cancer. It can be used as an indicator of drug resistance while using cell cycle inhibitors. The HOTAIR regulates the Rb- E2F pathway, thereby promoting the cell cycle passing through the restriction point during G1- S phase. In *in vitro* and *in vivo*, HOTAIR involves in the proliferation, migration, and invasion of lung cancer cells via the EMT and the β- catenin pathway. The resistant response to gefitinib through the dysregulated cell cycle mechanism correlates to high expression of HOTAIR (M. Liu et al., 2018). Other novel drug classes such as tyrosine kinase inhibitors, checkpoint inhibitors and immunotherapy including chimeric antigen receptor T cell for lung cancer may also contribute to the future vision of anticancer treatment (C. Zhang, Leighl, Wu, & Zhong, 2019).

Silk, which has the properties of biocompatibility and controlled degradation has been increasingly utilized as carriers for pulmonary drug delivery in lung cancer. The spray-dried or spray-freeze-dried silk fibroin particles can be delivered to the airways as dry powder inhalers. The *in vitro* lung deposition of the particles revealed high aerosolization comparable with commercially available dry powder inhalers. The particles were also found to be cytocompatible with A549 human lung epithelial cell line. Enhanced cytotoxic activity of cisplatin was observed when it was delivered as cross-linked silk-based particles (S. Y. Kim et al., 2015). Chemotherapeutic nanocarriers through aerosol delivery is an alternative therapy for lung cancer. The effectiveness of cross-linked gemcitabine (Gem)-loaded gelatin nanocarriers with genipin (Gem-GNCs) has been successfully evaluated as a nebulized treatment for lung cancer. The Gem-GNCs in nebulized form has satisfactory mass median aerodynamic diameter, geometric standard deviation, and fine particle fraction. The dynamic complex viscosity of mucus is reduced by that formulation consistent with raised mobility of nanoparticles.

For the evaluation in determination of release mechanism, Gem is released *in vitro* from Gem-GNCs in Dulbecco's phosphate-buffered saline and simulated lung fluid. Under different pH, the assessment of particle size stability is done. The differential scanning calorimetry is used to undergo determination of presence and stability of Gem-GNC components, while powder X-ray diffraction takes part in determining amorphization of Gem. The MTT assays are used to evaluate efficacy of Gem-GNC within A549 and H460 cells. The mucus rheology is treated by Gem-GNCs, lactose, and normal saline control and is measured after treatment (Youngren-Ortiz et al., 2017). Specific agents cause chemoprevention through reversion, suppression, or prevention of carcinogenesis. The trials of endpoint biomarker replacement have been carried out to support the

identification of agents for the trials in later-stage chemoprevention. It promotes the assessment of promising agents and decreases the need for large sample size, long duration, and expense (Szabo, Mao, Lam, Reid, & Keith, 2013).

Both primary lung tumour and metastasis develop from extrapulmonary neoplasms, and the development is facilitated by the lung microenvironment which involves blood vessel development, inflammatory process, modulation of immune system and body response to treatment. The processes of tumour microenvironment are significant in defining clinical biomarker and treatment for lung cancer. The development in the identification of key mutational change have resulted in major advances of molecular therapy, but the resistance mechanism limits its efficacy (Altorki et al., 2019). The overexpression of cullin 4A (Cul4A) is often observed in lung cancer cells. The shRNA can decline the Cul4A expression and then reduce the cancerous cell growth and proliferation. The body sensitivity to gemcitabine is raised in that effect. After the declined Cul4A expression, the expressions of TGF- β induced TIEG1 and TGFBI raise which correlate to raised chemosensitivity to gemcitabine. The relation between Cul4A and RNAi is important in the development of future lung cancer treatment (Hung et al., 2016).

4.5 Other Chronic Respiratory Diseases

In NCFBE, the ciprofloxacin trial revealed significant clinical benefits, while the aztreonam trial did not. Inhaled antibiotics are used to manage severe cases of NCFBE. They can be delivered to lung with minimal adverse effects and systemic toxicity compared to other routes of administration. The chronic infection stage requires long-term antibiotic therapy and associates with the mucoid

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strains of *P. aeruginosa* which are characterized by the formation of a protective polysaccharide biofilm. The sputum samples are collected from the patients. The density of bacteria in sputum is declined with inhaled ciprofloxacin. This reduction in bacteria which include *P. aeruginosa* is associated with decrease in the risk and frequency of exacerbations (Dhand, 2018).

