University of Southampton

FACULTY OF MEDICINE

Academic Unit of Clinical and Experimental Sciences

In vitro differentiation and characterisation of the phenotypic differences between mucociliary and squamous metaplastic airway epithelium in COPD

by

Graham Anthony Berreen

Thesis for the degree of Master of Philosophy

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ABSTRACT

FACULTY OF MEDICINE

Discipline

Thesis for the degree of Master of Philosophy

IN VITRO DIFFERENTIATION AND CHARACTERISATION OF THE PHENOTYPIC DIFFERENCES BETWEEN MUCOCILIARY AND SQAUMOUS METAPLASTIC AIRWAY EPITHELIUM IN COPD

Graham Anthony Berreen

Background: Chronic obstructive pulmonary disease (COPD) is one of the world's leading causes of death, mainly caused due to smoking. One of the complications of COPD is the development of Squamous Metaplasia (SM) of the airway epithelium, with prevalence correlating with the severity of COPD. Previous work has suggested that the absence of retinoic acid (RA) can induce a SM phenotype under *in vitro* culture conditions. Artificial induction of SM *in vitro* would allow for characterisation and classification in comparison to normal differentiated epithelium from various donor sources.

Methods: Primary bronchial epithelial cells from both healthy donors and COPD donors were cultured as differentiated air-liquid interface (ALI) cultures for 21 days in either the presence or absence of RA. These cultures were harvested at day 7, 14 and 21 for mRNA extraction and qPCR analysis, with some cultures being fixed for both immunohistochemistry and immunofluorescent staining. Further cultures from both donor groups, grown in the presence and absence of RA, were stimulated basally with 10ng/µl TNFα for 24 hours to induce a proinflammatory response, measured by mRNA extraction and qPCR analysis.

Results: Phenotypically, there is little difference between healthy and COPD donors when grown in the presence of RA, with similar levels of structural protein expression and cilia visible on the surface of both donor groups. COPD donors did exhibit a significantly lower expression of the gene SCGB1A1, encoding the protein CCSP that is secreted by Club cells. The removal of RA resulted in a significant increase in the Trans Epithelial Resistance (TEER) reading as well as visible thickening of the epithelium with a loss of cilia. Decreased expression of Cytokeratin-7 and elevated Involucrin support the structural changes seen with the removal of RA. The removal of RA also alters the epithelial immune response, resulting in an attenuated response for Interleukin (IL)-6, and a decrease in expression fold induction of IL-8 and RANTES. Removal of RA also reduces expression of the antioxidant gene GCLM, but significantly increase the expression of HO1, both at baseline and post stimulation.

Conclusion: The removal of RA from ALI culture medium during the 21 days of differentiation results in an epithelium that is phenotypically similar to SM found *in vivo*. Altered gene expression and changes in the physical characteristics of the epithelium suggest that the epithelium is initially reacting to environmental insult to protect from further damage. The ability to generate artificial SM *in vitro* warrants further research to determine its exact role in the pathogenesis of COPD.

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Declaration of Authorship

I, Graham Berreen, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

In vitro differentiation and characterisation of the phenotypic differences between mucociliary and squamous metaplastic airway epithelium in COPD

I confirm that:
This work was done wholly or mainly while in candidature for a research degree at this University;

Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

Where I have consulted the published work of others, this is always clearly attributed;

Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;

I have acknowledged all main sources of help;

Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

None of this work has been published before submission

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Abbreviations

α1AD	**Alpha-1-antitrypsin deficiency**
α1AT	**Alpha-1-antitrypsin**
ALI	**Air-liquid interface**
ATI	**Alveolar type one**
ATII	**Alveolar type two**
α-SMA	**Alpha smooth muscle actin**
BEBM	**Bronchial epithelial basal medium**
BEGM	**Bronchial epithelial growth medium**
BSA	**Bovine serum albumin**
CB	**Chronic bronchitis**
CBE1	**Ciliated bronchial epithelium 1**
CCSP	**Club cell secretory protein**
cDNA	**Complementary DNA**
CK	**Cytokeratin**
COPD	**Chronic obstructive pulmonary disease**
DE	**Diesel exhaust**
DMEM	**Dulbecco's modified Eagle's medium**
DNA	**Deoxyribonucleic acid**
dsDNA	**Double stranded DNA**
ECM	**Extra cellular matrix**
EGF	**Epidermal growth factor**
EGFR	**Epidermal growth factor receptor**
ELISA	**Enzyme-linked immunosorbant assay**
ETS	**Environmental tobacco smoke**
FEV1	**Forced expiratory volume in 1 second**
FITC	**Fluorescein isothiocyanate**
FVC	**Forced vital capacity**
GAPDH	**Glyceraldehyde 3-phosphate dehydrogenase**
GCL	**Glutamate-cysteine ligase**
GCLC	**Glutamate-cysteine ligase catalytic subunit**

GCLM	**Glutamate-cysteine ligase regulatory subunit**
GRO-α	**Growth related oncogene alpha**
GSH	**Glutathione (reduced form)**
HBSS	**Hanks' balanced salt solution**
HO-1	**Haem oxygenase-1**
HRU	**Histology research unit**
HRV	**Human rhinovirus**
IL	**Interleukin**
ITS	**Insulin, transferrin and sodium selenite**
MCP	**Monocyte chemotactic protein**
MMP	**Matrix metalloproteinases**
mRNA	**Messenger RNA**
MUC	**Mucin**
NE	**Neutrophil elastase**
Nrf2	**Nuclear factor (erythroid-derived 2)-like 2**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**
qPCR	**Quantitative polymerase chain reaction**
RANTES	**Regulated upon activation, normal T-cell expressed and secreted**
ROS	**Reactive oxygen species**
SCGB1A1	**Club Cell Secretory Protein Gene**
SHS	**Second hand smoke**
RNA	**Ribonucleic acid**
RSV	**Respiratory syncytial virus**
RT	**Reverse transcription**
TEER	**Transepithelial electrical resistance**
TEKT1	**Tektin 1 Gene**
TGF	**Transforming growth factor**
TIMPs	**Tissue inhibitor if metalloproteinases**
TPM	**Total particulate matter**
TNFα	**Tumour necrosis factor-α**
TSLP	**Thymic stromal lymphopoietin**
UBC	**Ubiquitin C**

1 Introduction

1.1 Clinical definition and Overview

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of morbidity and mortality in the world. In 2010, COPD was the third leading cause of death worldwide and responsible for 5.49% of all deaths (1). Future projections have estimated that COPD will result in 7.8% of all deaths by 2030 varying between 5.5%-12% depending on income (2). The Global Initiative for Obstructive Lung Disease (GOLD) defines COPD as a condition 'characterised by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung due to noxious inhalation of particles or gases'(3).

The main method that is used for the diagnosis of COPD is spirometry, which can assess the degree of airflow limitation present in the lungs of patients. Spirometry measures two parameters. First is the forced vital capacity (FVC), the volume of air that can be forcibly exhaled after maximal inspiration. The second is the forced expiratory volume in one second (FEV_1), the volume of air exhaled in the first second. These two measurements can be used to calculate the FEV_1/FVC ratio, with a ratio of <0.7 indicating airway obstruction as defined by the GOLD criteria. FEV1 can also be used to classify the severity of COPD (Table 1.1).

Governing Body	Disease State	Criteria Definition
GOLD	Stage I (Mild)	FEV_1/FVC <0.70 and FEV_1<80% predicted
	Stage II (Moderate)	FEV_1/FVC <0.70 and 50%≤ FEV_1<80% predicted
	Stage III (Severe)	FEV_1/FVC <0.70 and 30%≤ FEV_1<50% predicted
	Stage IV (V. Severe)	FEV_1/FVC <0.70 and FEV_1<30% predicted **or** FEV_1/FVC <0.70 and FEV_1<50% predicted and chronic respiratory failure

Table 1.1 Classification of COPD

Classification and severity staging according to the criteria of the Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD) (4)

1.2 Risk factors fo COPD development

COPD is a significant burden on health care systems across the world, and this is set to increase. It is therefore important too fully understand the risk factors that will influence whether or not an individual will develop COPD in their lifetime.

1.2.1 Cigarette smoking

The biggest risk factor that has been linked to COPD is cigarette smoking (3) with a causal relationship between the two being established (5). While smoking rates seem to have fallen in the UK (6), globally the number of smokers has risen from 721 million (1980) to 967 million (2012) (7). Cigarette smoke is highly toxic (8, 9) and has a specific effect on oxidative stress and inflammation in the airways (10, 11). For those that have COPD, approximately 90% of deaths will be as a consequence of smoking resulting in the development of COPD (12). Studies have shown an increased prevalence of COPD, in all GOLD categories, in smokers compared to non-smokers, when stratified for smoking status and age (13). Smoking leads to an accelerated decline in lung function as the rate of smoking increases (14). Smoking cessation has been shown to increase life expectancy, with the degree of impact dependent on the age at which the individual quit smoking. Those that quit early (25 to 34 years of age) gained approximately 10 years of life compared to those that continued to smoke. The gain in life expectancy from smoking cessation, decreases as the length of time spent smoking increases (15).

There is a large concern in regards to passive smoking, commonly known as second hand smoke (SHS) or environmental tobacco smoke (ETS), defined as the indirect inhalation of tobacco smoke generated by others. Logic dictates that if smoking can cause respiratory problems then exposure to the same environment will incur similar effects. In 2004, 603000 deaths worldwide were attributed to second hand smoke exposure, accounting for 1.0% of worldwide mortality (16).

1.2.2 Domestic smoke

Another source of particulate matter is from domestic exposure, with the burning of biomass fuels now being acknowledged as a potent source of particles. The use of biomass fuels in a domestic environment is common place with an estimate of around 50% of all households worldwide using biomass fuel as the main source of energy (17). Research has shown that there is a strong association between wood/charcoal smoke, and the development of COPD (18).

1.2.3 Other sources of particles

Whilst cigarette smoke is the most common risk factor, exposure to other sources of inhaled particle, such as air pollution, are often associated with the onset of symptomatic lung disorders and COPD. Research has found that the contribution of occupation towards the development of COPD had a population attributable risk (PAR) of 20-31% depending on the definition of COPD that was used (19, 20). In addition, studies have shown that pollution primarily from combustion of fossil fuels and automobiles is associated with a decrease in lung function in non-smokers (21) and an increase in the prevalence of COPD (22).

1.2.4 Age

Mannino et al. (2006) stratified data of COPD prevalence in the USA, using data from the NHANES III study, according to smoking status and age. The results show that while smoking does increase the percentage of COPD cases, with current and former smokers having higher rates of COPD, across all three groups the rates of COPD rise with age (13).

1.2.5 Gender Influences

Of the 15% of people who are diagnosed with COPD but have never smoked, nearly 80% of these are women (23). This finding may suggest that smoking rates alone are not entirely responsible for the differences seen between genders. While these differences may be biological, it could be that males and females are exposed to different risk factors, such as stereotypical occupations and domestic duties.

1.2.6 Genetic Influences

Whilst smoking is the main risk factor for COPD, only 15-20% of smokers will go on to develop COPD (24). This suggests a genetic component that might predispose an individual, in the presence of a risk factor, to the development of COPD.

The most documented genetic risk factor is alpha-1-antitrypsin deficiency (α1AD) that causes defective production of alpha-1-antitrypsin (α1AT). The main function of α1AT is to inhibit proteases, especially neutrophil elastase (NE), which breaks down elastin in the lung leading to the development of emphysema (25). While being diagnosed with α1AD can result in a faster than normal decline in lung function, this decline is greatly enhanced when combined with smoking (26). It has also been found that smoking cessation has very little to no effect on slowing the decline of FEV_1 in those that have α1AD (27).

Another genetic component may be transforming growth factor beta (TGF-β), a fibrogenic cytokine that has been found to be elevated in the small airways of patients with COPD (28). Research into the genetic components of COPD has highlighted an association between single nucleotide polymorphisms (SNPs) in or near the TGFβ1 gene and COPD phenotypes (29).

1.3 Anatomy of the lung

The lungs are divided into two sections, the conducting airways and the respiratory airways (Figure 1.1). The conducting airways comprise of the trachea, main bronchi and bronchioles that make up the branches 1-16 of the tracheobronchial tree. These upper airways are lined with pseudostratified epithelium in which all cells are in contact with the basement membrane (30). These epithelial cells generate hair-like projections called cilia that protrude into the lumen. Approximately 90% of the epithelium is ciliated (31). The purpose of the conducting airway is to humidify and warm the air whilst filtering out

particulate matter. Particles are trapped in mucus, secreted by interspersed goblet cells, which can be directionally transported away from the lower airways by the cilia (32). Basal cells are present throughout the conducting airway, reducing in number as the airway decreases in diameter.

The respiratory airways (branches 16-23) consist of the respiratory bronchioles, alveolar ducts and the alveolar sacs. The bronchioles are lined with ciliated columnar cells without pseudo-stratification, with a decreasing number of goblet cells but with an increasing number of Club cells (33). As the airway narrows into the respiratory bronchioles, the cells transition into simple cuboidal epithelium, comprising completely of ciliated cells and Club cells, eventually merging with the flattened epithelium of the alveolar ducts and the alveoli. Club cells are non-ciliated secretory cells that secrete Club cell secretory protein (CCSP) and surfactant proteins A, B and D (34). CCSP has been implicated in the regulation of normal lung secretions and homeostasis (35), while surfactant reduces surface tension to prevent lung collapse (36) as well as having a role in host defence (37).

The alveoli are hollow “balloon like” structures at the terminal end of the respiratory tree, branching out from the alveolar ducts. The alveoli comprise of two main types of cells, alveolar type I (ATI) and alveolar type II (ATII) cells. The ATI cells are squamous, very thin (25nm) with a large surface area, contributing about 90-95% of the alveolar surface. Their size enables optimal gas exchange with the capillary network that surrounds the alveoli. ATII cells are cuboidal in shape and are also responsible for the secretion of surfactant (38). They can also differentiate into ATI cells, providing a regenerative pool of cells to repair the lung (37). The alveoli have a complex interwoven network of collagen and elastin fibres that allow the alveoli to coordinate expansion and relaxation during normal respiration (39). Fibres from the alveolar ducts and septa connect with fibres in the septa and nearby blood vessels. Collagen fibres are present as waves in the collapsed lung but straighten in an inflated lung, allowing the alveoli to expand upon inspiration while offering a limit to the maximal expansion of the alveoli. The interwoven elastin fibres then allow for the alveoli to contract and recoil (40).

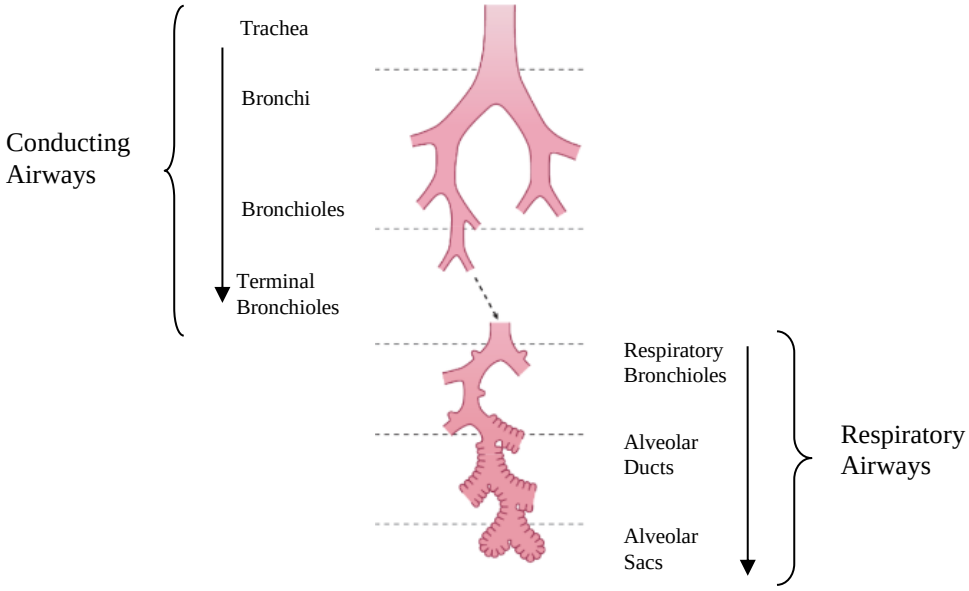

Figure 1.1 Diagram of the Tracheobronchial Tree

A schematic diagram of the tracheobronchial tree showing the 23 orders of branching. Gas exchange only occurs in the smallest respiratory bronchioles, alveolar ducts and the alveolar sacs. Adapted from (41)

1.4 Pathophysiology of COPD

COPD comprises of two distinct pathologic conditions, emphysema and chronic bronchitis. While both conditions can occur individually, they are often present together in varying degrees resulting in a wide range of presenting symptoms for COPD.

1.4.1 Emphysema

Emphysema is defined as "abnormal permanent dilation of the airspaces distal to the terminal bronchioles, accompanied by destruction of their walls without obvious fibrosis" (42). There are two main types of emphysema. Centriacinar emphysema involves the destruction of the respiratory bronchioles but the alveolar ducts and alveoli remain intact. Panacinar emphysema occurs when the alveolar ducts and alveoli are destroyed along with the respiratory bronchioles (Figure 1.2). This type of emphysema is most common with those individuals with α1AD (43).

The predominant theory is that emphysema is a result of an imbalance between proteases and anti-proteases within the lung. Cigarette smoke or external sources of irritation stimulates inflammatory cells to enter the lungs, leading to a large increase in proteases which cannot be counteracted (43). This theory also accounts for why those individuals with α1AD are highly likely to develop emphysema due to a genetically determined reduction in anti-proteases. Recently, research has looked at the possibility of emphysema being the result of increased apoptosis within the alveolar walls (44). Repeated acute lung injury could result in the chronic damage seen in emphysema. It has been shown that epithelial cells express cathespins B, L S, H and K (45), which upon apoptosis may be released, resulting in the destruction of the extracellular matrix (ECM).

The damage present in emphysema results in a reduction in the available area for effective gas exchange to occur. Furthermore there is a reduction in elastic recoil and increased surface tension due to loss of surfactant producing cells (ATII cells) and a decrease in the amount of elastin and collagen fibres preset (39). This leads

to a loss of alveolar patency during forced expiration (46) resulting in hyperinflation and hypoxia. It has been shown that chronic hypoxaemia can lead to activation of the immune system, in particular, the expression of TNF-α (47). Despite the accepted definition of emphysema describing "no obvious fibrosis", some research has pointed to a large increase in collagen deposition in the lungs of smokers with emphysema, increasing in parallel with the loss of airspace. These findings suggest that fibrosis may be evident in emphysema (48).

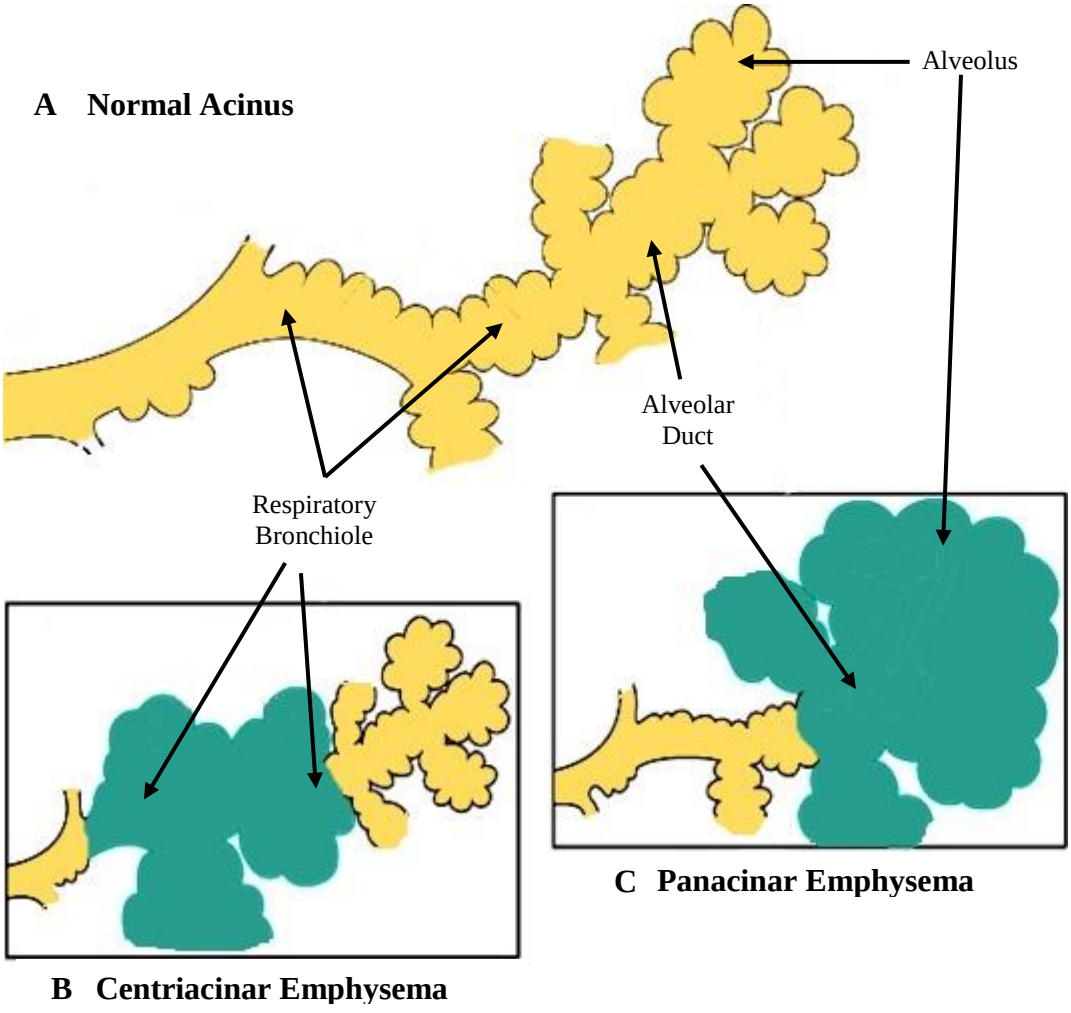

Figure 1.2 Illustration of Emphysema

An illustration of the two main types of emphysema observed in patients. A) A normal acinus, B) Centriacinar emphysema with damaged respiratory bronchioles, C) Panacinar emphysema with damaged alveolar and alveolus but intact respiratory bronchioles. Adapted from (49)

1.4.2 Chronic Bronchitis

Chronic bronchitis (CB) is defined as "the presence of chronic productive cough for 3 months in each of two successive years in a patient whom other causes of chronic cough have been excluded" (42). It is an independent disease that can either precede or follow the development of airflow obstruction but can also occur in individuals with normal spirometry (50).