Table 2: Extrapolation of outcomes from experimental models to clinical applications in human disease: variables and implications of *in vitro* experimental models

Context	**Dimension**	**Systems used to model ex vivo or in vitro**	**References**
Cell type	Authentication of cell type	Skewing conclusions if cell type tested is not correct	(Geraghty et al., 2014; Hughes, Marshall, Reid, Parkes, & Gelber, 2007)
Culture conditions	Media	The cell behaviour may be affected by the additives that are present in media	(Geraghty et al., 2014)
	Matrices	The cell phenotype may be affected by Matrices	(Bonvillain et al., 2013; Booth et al., 2012; Ghaedi et al., 2013; Gilpin et al., 2014;

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			Shojaie et al., 2015)
	Dimensions and mechanics	The behaviour of cells may change in two versus three dimensions, and on varying stiffness	(Guillame-Gentil et al., 2010; Maniotis, Chen, & Ingber, 1997; Nikolić & Rawlins, 2017; Rubashkin, Ou, & Weaver, 2014)

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5.0 Evaluation of Advanced Drug Delivery Systems as Novel Therapeutics for Chronic Respiratory Diseases Using *In Vitro* Experimental Models

Recent advances in nanotechnology, formulation strategies and drug delivery has made it possible for quick and reliable *in vitro* testing of emerging candidate drug including herbal single compound in various chronic respiratory disease model (Meenu Mehta, Saurabh Satija, et al., 2020; Keshav Raj Paudel, Nisha Panth, et al., 2020; Parteek Prasher et al., 2021). For example, the formulation of liquid crystalline nanoparticles offer versatility in testing therapeutic efficacy of different potential chemicals (Y. Chan, Mehta, et al., 2021). An *in vitro* study in human broncho alveolar epithelial cell line (BEAS-2B) stimulated with *P. aeruginosa* LPS was used to study the anti-oxidative potential of rutin-loaded liquid crystalline nanoparticles. The high level of reactive oxygen species and nitric oxide production induced by LPS was significantly attenuated by rutin-

LCNs formulation (K. R. Paudel et al., 2020). Further validation with PCR revealed that antioxidant activity was primarily due inhibition of oxidative stress related genes (*Nox4*, *Nox2B*) and upregulation of antioxidant genes (*GCLC*, *Nqo1*) (M. Mehta et al., 2021). Similarly, *in vitro* models also offer testing of compounds against lung cancer cell lines. Naringenin-LCNs (Wadhwa et al., 2021), rutin-LCNs (Paudel et al., 2021), boswellic acid-loaded chitosan nanoparticles (Solanki et al., 2020), and curcumin (Hardwick et al., 2021) have been investigated to inhibit the migration and proliferation of human lung cancer cell line (A549). *In vitro* experiments conducted in A549 and primary bronchial epithelial cells revealed that, IFN-β exerts robust and extended protection against rhinovirus infection thus suggesting IFN therapy can prevent COPD exacerbation (Gaajetaan et al., 2013). These advanced drug delivery systems target various pathways involved in chronic respiratory diseases such as MMPs (Meenu Mehta, Keshav R. Paudel, et al., 2020), NF-κB pathway (P. Prasher et al., 2021), phosphoinositide-3-kinase (Y. Chan, MacLoughlin, et al., 2021). Thus, these cellular signalling pathways can be targeted by nanocarrier-based drug delivery systems for the management of chronic respiratory diseases (M. Mehta et al., 2020).

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As discussed earlier, the elucidation of precise molecular mechanisms underlying the therapeutic actions of advanced drug delivery systems can be done by using modern three-dimensional microfluidic *in vitro* experimental models, as these models can maintain cell phenotypes and functions in a physiologically relevant way that closely mimics the human *in vivo* environment. Therefore, these represent important translational models for the evaluation of clinically relevant issues, the discovery and identification of novel therapeutic targets, as well as for the preliminary evaluation of novel therapeutics. As such, future studies on advanced drug delivery systems for

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chronic respiratory diseases should be performed on three-dimensional experimental models instead of two-dimensional models for an accurate simulation of tissue-specific physiological or pathophysiological disease-specific microenvironment in which cells would be able to aggregate, proliferate, and differentiate, providing researchers with insight on cell-to-cell, cell-to-matrix, and cell-to-mechanical forces interactions, as well as other conditions that are biologically relevant and closely recapitulate a particular disease phenotype. Nevertheless, although these experimental models closely resemble an *in vivo* environment, human clinical trials must still be performed to affirm the findings obtained from these experimental models to establish a clear profile on the mechanisms of action, efficacy, safety, and toxicity of advanced drug delivery systems prior to their clinical use, as the findings obtained from human trials may differ from those of preclinical studies due to the presence of a complex biological environment and interactions with other body systems that cannot be replicated in experimental models. In short, translation of these *in vitro* knowledge into *in vivo* pre-clinical and clinical models for the effective management of chronic respiratory diseases is the current necessity of the overall research and scientific community to address the growing threat of these diseases to public health.

6.0 Conclusion

There is a tremendous increase in the number of published *in vitro* research studies especially in the area of chronic respiratory diseases in recent years. These studies facilitate the identification and classification of chronic respiratory diseases into various groups based on their aetiology, molecular mechanisms, and pathogenesis, as well as their diagnosis and treatment options. An appropriate and detailed understanding of the aetiology and pathogenesis of chronic respiratory

diseases, as well as their classifications are very important as they can directly influence the decision of treatment and drug selection as well as the drug efficiency in patients. Thus far, several extended research studies have been carried out in *in* vitro, pre-clinical or animal models. As a future direction, such *in vitro* studies should be translated to clinical use. The approach of utilizing cell cultures or microbial culture can provide in depth knowledge of the disease pathology. Interestingly, *in vitro* systems, emerging from cultured cell lines to precision-cut tissue slices, to organoid cultures evoking three-dimensional structures that attempt to recapitulate tissue fluid flow and inter- or intra-cellular interactions may play a huge role in years to come. In all cases, the question of relevance to the *in vivo* setting remains an issue as cell heterogeneity and culture conditions may impede the translation of outcomes from *in vitro* to human disease. However, molecular techniques such as PCR or gene sequencing may be utilised to overcome such problems. Continued advances in the diagnostic approaches and therapeutic methods are required as chronic respiratory diseases are influenced by various unknown factors and causes that are yet to be investigated. Personalised medicine is another emerging strategy that is gaining much interest as a preferred treatment approach as disease progression is affected by many factors such as biological and environmental factors. However, it is yet to take up the shape of an established treatment option since research is still ongoing in many sub-groups of patients.

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Figure legends:

Figure 1: An overview of various *in vitro* experimental models currently available for the study of chronic respiratory diseases

Figure 2. *In vitro* cross-domain interactions between *Aspergillus* and *Streptococcus* which is predicted by the correlation network

Figure 3: Schematic representation of culture at the ALI, electron, and fluorescence microscopy examination of pediatric CF nasal epithelial cells