CB is caused by mucous metaplasia, overproduction of mucins due to inflammatory signals, often caused by external stimuli such as cigarette smoke (51, 52) and other stimulatory factors such as cytokines and growth factors (53). Hypersecretion of mucus by goblet cells within the airways can lead to airflow obstruction via mucus plugging. This is enhanced by poor mucociliary function (54), resulting in mucous that is difficult to remove from the airway. The buildup of mucus can also increase the risk of colonization and infection, leading to an increase in inflammatory mediators that can further worsen CB, and potentially cause an exacerbation of COPD. Goblet cell hypertrophy can lead to epithelial remodeling and the alteration of surface tension, both of which can lead to airway collapse (55). Chronic hypersecretion of mucus has been found to correlate with FEV_1 decline and hospitalization (56).

1.5 The immune response of COPD

The immune mechanism that drives both emphysema and chronic bronchitis are inextricably linked, involving the same pathways, inflammatory cells and mediators. Cigarette smoke and other inhaled irritants activate epithelial cells and initiate the recruitment of macrophages. Once activated, macrophages release inflammatory proteins that orchestrate the recruitment and activation of other immune cells, resulting in inflammation and damage to lung tissue (figure 1.3).

1.5.1 Macrophages

There is a correlation between the prevalence of macrophages and the severity of COPD (57). In addition, macrophages show strong localization to areas of alveolar damage in emphysema (58). They secrete a large number of cytokines and inflammatory mediators that can account for most of the clinical features seen in COPD (figure 6).

Macrophages release interleukin(IL)-8, a potent activator of the IL-8 receptor beta (CXCR2) found on neutrophils, resulting in recruitment of neutrophils to the site of inflammation (59). IL-8 release from macrophages has been shown to increase under hypoxic conditions, a factor that may be relevant in severe COPD exacerbations (60). Macrophages release growth related oncogene-α (GRO-α), which also activates the receptor CXCR2. While this receptor is primarily found on neutrophils, it is also found on 30% of monocytes (61).

Alveolar macrophages have been shown to express TGF-β1 and TGF- β3 (62) with mature TGF- β1 being localized primarily in the connective tissue of the airways (63). TGF-β1 can enhance fibrosis and induce proliferation of fibroblasts and alpha smooth muscle actin (α-SMA) (64) while TGF-alpha can induce fibrosis via epidermal growth factor receptor (EGFR) (65) it can also result in hypersecretion of mucus (66).

Alveolar macrophages can release several elastolytic enzymes such as matrix metalloproteinase (MMP)-2, MMP-9, MMP-12 and cathespins K, L and S (59). Macrophages can also release neutrophil elastase (NE) that has been taken up from neutrophils. These proteases break down the structural protein elastin that is vital for both elastic recoil and resisting negative pressure collapse in the respiratory airway (25, 67).

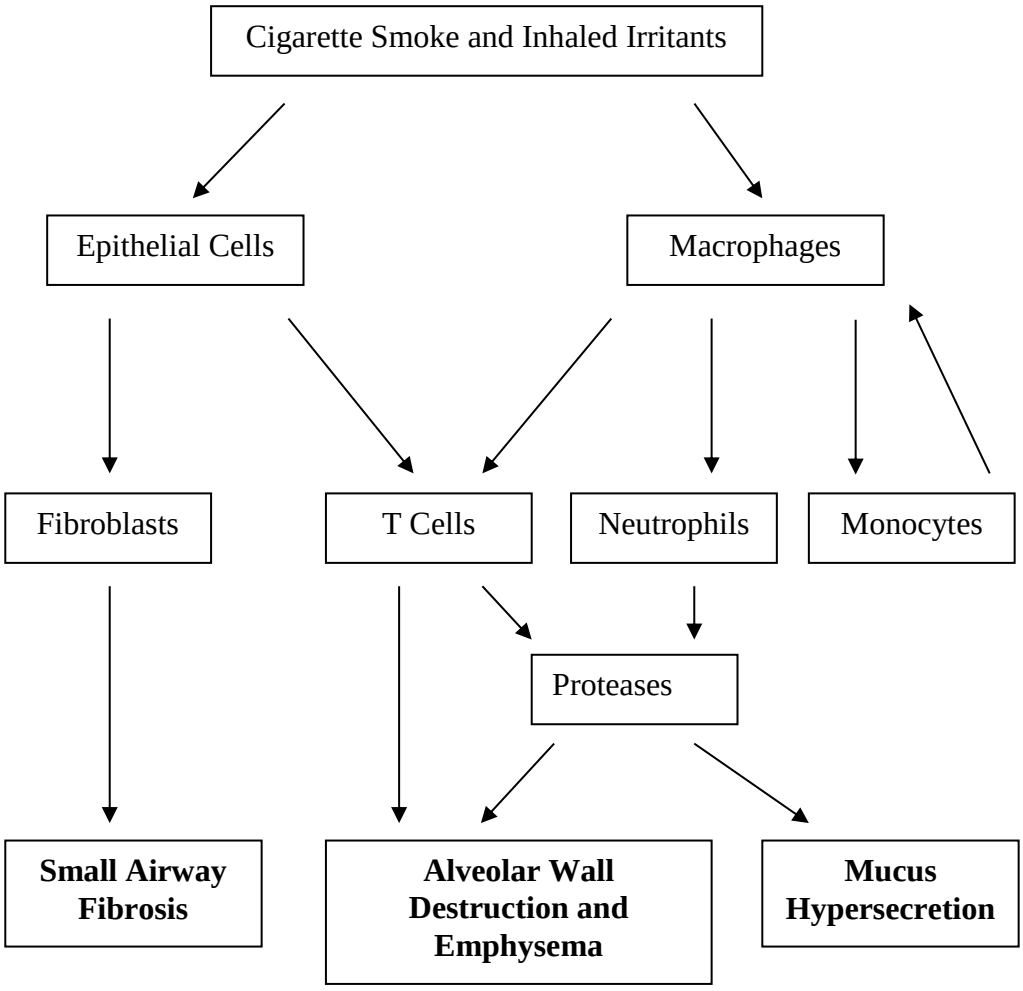

Figure 1.3. Immune response involved in COPD.

Inhaled irritants trigger an inflammatory response, activating epithelial cells and macrophages. Release of growth factors results in the proliferation of fibroblasts that causes the formation of fibrosis. Chemotactic factors results in the recruitment of further inflammatory mediator cells that release various proteases causing damage to epithelium and connective tissue, resulting in mucus hypersecretion (chronic bronchitis) or alveolar collapse (emphysema). Adapted from (61)

1.5.2 Neutrophils

Neutrophils are the initial source of NE, with activation resulting in granule exocytosis and NE release (68), as well as releasing cathespin, proteinase-3, MMP-8 and MMP-9 (69). Inhibition of NE occurs via α1AT, with a deficiency in α1AT resulting in enhanced destruction of elastin. NE also stimulates macrophages to release leukotriene B4 (LTB4), a chemotactic factor for neutrophils (70). This feedback loop means that a deficiency in α1AT further enhances the number of neutrophils present and an increase in NE released. Sputum counts of neutrophils has been shown to be elevated in individuals with COPD (71), increasing with COPD severity (72).

1.5.3 Monocytes and T lymphocytes

Recruited by macrophages via GRO-α and monocyte chemotactic protein-1 (MCP-1) (59), monocytes differentiate into either macrophages or dendritic cells (61). There is an overall increase in the number of T lymphocytes in the lung parenchyma, mainly CD8+ cytolytic T-cells (73). The CD8+ cells release cytotoxins such as perforin and granzymes that form pores in the cell membrane, resulting in apoptosis (74), which have been shown to be elevated in individuals with COPD (75)

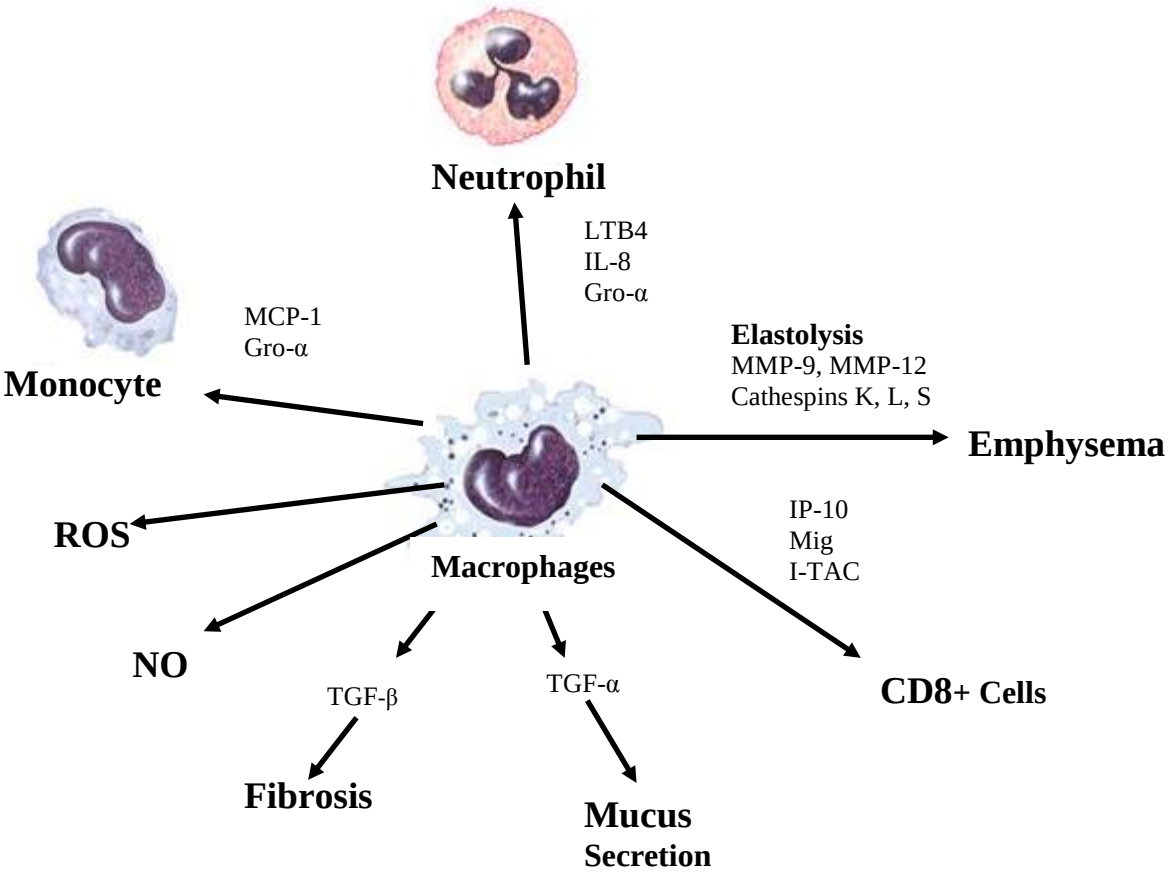

Figure 1.4. The role of alveolar macrophages in COPD.

The role of macrophages includes the release of chemotactic factors recruiting immune cells, pro-fibrotic factors inducing fibrosis and mucus secretion and elastolytic enzymes resulting in the breakdown of elastin. Adapted from (69).

1.6 The Epithelium

The epithelium plays an important role in the immune response of COPD by synthesizing and releasing a large number of pro-inflammatory mediators. This occurs in response to stimulation by other inflammatory mediators, amplifying ongoing inflammation, or initiating inflammation as a direct result of contact with noxious gases, such as CS (76). Chemotactic factors that are traditionally associated with immune cells such as IL-8 (neutrophils), RANTES (eosinophils) and MCP-1 (monocytes) have all been shown to be expressed by epithelial cells (77). In those patients with CB, exacerbations resulted in a large increase in RANTES expressed by epithelial cells, resulting in eosinophil migration into the airways (78).

Upon stimulation with CS, epithelial cells release epidermal growth factor (EGF) and increase expression of epidermal growth factor receptor (EGFR) (52) resulting in the up-regulation of MUC5AC a major mucin in the airway (79). Epithelial cells release TGF-β that can lead to the proliferation of fibroblasts. This uncontrolled proliferation can result in increased deposition of extracellular matrix (ECM) in the small airways, causing airflow obstruction via an increase in air resistance or occluding the airways completely (61).

1.7 Epithelial Structure and the Development of Squamous Metaplasia

The symptoms of COPD, are due to the altered structure and function of epithelial cells. Emphysema occurs due to the release of proteases (43) and apoptosis (44), both of which can result in the loss of connective tissue and the surrounding epithelium, creating a poor surface area for gaseous exchange and increasing the chance hyperinflation. However, in CB there is hypersecretion of mucus due to goblet cell hypertrophy. In addition, there is poor epithelial mucociliary clearance (54) due to decreased ciliary beat frequency and an increase in non-ciliated

epithelium, when compared to healthy controls (80).

One of the physical alterations that can occur to the epithelium during COPD is the development of squamous metaplasia (SM). This is the replacement of pseudostratified columnar epithelium with stratified squamous epithelium (Figure 1.5). Stratified squamous epithelium occurs in the body naturally in areas that experience high abrasion, presenting as either keratinized or non-keratinized epithelium. Keratinized stratified squamous epithelium occurs externally and protects from abrasion using a keratin layer. Non-keratinized stratified squamous epithelium is present internally, such as the oesophagus (81). The thick cell layer allows the sloughing of cells without exposure of the basement membrane. The incidence of SM found within the bronchus of individuals increases with the presence of COPD (82).

Research has shown that cigarette smoke induces the development of squamous metaplasia, transitioning from ciliated columnar epithelial cells (83). While this remodeling occurs in almost all smokers, only 10-20% of smokers go on to develop COPD (24, 84). Biopsies taken from smokers have shown a positive correlation between the number of pack years and the occurrence of squamous metaplasia (85). This is supported by serial analysis of the tracheobronchial tree from deceased patients that identified the overall percentage of squamous metaplasia. Those who did not smoke had 1.9% squamous metaplasia within the tracheobronchial tree. Those who smoked one pack of cigarettes a day increased to 6.3% and more than one pack a day had 9.5% squamous metaplasia (86). Smoking cessation of ≥3.5 years resulted in a decrease in SM observed, when compared to current smokers with COPD (87).

Whilst primarily thought to be a defense mechanism against physical irritation from inhaled particles, the development of SM also correlates with the severity of COPD (28). Severe hyperplasia and terminal SM that is irreversible has been shown to be associated with an up-regulation of TNFa, IL-1β and IL-6. This suggests that the development of SM results in an enhanced immune response that can further propagate the development of SM (84). The skin also expresses IL-1β, where it may represent activation of the epidermis in response to epithelial injury

and help explain the development of airway fibrosis. This elevated expression of IL-1β in SM may can result in increased activation of fibroblasts, leading to subepithelial fibrosis (88) that can contribute to airway narrowing and airflow limitation (3). This propogation of SM development, coupled with prolonged exposure to inhaled toxins, over time can lead to severe alterations in the epithelium, resulting in squamous cell carcinoma (89), with the detection of SM acting as a marker for a predisposition to the development of bronchogenic carcinoma (90).

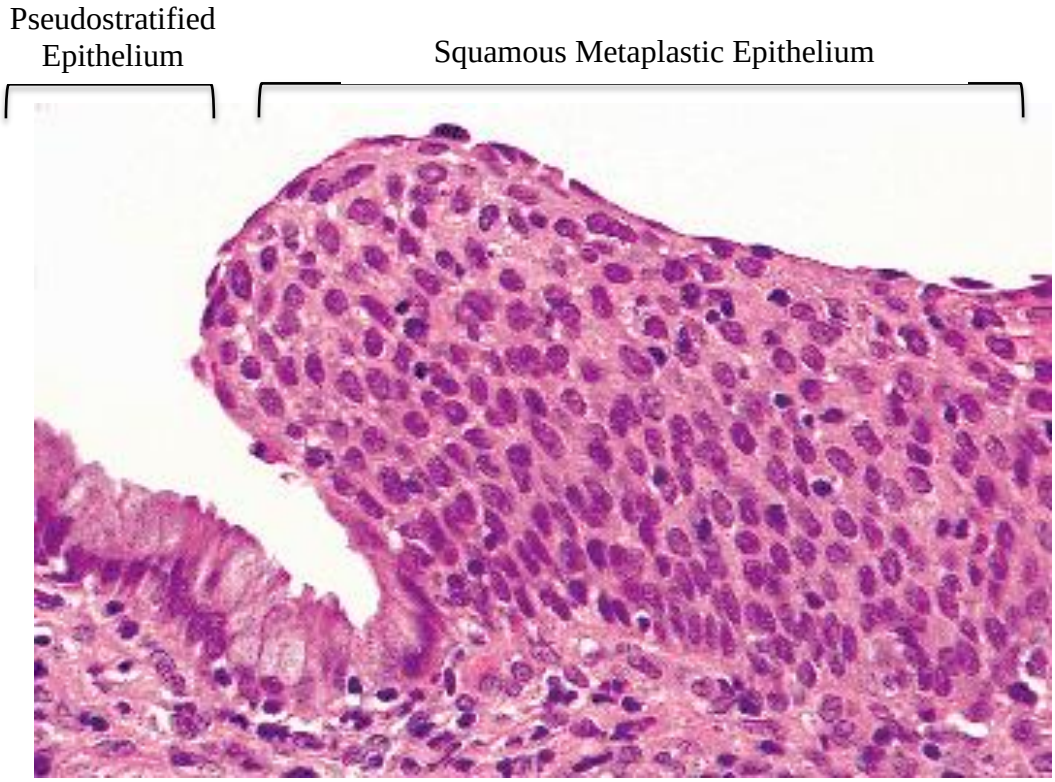

Figure 1.5. The development of Squamous Metaplasia

A histology sample demonstrating the transition of pseudostratified columnar ciliated epithelium to stratified squamous epithelium (91).

1.8 Vitamin A and Retinoic Acid Roles in Squamous Metaplasia

When culturing primary epithelium *in vitro*, vitamin A is a key culture requirement to develop fully differentiated mucus secreting epithelium. A lack of vitamin A in the diet of rats results in the replacement of the respiratory mucosa by keratinizing epithelium without the presence of exudate (92), a sign of squamous metaplasia. A vitamin A deficient diet in hamsters also resulted in the development of squamous metaplasia (93).

Vitamin A has three active forms, retinol, retinal and retinoic acid (RA). Circulating retinol is converted into either retinal (reversible) or oxidized into RA (irreversible) which is then involved in growth and epithelial differentiation (94). RA receptors (RARs) are present in bronchial epithelial cells (95) with stimulation resulting in suppression of involucrin (96), a key component in the development of stratified squamous epithelium (97). Studies using human derived tracheobronchial epithelial cells have shown that differentiation into a mucosal phenotype only occurred when RA was present, with the absence of RA resulting in squamous epithelium and a 300-900 fold decrease in mucin production (98). This dependence on the presence of RA to differentiate into the mucosal phenotype has not been found to occur with the other growth supplements present in the growth medium (99).

RA has been used in rats to alleviate induced emphysema (100) but was shown to have no effect in a mouse model (101). Human trials using patients with emphysema showed that treatment with RA can modulate levels of MMP-9 and tissue inhibitors of metalloproteinases (TIMPs), restoring the metalloproteinase-antiproteinase balance (102, 103).

1.9 Aims and Hypotheses

The pathogenesis of COPD is intrinsically linked to the respiratory epithelium, with damage and altered immune response resulting in the clinical features seen in both emphysema and chronic bronchitis. To try to determine the role that the epithelium plays in the pathogenesis of COPD, the primary aim of this research is to make direct comparisons between both healthy and COPD donor primary bronchial epithelial cells (PBECs).

In addition to this, research suggests shows that the removal of RA from the culture medium results in the induction of SM within PBEC cultures. Despite there being a strong association between SM and COPD symptoms, very little work has been carried out to make comparisons between the two. Therefore, the second aim of this study is to determine if SM can be induced by removing the RA, and to investigate how this changes the epithelium.

There are two hypotheses to be tested:

1. COPD donor cells will significantly differ from healthy donors

2. Removal of RA from ALI cultures results in the development of SM, regardless of cell donor source, with these cultures significantly differing from the normally differentiated epithelium

2 Materials and Methods

2.1 Materials

Reagent	Manufacturer	Code (if available)
Bovine serum albumin (BSA)	Sigma-Aldrich	A3059
Bronchial epithelial basal medium (BEBM)	Lonza (Slough, UK)	CC-3171
Bronchial epithelial growth medium (BEGM) Singlequots	Lonza (Slough, UK)	CC-4175
Chloroform	Sigma-Aldrich	C2432
DNase kit	Life Technologies (Paisley, UK)	AM1906
Dulbecco's Modified Eagle Medium (DMEM) – high glucose, no glutamine	Life Technologies	11960044
Ethanol >99.5%	Sigma Aldrich	32221
Foetal bovine serum	Life Technologies	10500-064
Glycogen	Sigma Aldrich	10902393001
Hank's Balance Salt Solution (HBSS) without Ca2+ and Mg2+	Life Technologies	14170-088
Isopropanol (propan-2-ol)	Sigma-Aldrich	I9516
100x ITS solution - 1mg/ml Insulin - 550µg/ml transferrin - 500ng/ml sodium selenite	Sigma-Aldrich	41400-045
Phosphate Buffered Saline (PBS) Tablets	Oxoid (Basingstoke, UK)	BR0014G
Precision reverse transcription kit	Primer Design (Southampton, UK)	RT-std
PureCol collagen	Advanced BioMatrix (Tucson, AZ, USA)	5005-B

Quantitative polymerase chain reaction (qPCR) mastermix	Primer Design	PrecisionC PrecisionC-SY
qPCR Primers (UBC/GAPDH, CBE1, CK7, CK13, GCLM, HO1, IL-6, IL-8, Involucrin, MUC5AC, MUC5B, NRF2, RANTES, SCGB1A1, TEKTIN1, TSLP)	Primer Design	PP-HU-600 SY-HU-600
Retinoic Acid (RA)	Sigma-Aldrich	R2625
Tumor Necrosis Factor Alpha (TNFα)	Sigma-Aldrich	T0157
Trizol lysis reagent	Life Technologies	15596-018
Trypan Blue Solution	Sigma Aldrich	93595
Trypsinethylenediaminetetraacetic acid (EDTA)	Life Technologies	15400054

2.2 Equipment

GraphPad Prism 7	GraphPad Software (San Diego, CA, USA)
iCycler CFX96 Thermal Cycler	Biorad (Hemel Hempstead, UK)
Improved Neubauer bright-line haemocytometer	Marienfeld (Lauda-Königshofen, Germany)
Leica DMI6000B light microscope and software	Leica Microsystems GmbH (Wetzlar, Germany)
Millicell ERS-2 epithelial volt-ohmmeter	Millipore UK Ltd (Watford, UK)
Nanodrop ND-1000 spectrophotometer	Nanodrop (Wilmington, DE, USA)
Nunc cell culture plates	Nalge Nunc (Rochester, NY, USA)
T100 Thermal Cycler	BioRad
Topmix vortexer	Fisher Scientific
Transwell culture apparatus	Thermo Fisher Scientific

2.3 Cell Culture

2.3.1 Primary Cell Culture and Seeding

All donors were recruited as part of the MRC/ABPI COPD cohort for the COPDMAP study. This study involved, but was not limited to, the harvesting of bronchial brushings for the isolation of primary epithelial cells from both healthy non-smokers, and those individuals with COPD. A total of 7 healthy donors and 13 COPD donors were used for this study (table 2.1).

	Healthy (N=7)	COPD (N=13)		
		GOLD Stage I	GOLD Stage II	GOLD Stage IV
Male	2	1	5	3
Female	4	0	2	1
Average Age	62.83	66	68.86	56.25
Average Pack Year History	-	49	55.67	30

Table 2.1. Patient information for the PBEC donors

Patient details for all but one healthy donor and one COPD donor whose details were not available. All of the healthy donors were non-smokers with zero pack year history. The COPD donors were either GOLD stage I, II or IV.

PBECs were expanded and cultured at air-liquid interface (ALI) according to the methods previously published (98). Cells were cultured until 70% confluent in tissue culture flasks (25cm^2 P0, 75cm^2 P^1) coated with 1% Bovine Collagen 1 (30µg/ml). Cells were grown in bronchial epithelial growth medium (BEGM) comprised of medium supplemented with supplied Singlequots (final concentration of 52 µg/ml bovine pituitary extract, 0.5 µg/ml epinephrine, 0.1% GA-1000 Solution (30mg/ml gentamycin and 15 µg/ml amphotericin), 0.5 ng/ml human epidermal growth factor, 0.5 µg/ml hydrocortisone, 5 µg/ml insulin, 10 µg/ml transferrin, 6.5 ng/ml triiodothyronine and RA of unknown concentration). Once 70-80% confluent, cells were washed twice with Hanks' balanced salt solution (HBSS –Ca^{2+}/-Mg^{2+}) and 3ml of 0.05% Trypsin-EDTA in HBSS was

added to the flask. Cells were incubated for 5 minutes until cell detachment, judged by light microscopy, had occurred. Once detached the cell suspension was removed and added to 10ml of serum containing medium (DMEM +10% FBS) to neutralise the trypsin. The flask was rinsed with 5ml DMEM +10% FBS and all media was pooled. The cell suspension was spun at 300 x g for 5 min to pellet the cells before being re-suspended in 1ml of BEGM. 10µl of cell suspension, 80µl PBS and 10µl Trypan blue stain were mixed and 10µl of the stained cells added to a Neubauer bright-line haemocytometer. Using a light microscope, cells in the four corner 1mm squares were counted and the mean calculated. This number was multiplied by 10,000 to calculate the number of cells per 1ml of cell suspension.

PBECs were seeded onto collagen-coated transwells at a density of 70,000 cells/well. Cells were diluted in BEGM to make 350,000 cells/ml, with 200ul of cell suspension being added to the apical compartment of the transwells. This was supplemented with 500ul of BEGM in the basal compartment. Cells were grown in this configuration for 24hrs until the cells were 100% confluent at which point they were taken to ALI by removal of the apical media. The basal compartment contained 300ul of ALI medium – equal volumes Dulbecco's modified Eagle's medium and 2xBEGM (containing twice the concentration of supplements compared to normal BEGM, no GA but 1.5µg/ml BSA), with 50nM retinoic acid being added to half of the cultures, with the other half receiving no RA. This generated two sub groups, those cultured with RA (+RA) and those cultured without RA (-RA).

Basal medium was changed every working day (Monday-Friday) with Transwells being cultured for 21 days. Transepithelial electrical resistance (TEER) was measured on day 7, 14 and 21 post ALI generation. 100µl HBSS was added apically and incubated for 15 minutes before the TEER was measured using a Millicell Voltmeter and chopstick electrodes.

2.3.2 Stimulation of Cells with TNFα

Starvation medium (BEBM with 1% ITS and 1.5ug/ml BSA) was applied to the basolateral compartment of all cultures 24hrs before stimulation. Stock TNFα 10μg/ml was added to starvation medium resulting in a final concentration of 10ng/ml, to generate stimulation medium. 10ng/ml of TNFα has previously be shown to be sufficient to stimulate PBECs (104). Cultures were washed in 1ml of PBS before stimulation medium was applied to the basolateral compartment. Once stimulation media was applied, 100μl of normal starvation media was added to the apical compartment of all cultures.

After 24hrs, apical and basal supernatants were collected and centrifuged at 7,000 RPM, 4°C for 7 minutes, to remove cellular debris. Supernatants were then aliquoted into fresh labelled tubes and stored at -80°C. Cultures were then either harvested for mRNA using Trizol, or fixed for immunofluorescence using 4% PFA.

2.4 Processing of Samples

2.4.1 GMA Embedding

The transwells were washed with PBS before the membrane was cut away from the plastic housing with a razor blade. Detached membranes were placed into a glass container of acetone containing 2mM phenyl methyl sulphonyl fluoride (35mg/100ml) and 20mM iodacetamide (370mg/100ml), supplied by the Histology Research Unit (HRU), University of Southampton, before being stored at -20°C overnight. The acetone solution was removed and replaced with pure acetone. Samples were incubated at room temperature for 15 minutes after which the acetone was removed and replaced with methylbenzoate. Samples were incubated for a further 15-minutes after which the pure methylbenzoate was replaced with 5% methylbenzoate (made using GMA solution A). All samples

were incubated for 6 hours in the fridge, with the 5% methylbenzoate being replaced with fresh every 2 hours.

35mg of benzoyl peroxide was weighed out and 5ml of GMA solution A added on top, mixing gently to fully dissolve before adding 125ul of GMA solution B. Labels were added to the plastic “coffins” followed by a small amount of GMA solution. The membranes were laid into the coffins and filled with GMA solution before being made airtight by the addition of liquid paraffin wax. The samples were stored at 4°C for 48hrs before being permanently stored in airtight boxes at -20°C.

GMA blocks containing samples were cut on a microtome to generate 2µm sections of the embedded sample. Each sample was stained using Toluidine Blue to visualise and evaluate the cellular structure of the epithelium, before being imaged on a light microscope.

2.4.2 Immunofluorescent Staining

The transwells were washed with PBS (3x5mins) before adding 1ml of 4% paraformaldehyde apically to each transwell. Samples were incubated at room temperature for 20 minutes before being re-washed with PBS (3x5mins) and 1ml of 0.05% sodium azide in H_2O was added. Plates containing the samples were wrapped with Parafilm and foil, and stored at 4°C until processing.

Samples were washed with PBS (3x5mins) before being permeablised with 1ml of 0.1% Triton X-100 in PBS for 15 minutes. Non-specific binding sites on each sample were blocked using blocking buffer (1% BSA, 0.1% Tween 20 in PBS) for 30 minutes. The sample was then removed from the plastic insert, halved and placed onto the microscope slides with the cell layer facing upwards. The FITC-β-tubulin antibody was diluted 1:50 in blocking buffer before 50µl was applied to each half sample. Samples were put in a humidified, lightproof, chamber and stored at 4°C for 24 hrs.

All samples were washed with 0.1% Tween 20 (4x5mins) and once with H_2O before being counterstained with a DAPI antibody (David Johnston, Biomedical Research Unit, University of Southampton) for 5 minutes followed by another round of washing with 0.1% Tween 20 (4x5mins). Individual transwells were picked up with forceps and moved to a fresh microscope slide. A single drop of mounting solution (David Johnston, Biomedical Research Unit, University of Southampton) was applied to each transwell before a cover slip was slowly lowered, using gentle pressure to make sure all air bubbles were removed. Slides were allowed to cure overnight at 4°C before being imaged on a fluorescent microscope.

2.5 RNA extraction and PCR

2.5.1 RNA Extraction and Purification

RNA was extracted from the Trizol/cell lysates using chloroform. Chloroform was added at a 1:5 ratio, shaken for 15 seconds to thoroughly mix and then incubated at room temperature for 10-15 minutes. Samples were centrifuged at 12,000 RPM for 15 minutes to generate two distinct layers, a colourless solution containing the RNA and a pink layer containing protein, both separated by a viscous white layer of DNA. The colourless solution was carefully removed to a fresh eppendorf tube and 1µl of a 20mg/ml glycogen solution was added to help visualise the RNA. Samples were vortexed to mix and an equal volume of ice-cold isopropanol was added before a final vortex for a few seconds. Samples were stored overnight at -80°C to allow for precipitation of the RNA.

The following day, samples were warmed to room temperature, before being centrifuged at 13,000RPM, 4°C for 30 minutes. Once spun, a small white pellet was visible at the bottom of the tube. The isopropanol was removed with a pipette, making sure not to disturb the pellet. A volume of 75% ethanol was then added, equal to the amount of Trizol that was present at the start (200ul). The samples were vortexed and re-spun at 8,000RPM, 4°C for 5 min. The ethanol was

removed using a pipette and the samples left to air dry; with the white pellet becoming transparent once dry.

Samples were treated with a DNase kit to ensure that the pellet is pure RNA with no DNA contamination. Pellets were resuspended in a mixture of 1µl of DNase, 2µl 10x Buffer and 17µl of RNA free H2O and incubated at 37°C for 45-60 minutes. After incubation, 5µl of DNase inhibitor was added to each tube before being thoroughly mixed and agitated twice more at 2-minute intervals. All samples were centrifuged at 13,000 RPM, 4°C for 2 minutes to pellet the DNase inhibitor. The aqueous solution (approx. 20ul) could then be removed and put into a fresh 0.5ml Eppendorf tube. Samples were stored at -80°C.

RNA concentration was determined using a NanoDrop 1000 spectrophotometer. RNA concentration was performed at a 260nm absorbance, the peak absorption of RNA, with DNA contamination calculated by the ratio of Abs_{260}:Abs_{280}, with 280nm being the peak absorption of DNA. A 260/280 ratio of 1.8-2.0 was considered adequately clear of DNA contaminants. The volume of solution containing 1µg of RNA was calculated by the following formula:

Volume containing 1ug RNA (µl) = 1000/RNA concentration(ng/µl)

2.5.2 RNA Reverse Transcription

RNA was reverse transcribed via a two step process to generate complementary DNA (cDNA). In the first step, 1µg of RNA was made up to a total volume of 12µl using ultrapure MilliQ water. To this was added 3µl of the annealing master mix (table 2.2)

Reagent	Volume for 1 Reaction
Oligo-dT Primers	1µl
Random Monomer Primers	1µl
Deoxyribosenucleotide triphosphate (dNTP) mix (10mM each dNTP)	1µl

Table 2.2. Reagents used in reverse transcription annealing mastermix.

The 15µl solution containing the 1µg of RNA and the annealing reagents were heated at 65°C for 5 minutes in a T100 Thermal Cycler. This heating step would denature the RNA, allowing for binding of the primers and dNTPs to the RNA. Once the 5 minute incubation had elapsed, samples were instantly "snap-cooled" using an ice/water solution to prevent further annealing. The second step involved extension of the now bound primers, with 5µl of the "extension mastermix" (table 2.3) being added to each sample (total 20ul).

Reagent	Volume for 1 Reaction
MMLV-RT enzyme	0.8µl
5x RT Buffer	4µl
Ultrapure MilliQ Water	0.2µl

Table 2.3. Reagents used in reverse transcription extension mastermix.

Extension was performed in the T100 Thermo Cycler, heating to 37°C for 10 minutes followed by an increase to 42°C for 60 minutes. Once completed, samples were diluted 1 in 10 using ultrapure MilliQ water (180µl added to 20 µl) and frozen down to -80°C until required.

2.5.3 Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction (qPCR) is the process by which cDNA is amplified to allow for the measurement of a specific sequence, usually a single gene on interest. Each well of a white 96-well plate would have mastermix (nucleotides and polymerase), primers (specific to the gene of interest), ultrapure MilliQ water and cDNA added (table 2.4). The Mastermix, water and primer was prepared and aliquoted into the 96 well plat. cDNA samples were then added to individual wells, in duplicate. The plates were centrifuged at 300 x g for 2 minutes to ensure all of the reaction mixture was at the bottom of the well before the plate was run on an iCycler CFX96 qPCR machine.

Reagent	Volume per reaction
Mastermix – specific to fluorescent system	5µl
MilliQ Water	2µl
Primer – specific to gene of interest and detection system	0.5µl
cDNA	2.5µl

Table 2.4. Reagents used in qPCR

Two systems are used, Perfect Probe and SYBR Green. The Perfect Probe system uses probes in the primer mix, complementary sequences that are labelled with a fluorophore and a quencher. Under normal circumstances, the quencher prevents any fluorescent signal from being detected. When the bound strand is extended, the probe is cleaved, separating the fluorophore and the quencher, allowing a fluorescent signal to be detected. Therefore, the level of fluorescence detected indicates the number of replicated strands.

SYBR Green involves the use of a fluorescent dye (found in the mastermix) which binds directly to double stranded (ds) DNA and fluoresces to create a detectable signal. However, since there is no sequence specific binding of a probe, the non specific nature of the SYBR Green system can create false signals. Mis-

amplified regions or primer-dimers that create dsDNA can result in a positive signal with an increase in fluorescence detected. To account for this, a melt curve is performed by which the sample is progressively heated to result in a separation of the double stranded DNA and a loss of fluorescent signal. The temperature at which the strands separate is unique to the base sequence; therefore, a single product should result in a single drop in fluorescence.

For each qPCR run, the threshold of fluorescence is set on a logarithmic scale of relative fluorescence units (RFU). Once a sample exceeds the threshold RFU, the number of PCR cycles that have been performed is read, called the Ct value. Ct values are calculated from two housekeeping genes, UBC and GAPDH, and a geometric mean is calculated between the two. Once the geometric mean Ct value of each gene of interest is calculated, the geometric mean of the house keeping genes is subtracted to generate the ΔCt value.

$$\Delta Ct = Ct_{\text{gene of interest}} - Ct_{\text{housekeeping gene}}$$

The ΔCt of the control sample is then subtracted from the ΔCt value of each treatment, generating the ΔΔCt value.

$$\Delta\Delta Ct = \Delta Ct_{\text{treatment}} - \Delta Ct_{\text{control}}$$

Fold induction is then calculated. The end result is a control value with a fold induction of 1, with samples resulting in either an increase of decrease in fold induction.

$$\text{Fold induction} = 2^{-\Delta\Delta Ct}$$

2.6 Statistical Analysis

All data analysis and graph drawing was done using GraphPad Prism 7 software. Normality was determined using Shapiro-Wilk normality test to determine whether parametric or non-parametric tests were required. A p-value<0.05 was used to determine if the data significantly deviated from a normal distribution, with significance resulting in the use of non-parametric tests. If p>0.05, parametric tests were used.

Comparisons between several groups with normally distributed data were made using a one-way ANOVA. For non-normally distributed data, a one-way ANOVA on ranks (Friedman test) was used. For analyses involving only two groups from the same donor, a paired t-test was used for normally-distributed data and a Wilcoxon matched-pairs signed rank test for those with non-normally distributed data. If the two groups were from different donors, a non-paired t-test or Mann-Whitney test would be used, depending on normality.

For all analyses, p<0.05 was considered to be significant.

3 Phenotypic Differentiation of PBECs

3.1 Introduction

As previously discussed, the growth of PBECs at ALI culture results in the pseudostratification of the epithelium, with the generation of a polarised cell layer that is representative of the bronchial epithelium *in vivo*. Differentiation can be monitored over the 21 days of culture by the analysis of gene expression. The transcription of a particular gene acts as a good indicator as to the protein expression levels of an epithelial cell layer. By measuring the mRNA levels of the epithelium at varying time points, we can determine how the expression profiles of particular donors varies over time, while allowing for a direct comparison between donor groups. To develop a detailed understanding of the expression profile of the different donor groups, genes were selected due to the expression of either cilia proteins, mucins or other secretary proteins that are expressed by normally differentiated epithelium. Comparisons between these genes would offer an insight as to how the epithelial cells from the different donor types may vary in terms of their structure and function within the respiratory system.

3.1.1 Cilia Genes

Cilliated Bronchial Epithelium 1 (CBE1) is a gene whose expression parallels the presence of ciliated cells in bronchial epithelium, being absent in other cells types where cilia are not present. CBE1 expression is detectable in bronchial brushings and is expressed when PBECs are grown at ALI culture. Due to the absence of a CBE1 homologue in invertebrates and microbes, where cilia are present, it suggests that CBE1 is involved in the differentiation or maintenance of ciliated cells in mammals as opposed to being a structural protein (105, 106). The expression of CBE1 mirrors that of another gene TEKT1 that encodes for the protein tektin (105). Tektins are structural proteins found in human bronchial

epithelial cells (107) that co-assemble with tubulins to form cilia in airway epithelial cells (108).

3.1.2 Mucins

One of the principal constituent parts of mucus within the respiratory tract are glycoproteins known as mucins. Heavy glycosylation results in the viscoelastic properties that make mucus a suitable protectant against a foreign insult. The secreted gel forming mucins come from four genes that form a cluster on chromosome 11p15.5, MUC2, MUC5AC, MUC5B and MUC6 (109). In airway tissue, MUC5AC is the predominant mucin expressed by goblet cells in normal healthy tissue, with MUC5B expression being restricted to submucosal glandular cells (110). Both MUC5AC and MUC5B are readily detectable in airway secretions of patients with COPD (111), and due to their abundance, make good markers to monitor epithelial mucin expression. *In vitro* research specifically looking at mucin production found that levels of MUC5A and MUC5B expression was influenced by the culture method, with ALI cultures exhibiting higher expression for both nasal and tracheal epithelium, when compared to 2D cultures on plastic (112).

3.1.3 Secretary proteins

Club Cell Secretory Protein (CCSP), encoded by the gene SCGB1A1, is a protein expressed by Club cells which are present in the bronchioles but are also among the basal cells of the larger airways (113). CCSP is a member of the secretoglobin family and has a role in modulation of the inflammatory response within the airway (114).