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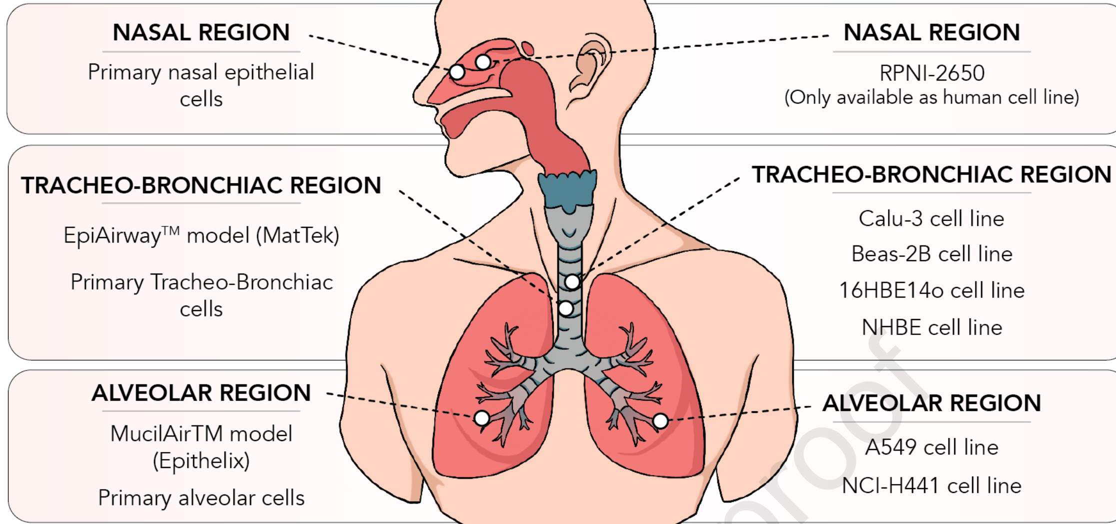

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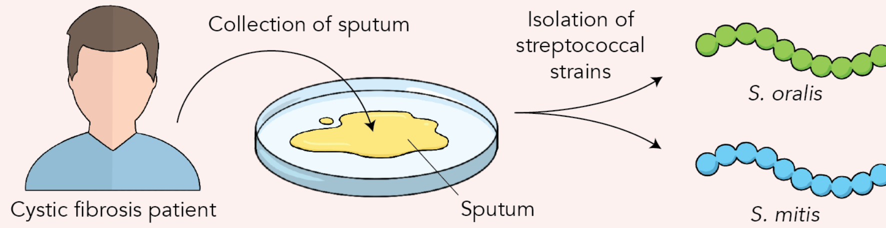