Glutathione (GSH) is an antioxidant, protecting against endogenous and exogenous reactive oxygen species and free radicals. GSH is synthesised by glutamate-cysteine ligase (GCL) which has two main subunits, a catalytic subunit (GCLC) and a modifier subunit (GCLM) (115). Genetically modified mice that had the GCLM gene removed show an overall decrease in GSH, due to a lower

rate of activity from GCL. The mice were also more sensitive to chemical oxidants (116). While this study used transgenic mice, it highlights the importance of GCLM, in modulating the activity of GCL and in the overall production of GSH.

3.2 Aims and Hypothesis

Research has shown that there are fundamental differences in terms of gene expression, and ultimately protein expression. Therefore, the aim of this study is to determine any significant differences in expression of the aforementioned genes of interest during epithelial differentiation of PBEC cultures grown both with and without RA, using qPCR.

The hypothesis is that there will be a significant difference between the healthy and COPD donor group's expressions of individual genes, with the removal of RA having a significant effect on gene expression within both groups.

3.3 Results

3.3.1 Comparisons between gene expression profiles of Healthy and COPD Donors with RA

Primary epithelial cells from both Healthy and COPD donors were seeded at $7x10^5$ cells per transwell insert, before being cultured for a total of 21 days. At days, 7, 14 and 21 transwells were isolated and the epithelial layer harvested using Trizol to allow for RNA extraction. After reverse transcription to cDNA, gene expression was measured using qPCR. While the three different time points allow for the tracing of gene expression through differentiation, it also allows for comparisons between the two donor groups to determine if the two cell populations differ in their differentiation and function.

Cells from healthy donors grown with RA showed an overall increase in CBE1 fold induction over the 21 days ($p<0.05$), with significance lying between day 7 and 21. Cells from COPD donors showed the same pattern of expression, with an increase in CBE1 fold induction over the course of 21 days ($p<0.05$), specifically between day 7 and 21. When comparing between the two donor groups, there is a significant difference between the fold induction at day 21 ($p<0.05$), with the healthy donors exhibiting higher expression of CBE1. While there was a trend for higher expression of CBE1 in the healthy donors at day 14 and less so at day 7, neither of these was statistically significant when compared to the COPD donors. Healthy donor cells with RA exhibited an overall increase in TEKT1 expression over the 21 days ($p<0.05$) with significant difference between day 7 and 21. COPD donor cells grown under the same conditions exhibited the same pattern, with an overall increase in TEKT1 expression ($p<0.05$). Comparisons between the two donor groups highlighted no differences across the three time points (Figure 3.1).

The fold induction of MUC5AC did not significantly vary over any of the time points for either donor group, whilst comparisons between the two donor groups

also highlighted no significant differences. When analysing the expression of MUC5B, only the healthy donors exhibited a significant increase in expression, between days 7 and 14 ($p<0.05$). Like MUC5AC, there were no significant differences between the two donor groups at any time points. However, the fold induction for both mucin genes is higher at all three time points then the day 0 base line (fold induction of 1) that the cultures are normalised to, for both donor groups.

Expression of SCGB1A1 did not significantly alter in either donor group over the 21 days of culture. Comparing between the two donor groups showed that the healthy donors had significantly higher expression of SCGB1A1 at all three time points ($p<0.05$). GCLM expression increased in healthy donors over the 21 days of culture ($p<0.005$). Despite this increase not being reflected in the COPD donors, there was no significant difference between the two donor groups at any time points.

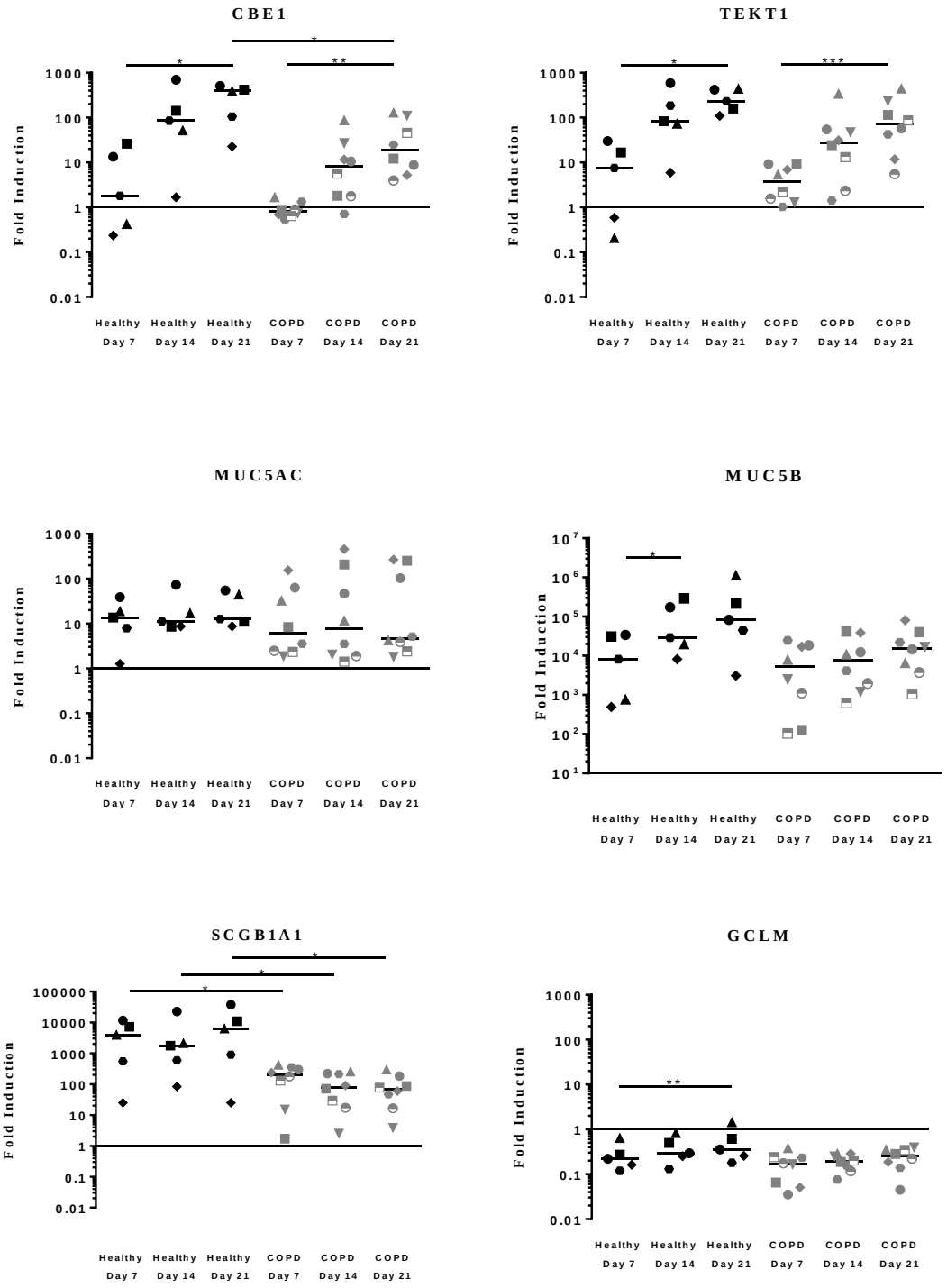

Figure 3.1. Phenotypic gene expression profiles over 21-day differentiation. Healthy and COPD PBEC ALI cultures grown over 21 days, with RNA being harvested at days 7, 14 and 21. Data calculated by the ΔΔCt method, normalising to housekeeping genes (UBC/GAPDH) expression, and then to a day 0 +RA control culture. Each data point represents a single donor, with the group median displayed. Significance was calculated using non-parametric testing, either the Friedman test or Wilcoxon test for inter-donor samples, or Mann-Whitney test for between donor testing. Significance is denoted by asterisk (*=p<0.05, **=p<0.01, ***=p<0.001). n = 5 healthy donors, 8 COPD donors

3.3.2 GMA and Fluorescent Images of Cultures grown with RA

The results suggest that there is very little difference between the healthy and COPD donors in terms of gene expression over the course of differentiation. The gene SCGB1A1 is the only gene that shows a significant difference between the two donor groups that is consistent over the 21 days of culture.

While there are small differences at the RNA level, it is yet to be determined if this translates into a difference at the protein level. Therefore, healthy and COPD donor cells cultured at ALI were fixed, embedded in GMA, and sectioned for staining. Cultures were also fixed for immunofluorescenct staining and imaging.

GMA sections of both donor groups show that at a structural level, there are no phenotypic differences between the two donor groups. Cultures from both donor groups start as a simple squamous layer that expand and differentiate into pseudostratified columnar epithelium (Figure 3.2). While it is not possible to see any extracellular structures such as cilia on the GMA sections, immunofluorescence staining of β-tubulin shows that both the Healthy and COPD donors have cilia on the apical surface (Figure 3.3).

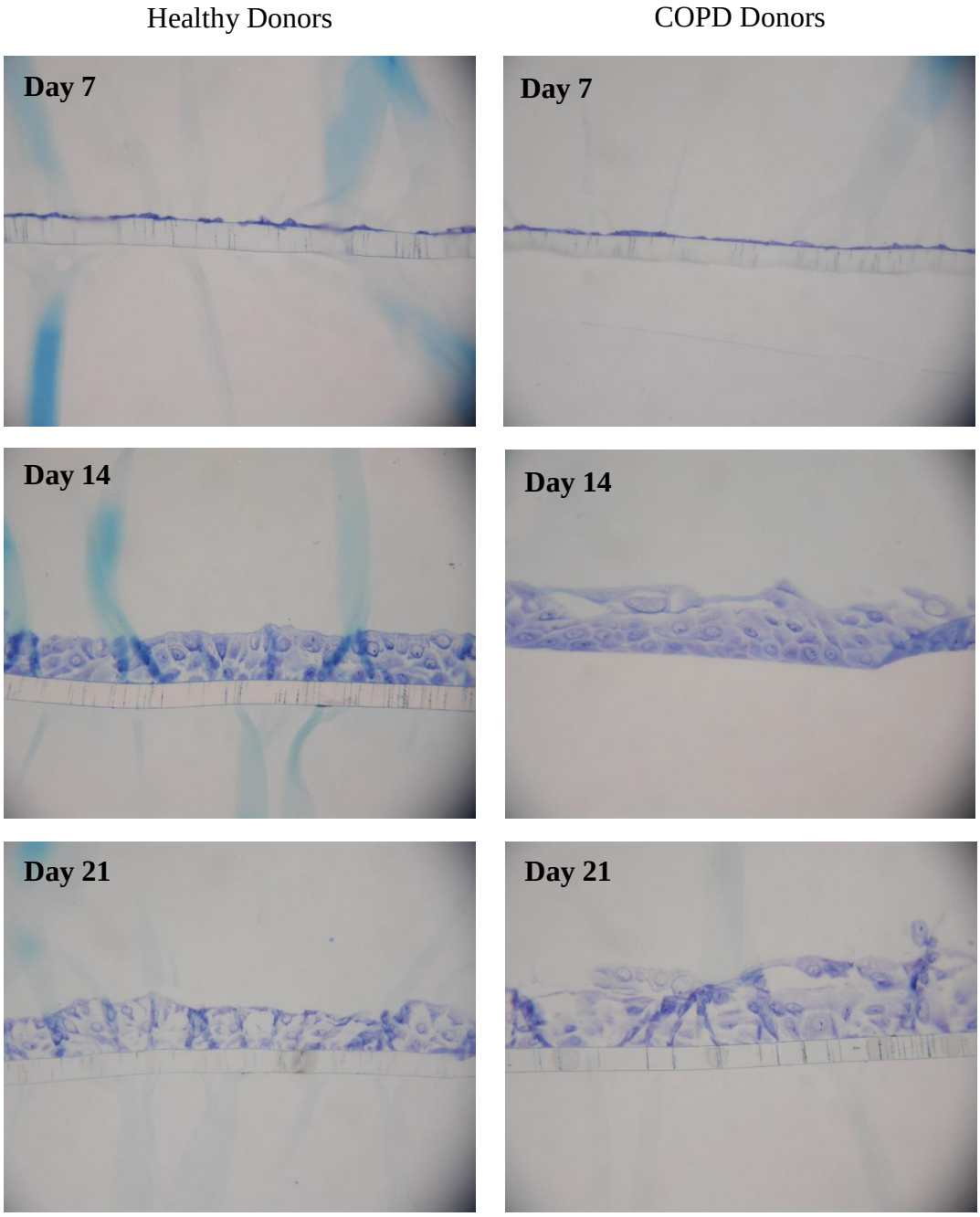

Figure 3.2. Representative GMA sections of differentiating epithelium for both Healthy and COPD donors.
PBEC cultures were fixed and embedded in GMA at day 7, day 14 and day 21. Sections were cut and stained with Toluidine Blue to visualise the cellular structure of the epithelium.

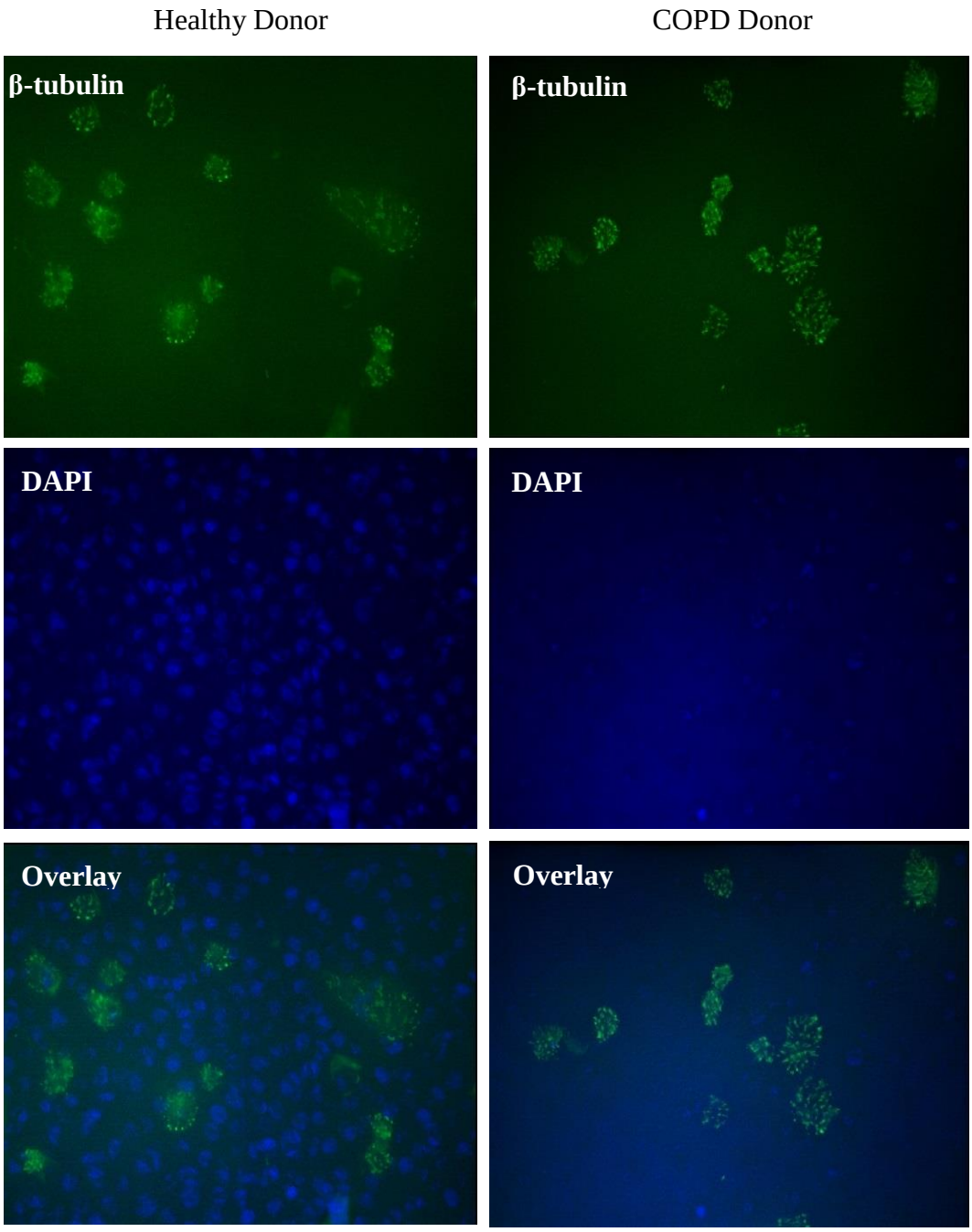

Figure 3.3. Representative images of immunofluorescent staining for β-tubulin for both healthy and COPD donors.
PBEC cultures were fixed with paraformaldehyde and dual stained using DAPI and a fluorescently tagged antibody for β-tubulin. Images were taken for each stain separately and then overlaid.

3.3.3 Comparisons between cultures grown with RA and those grown without RA

Thus far, there is very little difference between the Healthy and COPD cultures grown in the presence of RA in terms of both RNA expression as well as the physical structure of the epithelial barrier. To test the hypothesis that the removal of RA from the culture medium results in the development of SM, ALI cultures from both Healthy and COPD donors were grown from day 0 in ALI medium without the RA supplement being added. Cultures were harvested at days 7, day 14 and day 21, with RNA being extracted using Trizol. qPCR was performed to analyse the expression of the same aforementioned genes (Figure 3.4).

PBECs grown without RA showed no significant increases in fold induction of CBE1 over the 21 days of culture, in either the Healthy or COPD donor groups. COPD donors exhibited a significant increase in TEKT1 expression between day 7 and 21 ($p<0.05$). Whilst this pattern of increase is not seen in the cells taken from healthy donors, there was no significant difference between the two donor groups. Both MUC5AC and MUC5B gene expression showed no alteration in fold induction over the 21 days of culture for either the Healthy or the COPD donors.

Over time, there was a significant decrease in SCGB1A1 fold induction in both the Healthy ($p<0.05$) and the COPD donors ($p<0.005$). The Healthy donors had a significant decrease in fold induction between days 7 and 14 ($p<0.05$) while the COPD donors had a significant decrease in fold induction between days 7 and 14 ($p<0.05$) and day 7 to day 21 ($p<0.005$). Comparisons between the Healthy and COPD donors grown without RA showed significant differences at day 14 and day 21 ($p<0.005$) with the healthy donors exhibiting a higher fold induction of SCGB1A1 than the COPD donors. The absence of RA from the culture medium results in a slight decrease in fold induction of GCLM over the course of the 21 days in both donor groups, only proving significant between day 7 and 21 of the COPD donors($p<0.05$). When comparisons are made between the two donor groups, the healthy donors have significantly higher expression then the COPD donors at both day 14 ($p<0.005$) and day 21 ($p<0.05$).

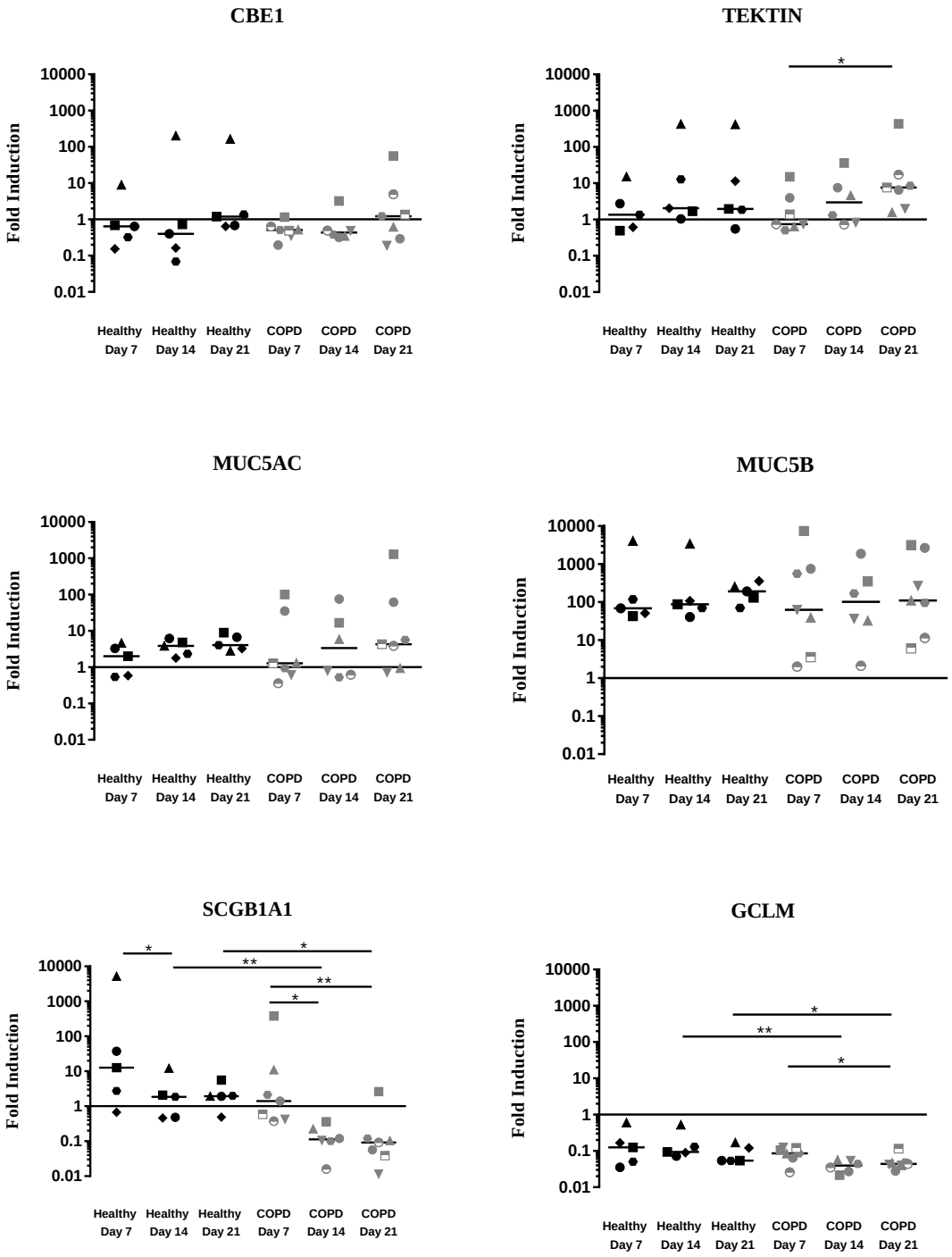

Figure 3.4. Phenotypic gene expression profiles over 21-day differentiation without RA.

Healthy and COPD PBEC ALI cultures grown over 21 days in the absence of RA, with RNA being harvested at days 7, 14 and 21. Data calculated by the ΔΔCt method, normalising to housekeeping genes (UBC/GAPDH) expression, and then to a day 0 +RA control culture. Each data point represents a single donor, with the group median displayed. Significance was calculated using non-parametric testing, either the Friedman test or Wilcoxon test for inter-donor samples, or Mann-Whitney test for between donor testing. Significance is denoted by asterisk (*=p<0.05, **=p<0.01, ***=p<0.001). n = 5 helathy donors, 7 COPD donors

3.3.4 GMA and Fluorescent Images of Cultures grown without RA

From the qPCR data, it is evident that there is very little change in the expression of cilia related genes and those genes associated with the production and secretion of mucus, over the course of differentiation in cultures grown without RA. The main difference in expression occurs in the gene encoding for CCSP, with expression decreasing in both cultures but lower expression occurring in the COPD donors. As with the cultures grown in the presence of RA, ALI cultures grown without RA were fixed, for either embedding in GMA or for immunofluorescence staining.

GMA sections from cultures grown without RA exhibit a different pattern of development from those cultures grown with RA. At day 7, the epithelial layer is a simple cuboidal epithelium. By day 14, this changes into a stratified squamous epithelium with a keratin layer on the apical surface. This architecture persists until day 21, with the cell layer thickening slightly and the keratin layer becoming more pronounced as the culture develops, with increasing signs of cell hyperplasia (Figure 3.5).

Whilst the GMA sections suggest no apical features that are consistent with a well-differentiated pseudostratified epithelium, staining with an immunofluorescent antibody for β-tubulin confirmed the absence of cilia on the apical surface (Figure 3.6).

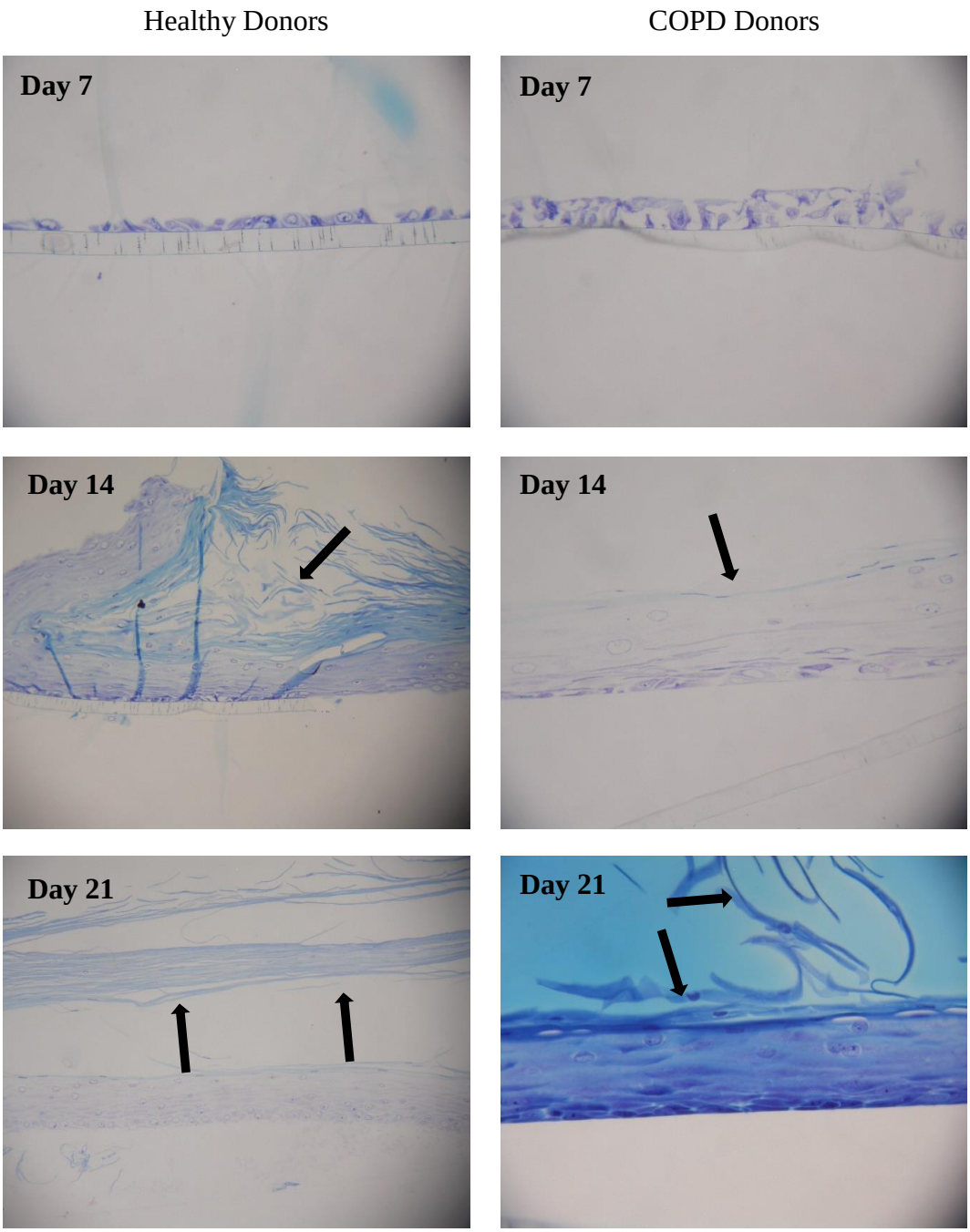

Figure 3.5. Representative GMA sections of differentiating epithelium for both Healthy and COPD donors grown in the absence of RA.
PBEC cultures grown in the absence of RA were fixed and embedded in GMA at day 7, day 14 and day 21. Sections were cut and stained with Toluidine Blue to visualise the cellular structure of the epithelium. Arrows indicate areas of keratin build up.

Healthy and COPD Donors

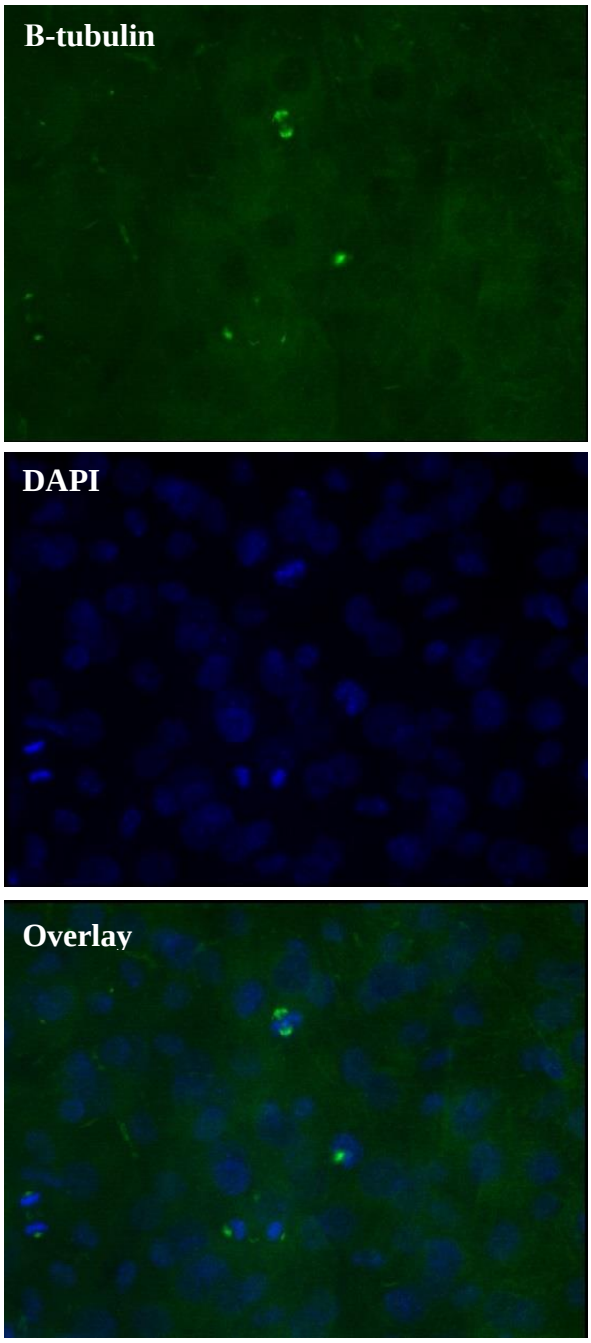

Figure 3.6. Representative image of immunofluorescence staining for β-tubulin of both healthy and COPD donors grown without RA.
PBEC cultures grown in the absence of RA were fixed with paraformaldehyde and dual stained using DAPI and a fluorescently tagged antibody for β-tubulin. Images were taken for each stain separately and then overlaid.

3.3.5 Comparison of TEER readings between cultures grown with RA, and those grown without RA

As can be seen from the GMA images, there is a marked difference between epithelial thicknesses when RA is removed from the growth medium. This difference in epithelial thickness can be measured indirectly by the use of chopstick electrodes, measuring the electrical resistance over the epithelial barrier. A thicker epithelium results in an increase in resistance, allowing for comparisons between cultures.

The removal of RA results in an increase in the TEER readings at all three time points, for both donor types. While those cultures grown with RA displayed a significant increase in TEER reading over the 21 days, this increase was also seen in the cultures grown without RA. By day 21, both donor groups grown without RA displayed a 2.9-5.5 fold increase in TEER when compared to those grown with RA. When comparisons are made between the two donor groups, there are no significant differences between the TEER readings for those cultures grown in the presence of RA or without RA.

This difference in TEER reading shows that the differences of the epithelial thickness seen in the GMA images are consistent across all donors, despite the large spread in the TEER readings within the donor groups (Figure 3.7).

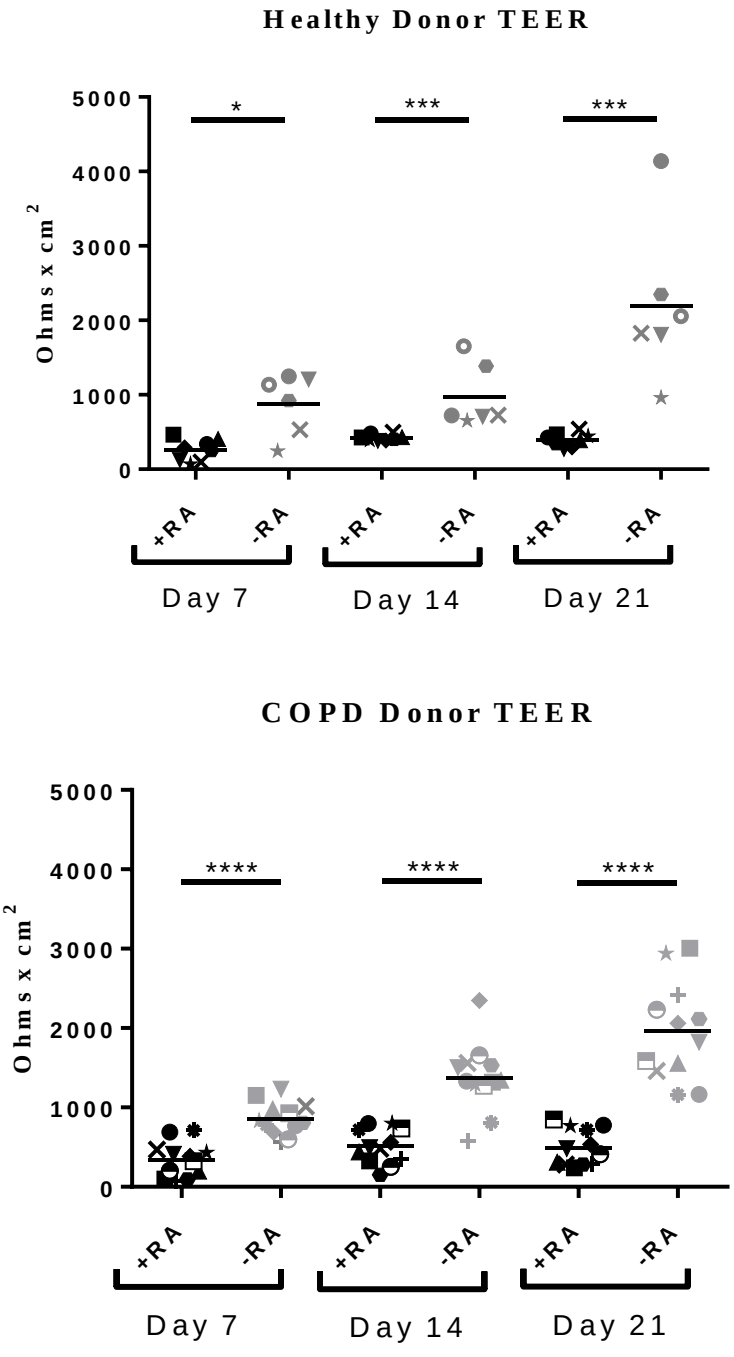

Figure 3.7. TEER readings of Healthy and COPD cultures, grown in the presence and absence of RA.

Cultures had the TEER measured over 21 days of culture, either in the presence or absence of RA. The removal of RA results in a large increase of TEER reading, regardless of donor type. Each data point represents a single donor, with the group median displayed. Statistics was performed using the Mann-Whitney test. Significance is denoted by asterisk (*=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001). n = 8 healthy +RA, 6 healthy –RA, 12 COPD +/- RA.

3.3.6 Cytokeratin and Involucrin expression profiles as an indicator of SM development

As previously discussed, the expression profile of cytokeratins is tissue specific and can be used to identify the source of epithelium, as well as being able to distinguish between the type of epithelium. Therefore, by looking at the expression profiles of CK7 and CK13, in conjugation with the expression of the SM marker involucrin, we can assess whether or not SM has developed when the PBECs are grown in the absence RA.

PBECs from healthy donors grown without RA show a trend for a decrease in CK7 expression, but this was not significant. However, PBECs from COPD donors grown under the same conditions exhibited a significant decrease in fold induction of CK7 expression between day 7 and 21 as well as between day 14 and 21 ($p<0.05$). When these cultures were compared to those grown with RA, the cultures without RA showed a significant decrease in CK7 expression at day 21 for healthy donors, and day 14 and 21 for COPD donors ($p<0.05$). The fold induction of CK13 in cultures grown without RA shows an overall decrease over the 21 days for both Healthy ($p<0.05$) and COPD donors ($p<0.01$), with a significant difference between days 7 and 21 for both. Despite this decrease, there was no significant differences at any time point when compared to those cultures grown in the presence of RA.

Healthy donors exhibited a statistically significant increase in the fold induction of Involucrin over the 21 days of culture ($p<0.001$), with a significant difference between day 7 and 21 ($p<0.005$). COPD donors grown without RA have the same pattern of increase of involucrin expression but it is not significant at any time point. Comparisons of involucrin expression between those cells grown with RA and those grown without RA, highlights a significantly higher fold induction in the COPD cells grown without RA at both day 14 ($p<0.05$) and day 21 ($p<0.05$).

There was no significant differences between the expression of either CK7, CK13 or involucrin at any time point, between the Healthy and COPD donors when grown in the presence of RA (Figure 3.8).

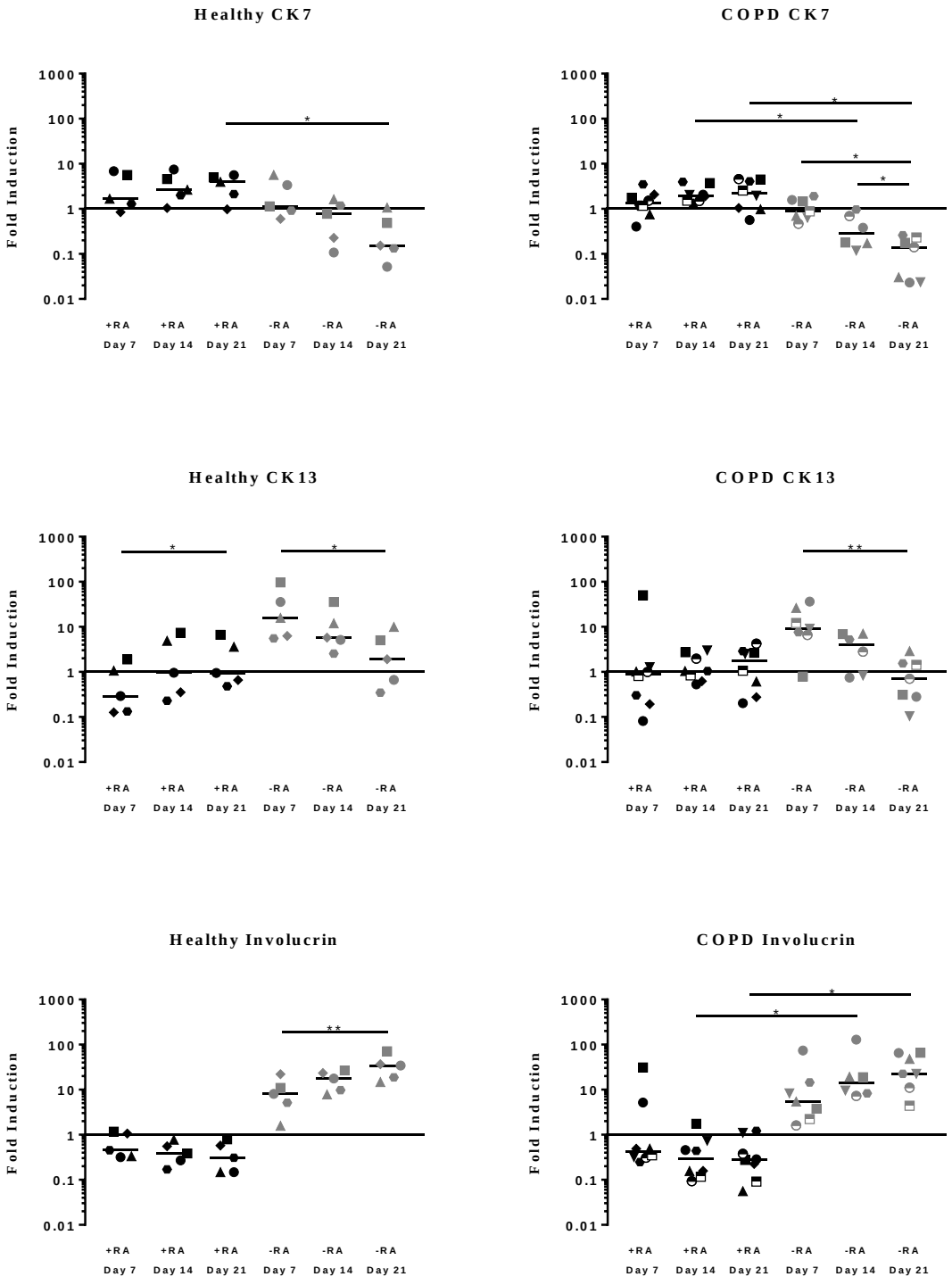

Figure 3.8. Healthy and COPD PBEC ALI cultures grown over 21 days, both in the presence and absence of RA.

RNA was harvested at days 7, 14 and 21. Data calculated by the ΔΔCt method, normalising to housekeeping genes (UBC/GAPDH) expression, and then to a day 0 +RA control culture. Each data point represents a single donor, with the group median displayed. Significance was calculated using non-parametric testing, either the Friedman test or Wilcoxon test for inter-donor samples, or Mann-Whitney test for between donor testing. Significance is denoted by asterisk (*=$p<0.05$, **=$p<0.01$). n = 5 healthy, 8 COPD

3.4 Discussion

3.4.1 Comparisons between healthy donors and COPD donors

This series of experiments examined the differences in gene expression between PBEC cultures from Healthy and COPD donors, grown both with RA and without RA, over the course of 21 days of culture. The qPCR data is supported with both GMA sections of embedded transwells, as well as immunofluorescence staining for the cilia protein β-tubulin.