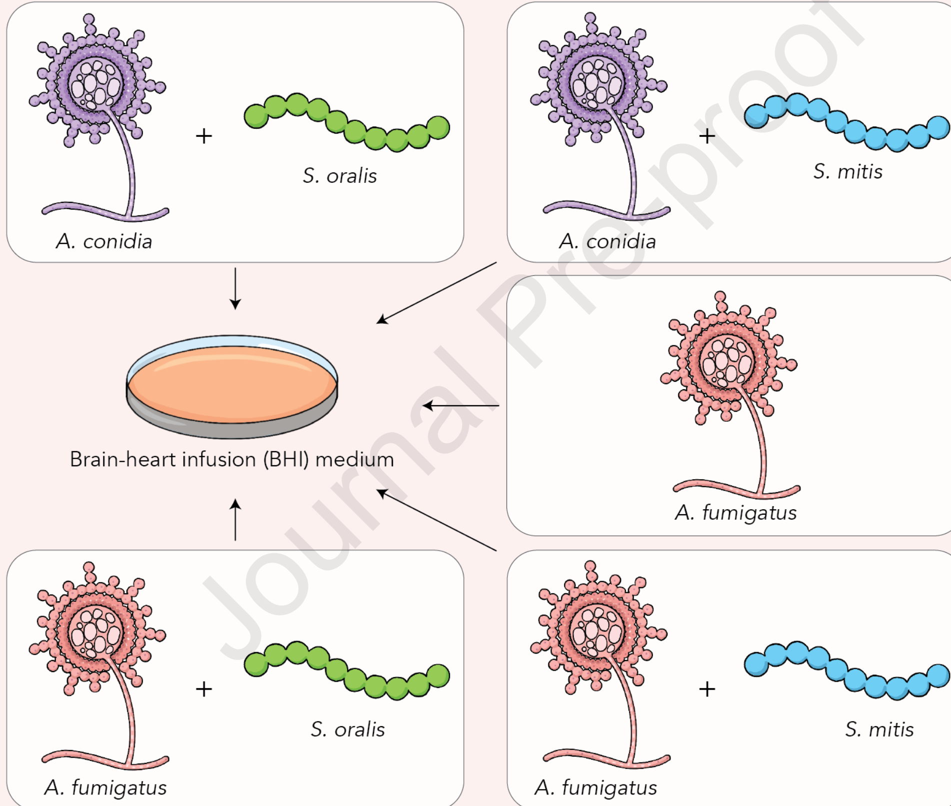

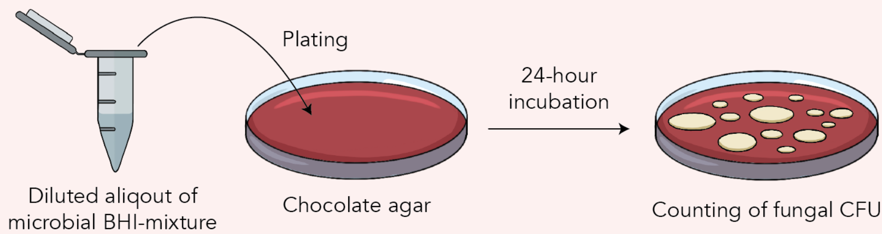

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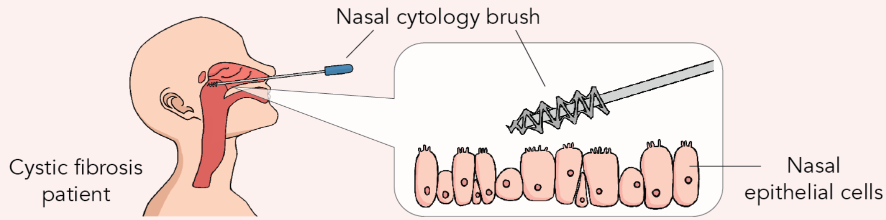

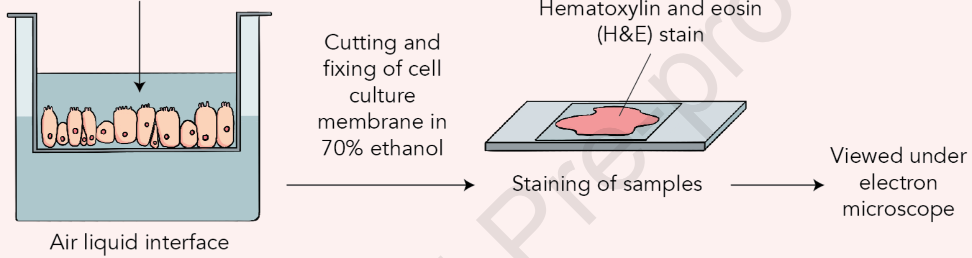

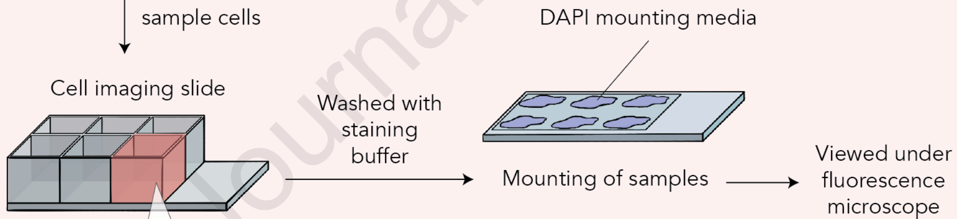

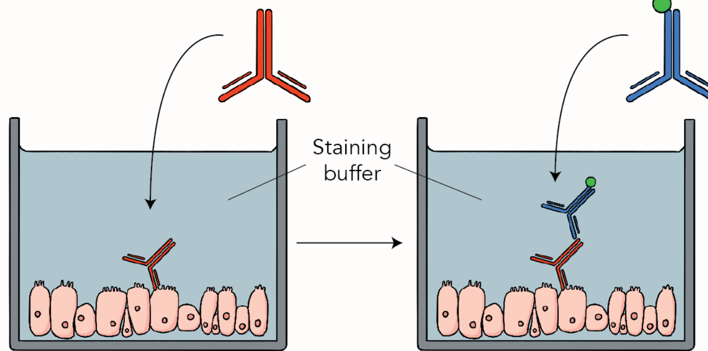

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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