Both CBE1 and TEKT1 displayed an increase in expression over the 21 days, for both COPD and healthy donors. This pairwise expression of these two genes has been demonstrated before (105) and suggests that despite being taken from a COPD donor, the epithelial cells are still capable of generating cilia. Previous studies suggest that smoking reduces the amount of cilia present (117) with cilia shortening present in individuals who smoke, and further shortening in those with COPD (118). While this shortening may be present in these cultures, it might occur at the protein level or once the cilia have formed, and therefore any differences are undetectable at the mRNA level. While cilia are not visible via Toluidine blue staining of GMA sections, and therefore the length cannot be measured, staining with a fluorescently tagged β-tubulin antibody showed that both the COPD and healthy donors exhibited clearly visible cilia on the apical surface. While there is evidence that cigarette smoke (CS) can suppress levels of TEKT1 expression (119), there is no CS present in the ALI culture system. Despite both donor groups exhibiting an increase in CBE1 expression, the healthy donors exhibit a trend for higher expression of CBE1, becoming significant by day 21. This suggests that while both cell populations are able to produce cilia, the modulation of the ciliated epithelium may be different between the two groups.

The qPCR data highlights no change in the expression of MUC5AC over the 21 days of culture for either donor group. Previous research used in vivo sampling from patients with COPD who continued to smoke, to show an increase in

MUC5AC expression in the bronchial epithelium (120). The persistence of CS may be the reason for the increased levels of MUC5AC expression, with inflammation associated with cigarette smoke and pollutants appearing to mediate secretory cell hyperplasia (110). While there was no change in expression from day 7 to day 21, it is worth noting that fold induction was higher than the day 0 values that comprised the baseline. Previous research had shown that MUC5AC expression reached a plateau by day 7 of culture (121). Unlike MUC5AC, MUC5B expression showed an increase in the healthy donors over the 21 days of culture, significantly increasing from day 7 to day 14. Despite this increase in expression, there were no significant differences between the two donor groups. This result contradicts some of the literature that has shown MUC5B to have a higher mRNA expression in individuals with airway inflammation (122), with protein expression correlating with pack years (120).

The expression of the gene SCGB1A1, that encodes for the protein CCSP, did not significantly alter over the 21 days of culture in either donor group, when grown in the presence of RA. While the expression did not change for the healthy donors, there was significantly higher expression when compared to the COPD donors. These findings are in accordance with previous research that has shown that CCSP expression is lower in patients with COPD (123, 124), with fewer CCSP positive cells being found in tissue samples taken from patients with COPD (125). It suggests that the expression of CCSP may be under the regulation of endogenous transcription factors as opposed to expression primarily being influenced by the environment. If the later were true then we could expect the levels of CCSP expression to be the same between both donor groups due to the absence of environmental stimuli such as CS in this culture model. It has been suggested that CCSP can been used as a predictive biomarker of both lung function decline and the development of COPD (126), an idea that is supported by this data, with lower levels of CCSP expression found in those with COPD.

The expression for GCLM differs only in the healthy donor group, significantly increasing over the 21 days of culture. Despite this increase, there is no difference between the two donor groups. While there is a significant increase over the 21 days for the healthy cultures, the increase is very small, with the median being

below the baseline fold induction of a day 0 culture. This suggests that at day 0, freshly plated cells are under more oxidative stress compared to a fully differentiated epithelial barrier. It has been shown that bronchial brushings from healthy-smokers had a higher expression of many antioxidants, including GCLC and GCLM, when compared to those who did not smoke. This difference in expression becomes more pronounced in those with mild and severe COPD, with expression increasing as disease severity worsens (127). This is counter to the results shown here, with there being no significant difference between the Healthy and COPD donors.

3.4.2 The Removal of RA

The removal of RA has an effect on the epithelial phenotype in both Healthy and COPD PBECs. The removal of RA attenuates the increase in expression of CBE1 in both the Healthy and COPD donors. However, there is an increase in expression of TEKT1 by the COPD donors, grown without RA. While this increase is not seen in the healthy donors, there are no significant differences between the two donor groups. When compared to comparable cultures that are grown in the presence of RA, there is a trend for lower expression in the cultures grown without RA. The increase in TEKT1 expression in the cultures without RA is counterintuitive, when both the GMA sections and immunofluorescence images show that there is an absence of cilia on the apical surface. However, it could be that in this instance, an increase in RNA expression does not necessarily equate to an increase in protein.

MUC5AC expression did not differ between the two donor groups when cultures were grown without RA. When these cultures were compared to those grown in the presence of RA, there was only a trend for decreased expression in the healthy donors grown without RA. This trend for a decrease in MUC5AC expression correlates with previous findings that MUCAC is downregulated in SM (128). The expression of MUC5B follows a similar pattern, with no differences between the two donor groups grown without RA. Comparisons with those cultures grown in the presence of RA show a trend for lower expression in the healthy donors and

a significantly lower expression in COPD donors grown without RA at days 14 and 21. The suggestion that MUC5B expression is lower is SM is supported by *in vitro* research looking at mucin expression profiles of cells cultured both at ALI and fully submerged 2D cultures that result in a squamous phenotype (112). These findings show that the expression of mucins is dependent on whether or not the epithelium has fully differentiated or has developed into SM. However, the differences in MUC5AC fold induction are not as big as has been previously reported (98).

Both donor groups exhibited a decrease in SCGB1A1 expression over the 21 days of culture when cultured without RA. While both donors show a decrease in expression, the healthy donors show a consistently higher expression, becoming significant at day 14 and 21. This difference in expression parallels the cultures grown with RA, where the healthy donors exhibited higher expression then their COPD counterparts. Comparisons made between the two culture conditions show that there is a trend for those cells grown without RA to have lower expression of SCGB1A1, becoming significantly lower for the COPD cells by day 14, persisting until day 21. As SCGB1A1 is expressed in club cells, it is logical that as the epithelium terminally differentiates into SM that there is an overall reduction in the number of CCSP secreting cells, leading to a decrease in expression.

The expression of GCLM is significantly lower in the COPD donors cultured without RA when compared to healthy donors in the same culture conditions. This low expression remains when samples are compared to those grown in the presence of RA, resulting in a significant difference between the two culture methods for the COPD donors, and only a trend for a lower expression in the healthy donors grown without RA. These results suggest that, due to the dependant relationship of GCL activity on the presence of GCLM, that the SM cultures have an inhibited anti-oxidant response with decreased activity of GCL, producing lower levels of GSH.

There was a significant decrease in CK7 expression in COPD cultures grown without RA over the 21 days of culture, while the healthy donors exhibited a trend for the same decrease in expression. When compared to those cultures grown with

RA, there is a significantly lower expression at day 14 and 21 for COPD donors and day 21 for healthy donors. This shows that in both donor groups, the same pattern of decreased expression for CK7 occurs when RA is removed from the culture medium. The same pattern of decrease occurs for CK13, decreasing over the 21 days in cultures grown without RA for both donor groups. However, comparisons made with cultures grown with RA highlighted no differences in expression. Involucrin expression showed the opposite pattern to that of CK7 and CK13, increasing in both donor groups grown without RA, but only significant in the healthy donors. When compared to those cultures grown with RA, only the COPD cultures had significantly higher expression at day 14 and day 21. The healthy donors grown without RA, while not significant, still displayed higher expression of involucrin when compared to their counterparts grown in the presence of RA.

The expression of these three structural genes has been used before in the identification of SM in endobronchial biopsies from smokers (129). The lower expression of CK7 in those cultures grown without RA is a good indicator of the presence of SM, with CK7 expression found to be strong in respiratory epithelium when compared to SM (130). While the expression of CK13 is initially much higher in cultures grown without RA, expression lowers over time. As we are looking at the expression of a gene rather than the total amount of protein present, a down regulation of expression over time could represent saturation of that particular protein. Counter to this is the expression of involucrin, with expression rising over the 21 days. As involucrin is a protein that cross links to form the cornified cell envelope (131, 132), continued increase in expression could represent a further thickening of the cell envelope, as seen in the cultures grown without RA (figure 3.5).

Despite the qPCR data being supported with GMA sections and immunofluorescence imaging, there are further analyses that could be performed to further understand the changes that are occurring during differentiation when grown either with or without RA. Epithelial samples could be harvested for protein extraction and analysis via Western blot. This would allow for the detection, as well as the quantification, of proteins expressed by the epithelium.

Further work using the GMA sections and specific antibody staining could identify differences, not just in expression levels, but also how those proteins may differ in their localized expression. Staining can also identify key cellular structures that may not be apparent from Toluidine Blue staining, or that may be loss due to the GMA embedding process. Enzyme linked immunosorbent assays (ELISAs) can assess the amount of protein that is secreted into the medium. Through collection of the basal and apical washes, secretion of proteins such as Mucins can be analysed over time, potentially depicting a much more accurate pattern of expression. While the qPCR data in this study allows for the quantification of RNA expression, these further methods would elucidate how much of the transcribed RNA is translated into protein.

3.5 Conclusion

Overall, the data suggests that there is very little different between the two donor groups when grown in the presence of RA. From a structural point of view, the cells are phenotypically very similar. While there was a small amount of variation in expression over the 21 days of culture, comparisons between the two donor groups only highlighted a significant difference in the expression of CBE1, with healthy donors exhibiting a greater increase over the 21 days then the COPD donors, whose expression also significantly increased. This difference in expression of CBE1 does not appear to result in a difference in cilia present on the apical surface, as seen with the immunofluorescence imaging. In terms of secretary proteins, only CCSP exhibited a difference between the two donor groups, with the healthy donors exhibiting greater expression, a finding previously reported.

The removal of RA has a profound effect on the structure of the cells, the cytokeratin and involucrin profile is altered, presenting a phenotype that is very similar to that of SM. The expression of cilia markers does alter over the 21 days of culture but tends to be downregulated when compared to those cultures grown with RA. The expression profile of secreted genes is also altered when RA is removed, resulting in the down regulation of CCSP and mucin genes.

This preliminary data seems to suggest that there is very little difference between healthy and COPD donors in terms of structure and their secretary profile in terms of mRNA expression. Only the removal of RA and the induction of SM results in any real change.

4 Basal Stimulation of a fully differentiated epithelium with the addition of TNFα

4.1 Introduction

The results so far suggest that epithelial cells from both healthy and COPD, cultured in the presence of RA, will develop into functional, fully differentiated epithelium, with similar genetic expression profiles. Removal of RA from the culture induces a change to the expression profile, resulting in terminally differentiated epithelium that displays some of the characteristics of SM.

The next line of investigation is to determine if these cultures show any differences in their response to inflammation. The addition of a pro-inflammatory mediator to the epithelium will simulate an *in vivo* insult such as CS. Monitoring the genetic response to this challenge will highlight any nuances between the different donor groups as well as the culture method.

4.1.1 TNFα as a pro-inflammatory cytokine

The greatest risk factor for the development of COPD is CS, resulting in a large increase in the number of inflammatory cells and inflammatory mediators that are released (10), dependent on smoking intensity (133). One inflammatory mediator that has been studied in detail is tumour necrosis factor alpha (TNFα). The addition of CS to a mouse model shows an increase in TNFα expression in mice in the first few hours, playing a role in the acute phase of inflammation (134). CS exposure in guinea pigs also resulted in an increase of TNFα in the first 2 hours of exposure, with long-term exposure resulting in an increase in plasma TNFα (135).

There is evidence that the action of TNFα is linked directly to the development of COPD. The expression of TNFα in human biopsy samples increases in parallel to the severity of hyperplasia and subsequent metaplasia that occurs in individuals who smoke (84). Artificial overexpression of TNFα in Mice results in pathological changes similar to that of emphysema, enlargement and loss of airspaces within the lung (136). Likewise, if the receptor for TNFα is knocked out in genetically modified mice, the emphysema-like effects of pancreatic elastase do not occur (137). These results show that TNFα *in vivo*, is implicated in the development of emphysema and thus in the pathogenesis of COPD. *In vitro* work using normal Human Bronchial Epithelial Cells has shown that increasing pro-inflammatory cytokines, including TNFα, resulted in basal cell hyperplasia and the development of SM.

The addition of a single pro-inflammatory mediator, in this instance TNFα, means that cause and effect are more easily determined. By monitoring the expression of genes that are involved in inflammation and those that respond to oxidative stress, we can monitor how epithelial cells from different donor populations, as well as different culture methods, differ in their gene expression when stimulated.

For this experiment, genes of interest have been split into two groups, those that are involved in antioxidant pathways, and those that are involved in the proinflammatory pathway.

4.1.2 Inflammatory Genes of Interest

4.1.2.1 Antioxidant Genes

Haem oxygenases (HO) are enzymes that catalyse the breakdown of haem into bile pigments (138). The two isoforms, HO-1 and HO-2, exhibit different expression patterns, with HO-1 being the main candidate for adaption to new environmental stressors (139) being inducible by not just haem but a host of inducers such as heavy metals and inflammatory cytokines (140). Work with mice has shown that the addition of TNF or IL-8 to mice resulted in a rapid increase in the levels of HO activity (141).

NRF2 is a central transcription factor that regulates the antioxidant defence system and is there for implicated in inflammatory lung diseases (142). Upon activation, NRF2 binds to the Antioxidant Response Element (ARE), an enhancer sequence that results in the expression of certain antioxidant genes (143). CS in humans results in an increase of NRF2 expression in the lungs and an increase in the expression of NRF2 regulated genes (144). The removal of the NRF2 gene from mice results in a higher susceptibility to the development of CS induced emphysema, with mice exhibiting increased apoptosis and alveolar oxidative stress (145).

One of the genes that is under the control of NRF2 is GCLM (142). As previously described, the GLCM subunit is involved in the production of the antioxidant GSH (115). While we have shown no significant difference in the expression of GCLM between COPD and healthy donors grown in the presence of RA, measuring the levels of GCLM post TNFα stimulation will add credence to any alterations in the expression of NRF2.

4.1.2.2 Proinflammatory Genes

IL-6 and IL-8 are both proinflammatory cytokines that play important roles in the immune response of the lungs. IL-6 is involved in the regulation of immune cells,

including the terminal differentiation of B cells. Whilst IL-6 is secreted by inflammatory cells, it has also been shown to be expressed in the bronchial epithelium (146). Previous studies have shown that levels of IL-6 are elevated in individuals with COPD, in the blood plasma (147), serum (148) and in breath exhaled condensates (149). It also appears that the measurable levels of IL-6 differ depending on disease state, increasing upon an exacerbation of COPD (150), and disease progression, with expression increasing with the severity of COPD (147). IL-8 is a neutrophil chemoattractant, resulting in an influx and activation of neutrophils and other leukocytes to the lungs upon stimulation (151). Basal levels of IL-8 are found to be higher in the lungs of patients with COPD when compared to those with normal lung function (152) or those with asthma (153, 154). Both IL-6 and IL-8 expression is inducible in epithelial tissue with the addition of TNFa (146, 151).

The protein RANTES (also known as CCL5) is a chemotactic cytokine leukocytes, playing a role in immune cell recruitment during inflammation in the lung. Due to this chemotaxis, induction of RANTES is triggered by invading pathogens, both bacterial and viral (155), which results in the expression of RANTES by the respiratory epithelium (156). Studies have shown that while patients with severe COPD have elevated levels of RANTES in the bronchial epithelium (157), the addition of CS to cell cultures resulted in RANTES repression when further stimulated with Rhino Virus (RV) infection (158). Any effect that the addition of a proinflammatory mediator such as TNFα may have on the expression of RANTES would be of interest, to understand the role that it may play in the pathogenesis of COPD. Initial evidence suggests that the addition of TNFα will result in a dose dependant release of RANTES from cultured PBECs (159).

Thymic stromal lymphopoietin (TSLP) is a protein involved in the activation of dendritic cells resulting in a proinflammatory cascade, with the activation of Th2 pro inflammatory cells resulting in the release of cytokines, including TNFα (160). TNFα has also been shown to be an inducer of TSLP (161). The levels of TSLP mRNA was elevated in the epithelium of COPD patients as well as protein levels in the BAL fluid when compared to healthy controls (162).

4.1.3 Aims and Hypothesis

The first aim of the research in this chapter is to determine if cells taken from healthy and COPD individuals exhibit differing patterns of inflammatory gene expression when stimulated with TNFα. The second aim is to observe the impact that the growth supplement RA will have on the inflammatory response in cultures from both COPD and healthy donors.

The first hypothesis to be tested is that there will be a more pronounced inflammatory response in cultures from COPD donors when compared to healthy donors. The second hypothesis to be tested is that the removal of RA from the culture medium will attenuate the inflammatory response in cultures grown without RA when compared to those grown with RA, regardless of donor type.

4.2 Results

4.2.1 Comparisons between the gene expression profile of Healthy and COPD donors when stimulated with TNFα

Using the same methodology as in the previous study, PBECs from both Healthy and COPD donors were seeded at $7x10^5$ cells per transwell and cultured for 21 days at ALI. Once fully grown, cultures had the basal medium replaced with starvation media for 24 hours. All except one culture per donor was stimulated with the addition of 10ng/ml of TNFα to the basal compartment and incubated at 37°c. The negative control culture (un-stimulated) just had starvation media added to the basal compartment. After 24 hours, the epithelium was harvested with Trizol to allow for RNA extraction. After reverse transcription, gene expression was measured by qPCR.

Stimulation with TNFα resulted in a significant increase in IL-8 expression for both Healthy ($p<0.01$) and COPD ($p<0.001$) donors when compared to baseline expression. Despite the COPD donors exhibiting a higher level of gene expression then the healthy donors, there was no significant difference between the two donor groups. While both donor groups had increased levels of IL-6, only the healthy donors had a significant increase when compared to baseline ($p<0.01$), with a significant difference occurring between the two donor groups ($p<0.05$).

The expression of RANTES exhibited the same pattern as that of IL-8. Both donors exhibited an increase in expression with TNFα stimulation ($p=0.01$) with higher expression in the COPD donors when compared to the Healthy donors. Stimulation with TNFα resulted in no alteration in the expression of TSLP when compared to baseline for the healthy donors. In comparison, the COPD donors exhibited a significant decline in the expression of TSLP ($p<0.01$). Out of the three antioxidant genes that were analysed, there was only a significant change in the expression of HO1 in the COPD donors, increasing when compared to the baseline ($p=<0.05$). There were no significant alterations in expression levels for

either NRF2 or GCLM in either donor groups when simulated with TNFα (Figure 4.1).

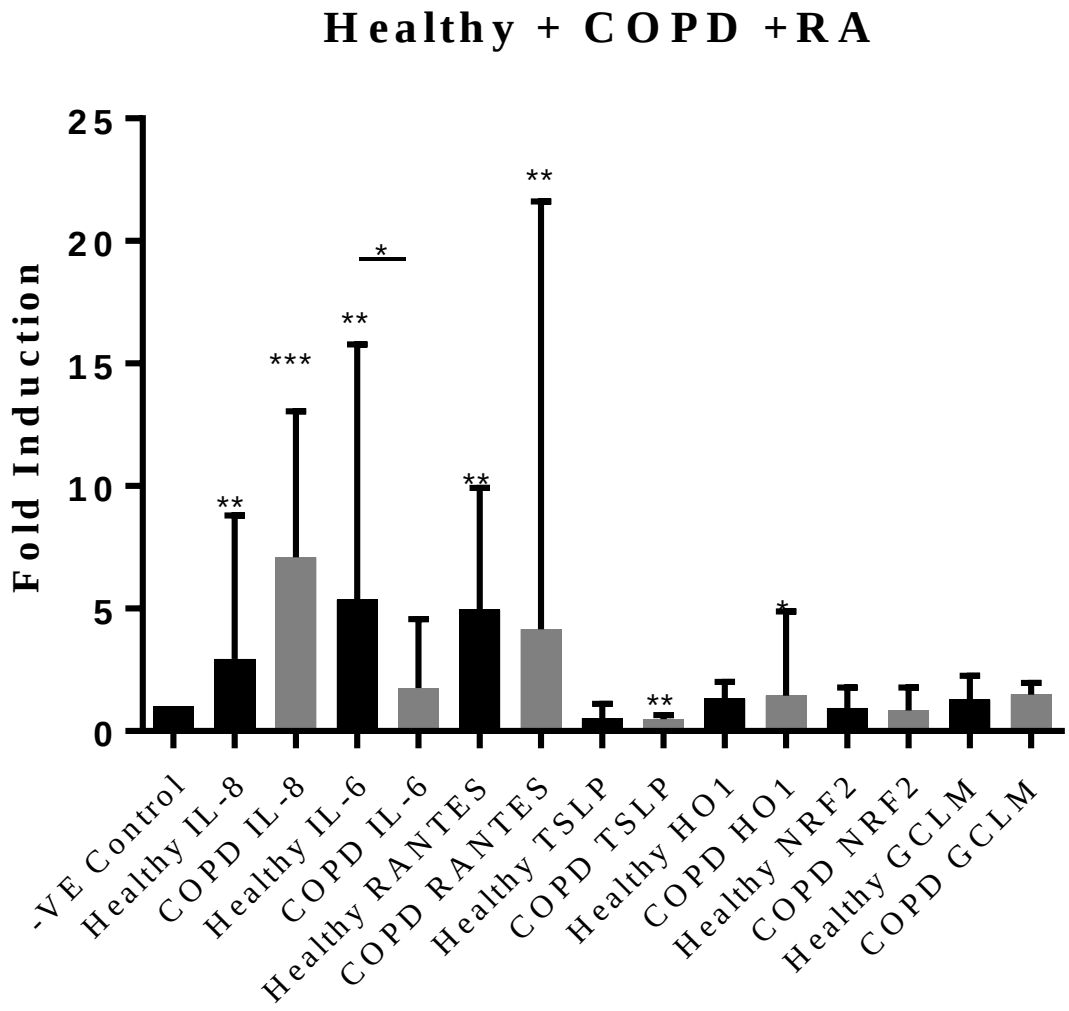

Figure 4.1. Pro-inflammatory and antioxidant gene expression after 24 hours of stimulation with TNFα.

Healthy and COPD PBEC ALI cultures grown over 21 days in the presence of RA, before being basally stimulated with TNFα with 24 hours and RNA was harvested. Data was calculated by the ΔΔCt method, normalising to housekeeping genes (UBC/GAPDH) expression, and then to an un-stimulated control transwell from each donor. Data displayed as the group median with 95% confidence intervals. Significance calculated using the Kruskal-Wallis test. Statistical significance from baseline is denoted by an asterisk (*=p<0.05, **=p<0.01, ***=p<0.001), with horizontal bars representing significance between donor groups. n = 7 Healthy, 7 COPD.

4.2.2 Analysis of inter donor variability of cultures grown with RA, in response to stimulation with TNFα

While the data so far offers a useful insight as to the epithelial response to stimulation with TNFα differs between the two different donor groups, it does not take into account inherent differences within the donor group, or differences of the baseline expression between the two groups.

As has been shown in the previous study, the expression of particular makers of differentiation such as CBE1 and TEKTIN vary a great deal within donor groups. While the overall trend may be significant in the alteration of expression, individual donors exhibit their own pattern of expression unique to themselves. The same would apply to individual donors that are having a stimulated response normalised to their own baseline expression of a particular gene. While all donors may show an increase in expression upon stimulation, the fold induction relies heavily on the inherent expression at baseline to determine the magnitude of the change. Likewise, no comparison between the baseline expression can be drawn between the two donor groups. If baseline expression of a gene is naturally very high or very low, it may mask the alteration of gene expression upon stimulation, either under or over exaggerating the relative fold induction of that gene.

To try to analyse this, qPCR data for gene expression at the baseline for all healthy donors were averaged to generate a new ΔΔCt value for that particular gene. The new ΔΔCt value was then applied across all donors, both Healthy and COPD, to generate new fold induction values for each gene. This new data set allows for the analyses of baseline expression between donor groups, as well as allowing for a more accurate interpretation of gene fold induction upon stimulation with TNFα.

Both the Healthy and COPD donors exhibited a significant increase in the expression of IL-8 when compared to baseline expression. When compared to their own baseline expression, the COPD donors had a lower P value of significance ($p<0.01$) then the Healthy donors ($p<0.05$). This difference in

significance is due to the baseline expression of the healthy donors being higher than that of the COPD donors, despite there being no statistical difference between the two. The same pattern of expression is seen in IL-6, with a significant increase in both the healthy and COPD donors ($p<0.05$). This is a change from the original data; with the COPD donors now displaying a significant increase in expression when before there was no significance. Likewise, the significant difference between the two donor groups in terms of IL-6 expression no longer exists, with both donor groups displaying similar levels of IL-6 expression.
Both donor groups displayed an increase in RANTES expression when stimulated with TNFα when compared to the baseline expression. The increase in expression for the COPD donors ($p<0.01$) is greater than the Healthy donors ($p<0.05$), a difference that is reflected in the original qPCR data. This similarity between the two qPCR data sets is reflected in the expression levels of TSLP, with a significant decrease in TSLP expression in the COPD donors when compared to the baseline expression. While there is a decrease in the expression of TSLP for the healthy donors, this is not significant (Figure 4.2).

The addition of TNFα to the basal media has no significant effect on the fold induction of the anti-inflammatory genes HO1, NRF2 and GCLM, in either donor group. Despite no significant increases from base line, there is a significance difference between the TNFα stimulated fold induction of the gene GCLM when comparing between the Healthy and COPD donors, with the Healthy donors exhibiting higher expression. By normalising to the mean Healthy donor ΔΔCt, the significant difference in fold induction of HO1 between the Healthy and COPD donors is lost (Figure 4.3).

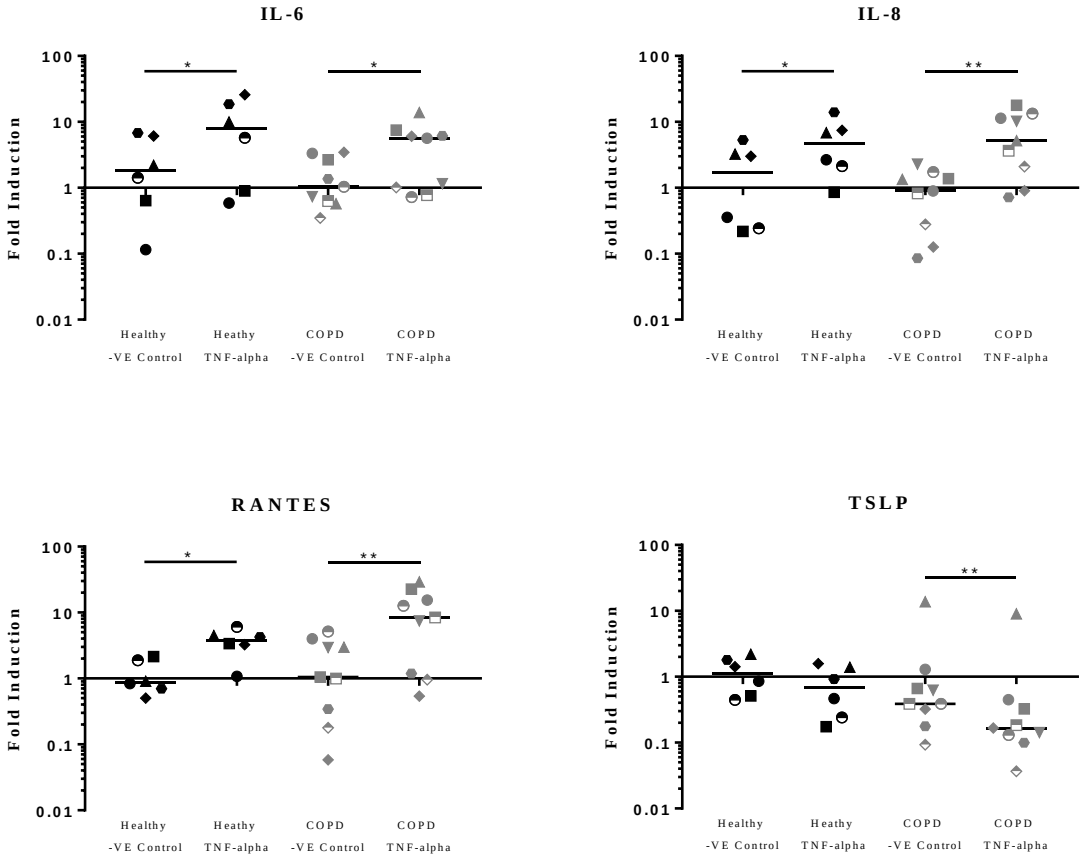

Figure 4.2. Pro-inflammatory gene expression after 24 hours of stimulation with TNFα.

Healthy and COPD PBEC ALI cultures grown over 21 days in the presence of RA, before being basally stimulated with TNFα with 24 hours and RNA harvested. Data was calculated by the ΔΔCt method, normalising to housekeeping genes (UBC/GAPDH) expression, and then to a ΔΔCt value generated by the mean of all Healthy donor negative control ALIs. Each data point represents a single donor, with the group median displayed. Significance was calculated using the Wilcoxon test and Unpaired t-tests. Significance is denoted by asterisk (*=$p<0.05$, **=$p<0.01$). n = 7 healthy, 9 COPD.

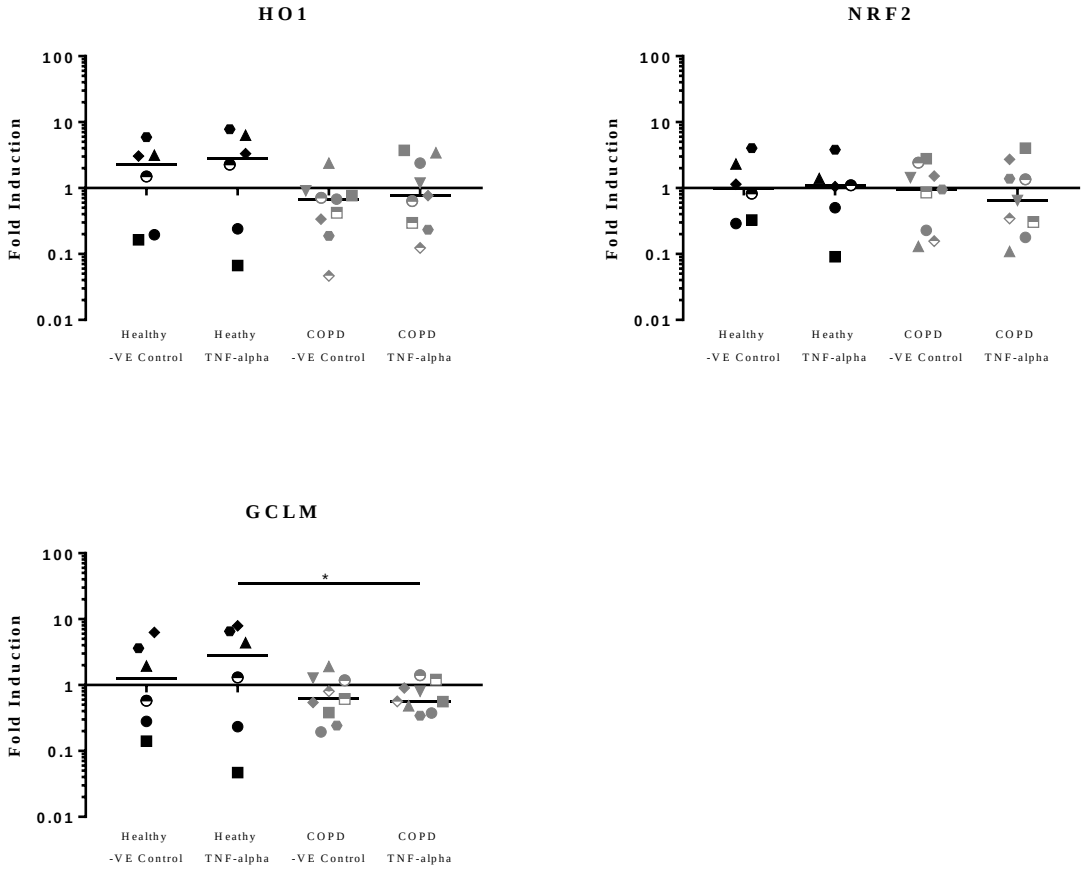

Figure 4.3. Anti-oxidant gene expression after 24 hours of stimulation with TNFα. Healthy and COPD PBEC ALI cultures grown over 21 days in the presence of RA, before being basally stimulated with TNFα with 24 hours and RNA harvested. Data was calculated by the ΔΔCt method, normalising to housekeeping genes (UBC/GAPDH) expression, and then to a ΔΔCt value generated by the mean of all Healthy donor negative control ALIs. Each data point represents a single donor, with the group median displayed. Significance was calculated using the Wilcoxon test and Unpaired t-tests. Significance is denoted by asterisk (*=$p<0.05$, **=$p<0.01$). n = 7 healthy, 9 COPD.

4.2.3 Alterations in pro-inflammatory and anti-oxidant gene responses in SM epithelium, in response to TNFα stimulation

The previous study highlighted that the gene expression profile is altered during differentiation of pseudo-stratified epithelium when compared to the development of SM. To see if this difference in phenotype results in an alteration of the immune response, ALI cultures grown in the absence of RA for 21 days were stimulated basally with TNFα. Cultures were harvested for mRNA extraction and qPCR was performed to analyse the expression of the previously mentioned genes of interest. The data for both the Healthy and COPD cultures grown without RA was normalised to negative control cultures from each individual donor, to determine the response to TNFα stimulation.

The stimulation with TNFα resulted in a significant increase in the expression of IL-8 when compared to baseline expression levels for the Healthy donors grown without RA ($p<0.05$). While the COPD donors exhibited an increase in expression upon stimulation, this increase was not significant, nor was there a significant difference between the two donor groups. Stimulation had no effect on the expression of IL-6 or RANTES for the Healthy donors when compared to baseline expression. For the COPD donors, there was a significant increase in the expression of RANTES with stimulation ($p<0.05$), but like IL-8, there was no significance between the two donor groups. Stimulation with TNFα had no effect on the expression of either TSLP, HO1, NRF2 or GCLM in either donor group (Figure 4.4).

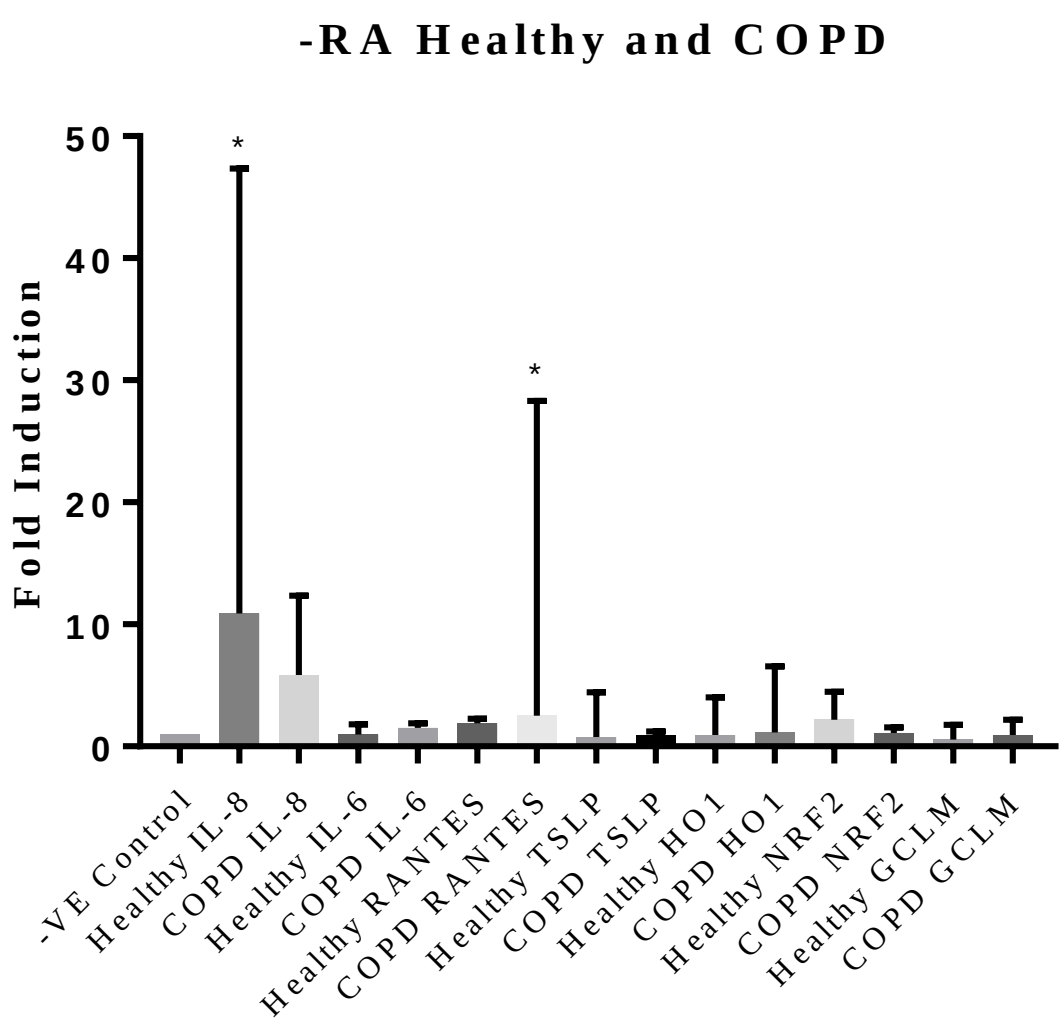

Figure 4.4. Pro-inflammatory and antioxidant gene expression of culture grown without RA, after 24 hours of stimulation with TNFα.

Healthy and COPD PBEC ALI cultures grown over 21 days in the absence of RA, before being basally stimulated with TNFα with 24 hours and RNA was harvested. Data was calculated by the ΔΔCt method, normalising to housekeeping genes (UBC/GAPDH) expression, and then to an un-stimulated control transwell from each donor. Data displayed as the group median with 95% confidence intervals. Significance calculated using the Kruskal-Wallis test. Statistical significance from baseline is denoted by an asterisk (*=$p<0.05$, **=$p<0.01$), with horizontal bars representing significance between donor groups. n = 5 healthy, 7 COPD.

4.2.4 Analysis of inter donor variability in cultures grown without RA, in response to stimulation with TNFα

This initial data suggests that the removal of RA from the culture medium, and the implicated development of SM, has an attenuating effect on the pro-inflammatory expression profile that was exhibited by those cultures that were grown in the presence of RA. While there is still an increase in expression of IL-8 in the Healthy donors and RANTES from the COPD donors, these increases were not reflected in the alternate donor group, with both donor groups exhibiting a complete loss of the IL-6 response. Those increases that are significant also have a very large 95% confidence intervals, indicating that the increases seen are not necessarily exhibited by all individual donors.

To better understand the effect that the removal of RA has on each donor's ability to respond to TNFα stimulation, the qPCR data was normalised to an external value. As with the data for the cultures grown with RA, the qPCR data was normalised to the mean ΔΔCt value of the healthy donor negative controls grown in the presence of RA. This will allow for analysis of the differences in baseline expression levels for both donor groups as well as the response to TNFα stimulation.

Re-normalisation of the data shows that while both donor groups exhibit an increase in IL-8 expression, it is only significant for the COPD donors ($p<0.05$). While the COPD donors still exhibit the increase in RANTES fold induction, the increase is not significant when compared to baseline expression. Neither donor group exhibits any alteration in IL-6 nor TSLP fold induction when stimulated with TNFα (Figure 4.5). While the addition of TNFα has no effect on the fold induction of HO1, NRF2 or GCLM, both donors exhibit an elevated baseline expression of HO1 that is maintained post stimulation. Comparisons between the two donor groups show that the healthy donors exhibit a higher level of HO1 expression both at baseline ($p<0.05$) and after TNFα stimulation ($p<0.01$) (Figure 4.6).

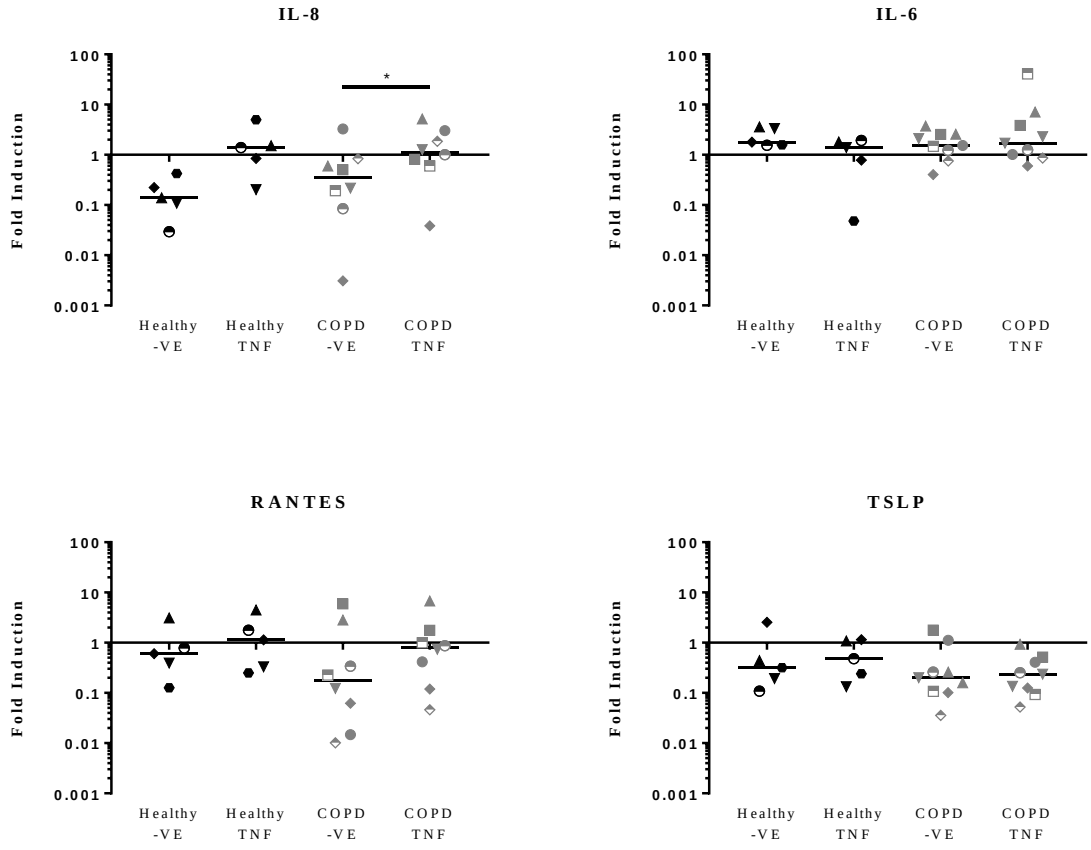

Figure 4.5. Pro-inflammatory gene expression of cultures grown without RA after 24 hours of stimulation with TNF*α*.

Healthy and COPD PBEC ALI cultures grown over 21 days in the absence of RA, before being basally stimulated with TNFα for 24 hours and RNA harvested. Data was calculated by the ΔΔCt method, normalising to housekeeping genes (UBC/GAPDH) expression, and then to a ΔΔCt value generated by the mean of all Healthy donor negative control ALIs grown with RA. Each data point represents a single donor, with the group median displayed. Significance was calculated using the Wilcoxon test and Unpaired t-tests. Significance is denoted by asterisk (*=$p<0.05$). n = 5 healthy, 8 COPD.

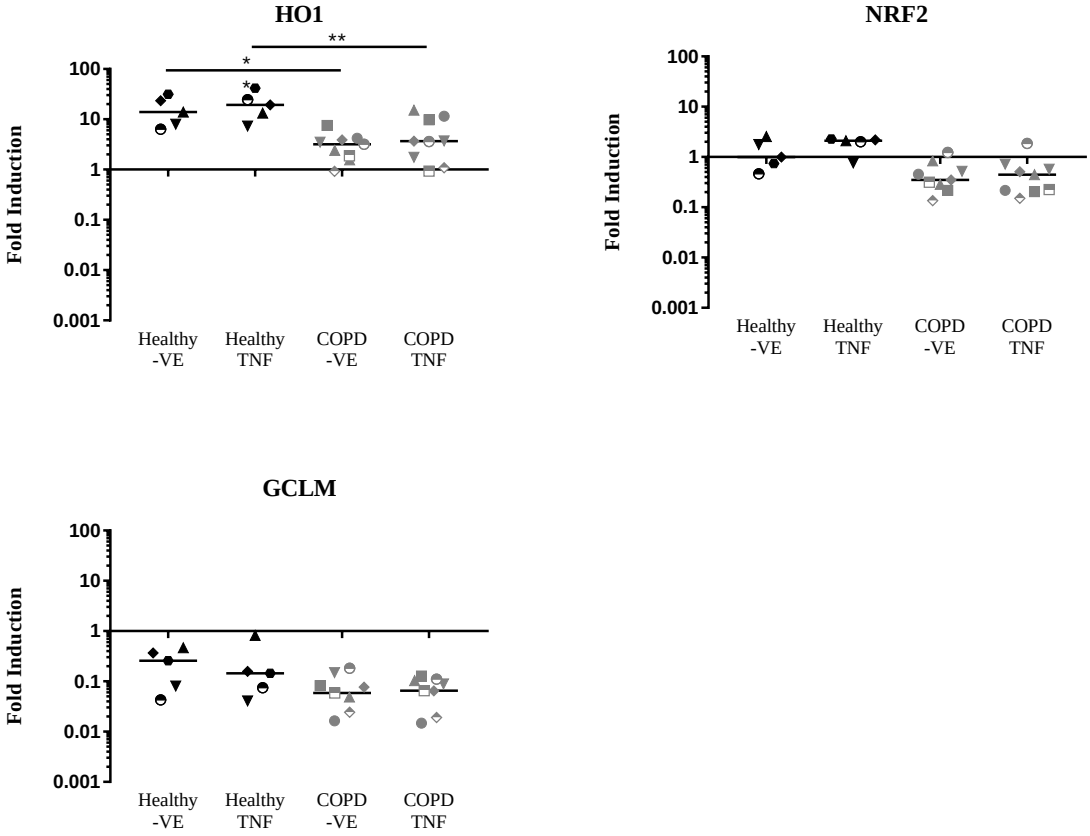

Figure 4.6. Antioxidant gene expression of cultures grown without RA after 24 hours of stimulation with TNFα.

Healthy and COPD PBEC ALI cultures grown over 21 days in the absence of RA, before being basally stimulated with TNFα for 24 hours and RNA harvested. Data was calculated by the ΔΔCt method, normalising to housekeeping genes (UBC/GAPDH) expression, and then to a ΔΔCt value generated by the mean of all Healthy donor negative control ALIs grown with RA. Each data point represents a single donor, with the group median displayed. Significance was calculated using the Wilcoxon test and Unpaired t-tests. Significance is denoted by asterisk (*=$p<0.05$, **=$p<0.01$). n = 5 healthy, 8 COPD.

4.2.5 Direct comparison of the effect of removing RA within donor groups

Since the qPCR data for cultures grown in the presence of RA, and those grown in the absence of RA, were normalised to the same mean ΔΔCt, a direct comparison can be made between the two different culture methods.

The removal of RA from the culture medium results in lower levels of IL-8 expression from both the Healthy and COPD donors. Both donor groups exhibit a trend for lower expression both at baseline and after TNFα stimulation, proving to be significantly lower for the COPD donors post stimulation ($p<0.01$). The same pattern can be seen in the expression of RANTES, with the removal or RA resulting in an overall trend for a decrease in expression levels in both donor groups, while still increasing upon stimulation. As with the IL-8 expression, the only significant difference in expression is between the COPD donors grown with RA and those grown without, post stimulation ($p<0.01$). The pattern of expression for IL-6 differs from that of IL-8 and RANTES, in that the basal expression is not affected by the removal of RA. However, the removal of RA removes the significant increase in expression seen in both the Healthy and COPD cultures when grown with RA. Despite the lack of increase in expression in the cultures grown without RA, the difference is not significant (Figure 4.7). The removal of RA has no significant effect on the expression of TSLP, with both donor groups exhibiting a very slight trend for a decrease in expression one the RA has been removed from the culture medium (figure 4.8).

The removal of RA from the culture medium results in a significant increase in the expression of HO1 from both the Healthy ($p<0.05$) and COPD donors ($p<0.01$) both at baseline and post TNFα stimulation. The addition of TNFα does not alter the expression levels of HO1 in either donor group. The opposite pattern is seen in the expression of GCLM, with the removal of RA resulting in a decrease in expression. While this reduction is seen in both donor groups, it is only significant for the COPD donors, at both baseline ($p<0.01$) and post stimulation ($p<0.001$), with the addition of TNFα having no effect on expression

in either donor group. The removal of RA or the addition of TNFα has no significant effect on the expression of NRF2 (Figure 4.9).

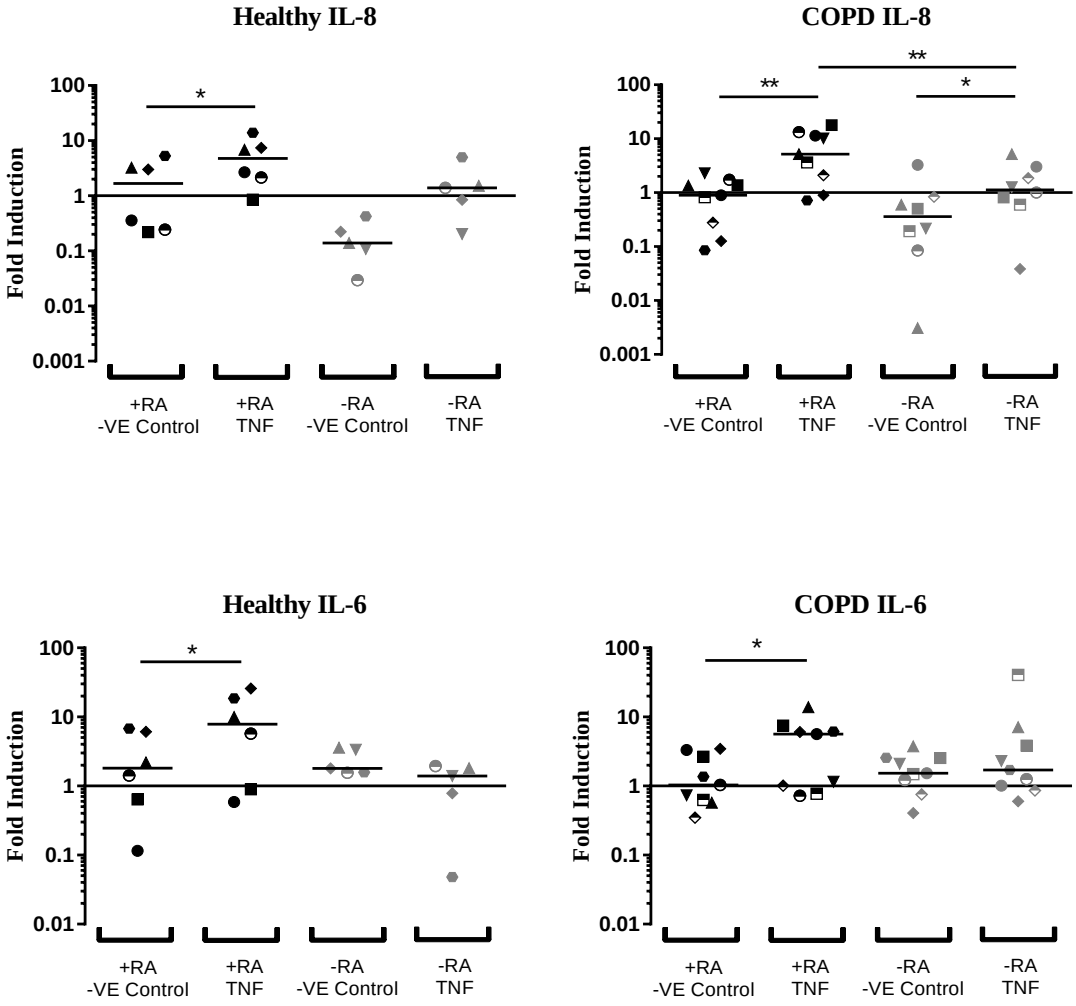

Figure 4.7. Pro-inflammatory gene expression of cultures grown both with and without RA after 24 hours of stimulation with TNFα.

Healthy and COPD PBEC ALI cultures grown over 21 days in the presence and absence of RA, before being basally stimulated with TNFα for 24 hours and RNA harvested. Data for IL-8 and IL-6 expression was calculated by the ΔΔCt method, normalising to housekeeping genes (UBC/GAPDH) expression, and then to a ΔΔCt value generated by the mean of all Healthy donor negative control ALIs grown with RA. Each data point represents a single donor, with the group median displayed. Significance was calculated using the Wilcoxon test and Unpaired t-tests. Significance is denoted by asterisk (*=p<0.05, **=p<0.01). n = 6 healthy +RA, 5 healthy –RA, 9 COPD +RA, 8 COPD – RA.

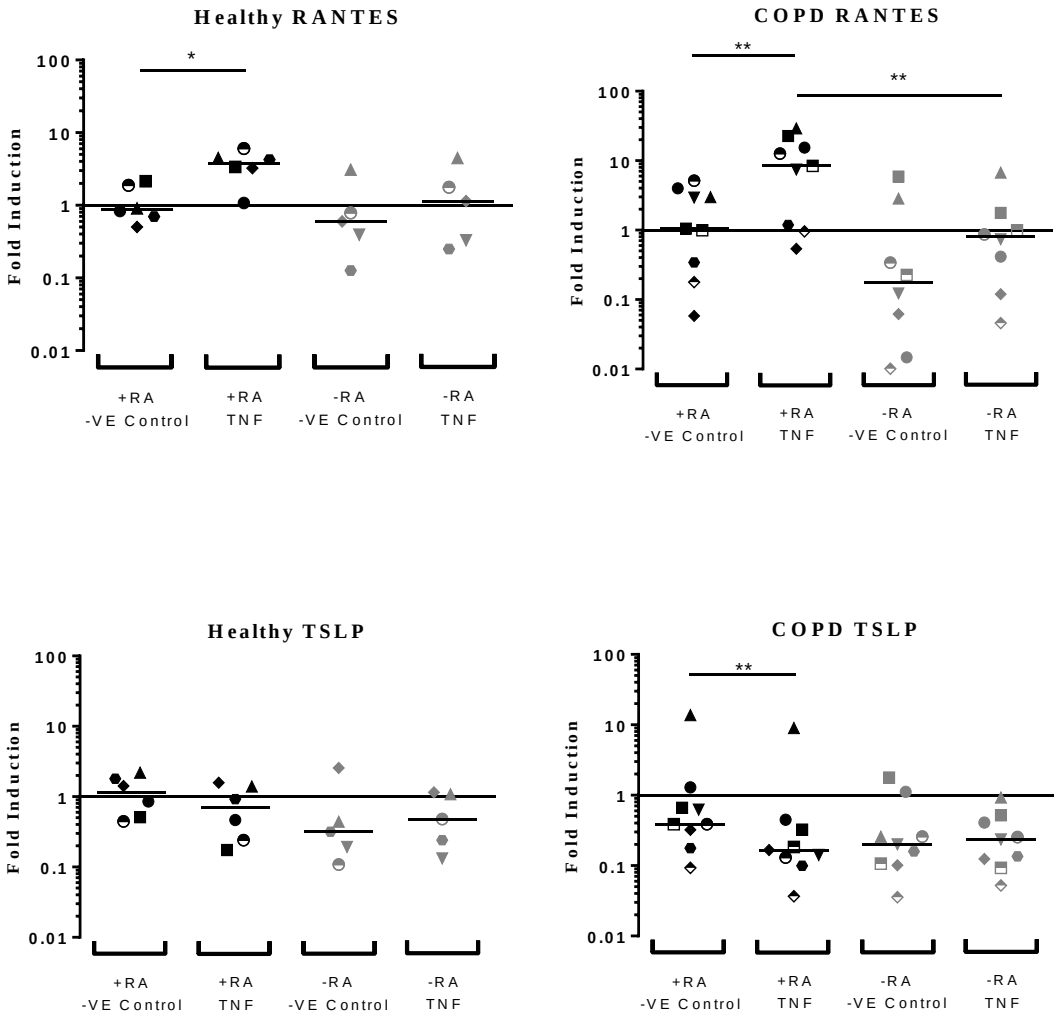

Figure 4.8. Pro-inflammatory gene expression of cultures grown both with and without RA after 24 hours of stimulation with TNFα.

Healthy and COPD PBEC ALI cultures grown over 21 days in the presence and absence of RA, before being basally stimulated with TNFα for 24 hours and RNA harvested. Data for RANTES and TSLP expression was calculated by the ΔΔCt method, normalising to housekeeping genes (UBC/GAPDH) expression, and then to a ΔΔCt value generated by the mean of all Healthy donor negative control ALIs grown with RA. Each data point represents a single donor, with the group median displayed. Significance was calculated using the Wilcoxon test and Unpaired t-tests. Significance is denoted by asterisk (*=$p<0.05$, **=$p<0.01$). n = 6 healthy +RA, 5 healthy –RA, 9 COPD +RA, 8 COPD -RA.

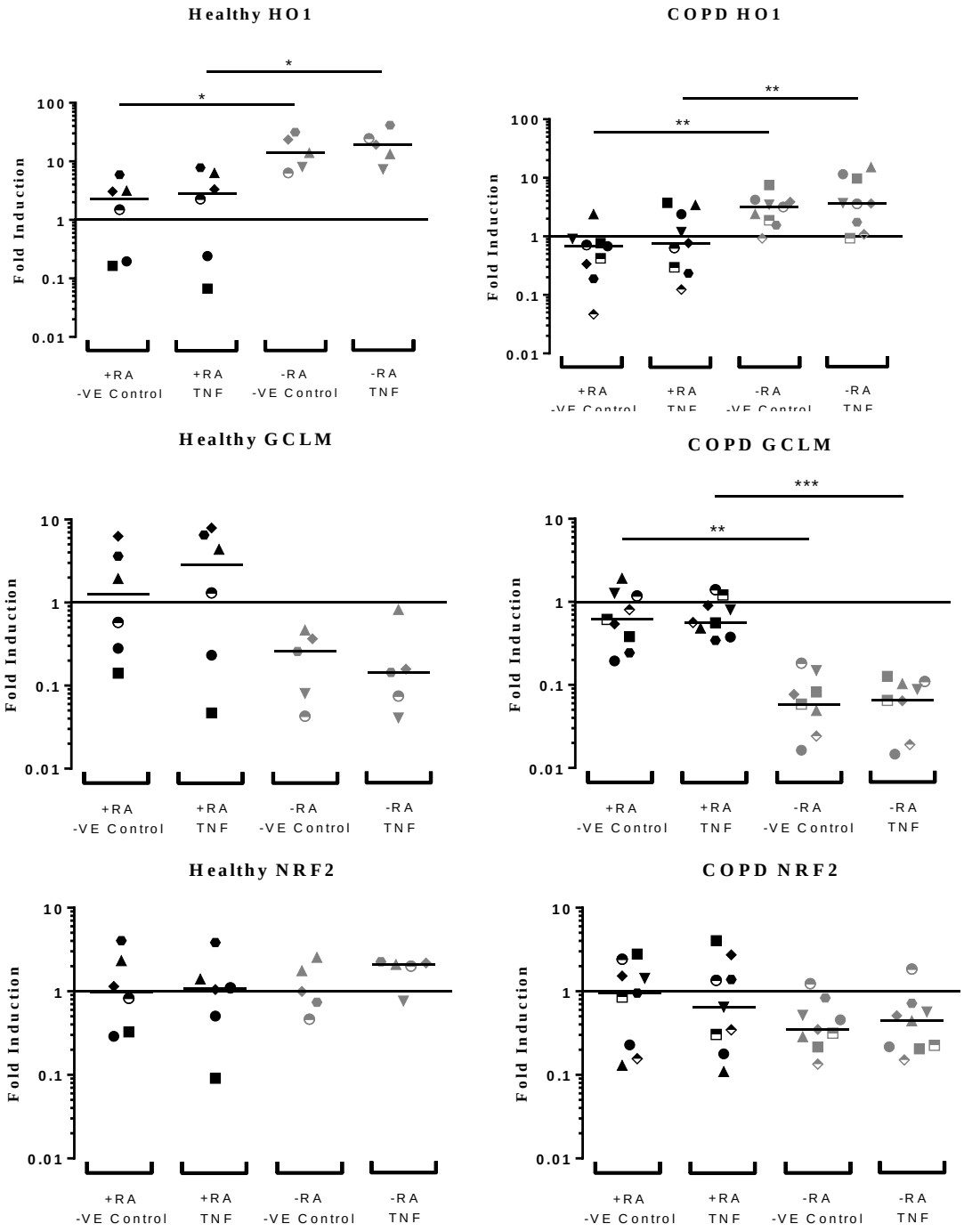

Figure 4.9. Antioxidant gene expression of cultures grown both with and without RA after 24 hours of stimulation with TNFα.

Healthy and COPD PBEC ALI cultures grown over 21 days in the presence and absence of RA, before being basally stimulated with TNFα for 24 hours and RNA harvested. Data for HO1, GCLM and NRF2 expression was calculated by the ΔΔCt method, normalising to housekeeping genes (UBC/GAPDH) expression, and then to a ΔΔCt value generated by the mean of all Healthy donor –VE control ALIs grown with RA. Each data point represents a single donor, with the group median displayed. Significance was calculated using the Wilcoxon test and Unpaired t-tests. Significance is denoted by asterisk (*=p<0.05, **=p<0.01, ***=p<0.001). n = 6 healthy +RA, 5 healthy –RA, 9 COPD +RA, 8 COPD –RA.

4.3 Discussion

This study examined the differences in gene expression between PBEC cultures from Healthy and COPD donors, grown both in the presence and absence of RA, after being basally stimulated with TNFα for 24 hours.

4.3.1 Comparisons between COPD and Healthy Donors

The baseline expression for the proinflammatory genes exhibited the same pattern, with no differences occurring between the two donor groups when grown with RA. The proinflammatory genes all exhibited altered expression with the addition of TNFα. Both IL-8 and IL-6 exhibited a significant increase in expression with the addition of TNFα, a finding previously reported (146, 151). The expression of RANTES was also significantly elevated after 24 hours of TNFα incubation in both donor groups. Despite RANTES primarily being involved in a pathogen response, this response to TNFα could imply that RANTES may have a wider role in the immune response to proinflammatory mediators.

Unlike the other proinflammatory genes looked at, TSLP significantly decreased in expression for the COPD donors in the presence of TNFα, with a trend for the same pattern in the healthy donors. This is counter to the literature that TSLP expression increases with the addition of TNFα (161). The reason for this down regulation is not apparent. It could well be that the expression of TSLP did increase when the TNFα was added but did so immediately and then was actively downregulated so that by 24 hours post stimulation, the relative expression of TSLP was significantly lower than baseline.

In terms of baseline expression of the antioxidant genes, there were no significant differences between cells taken from COPD donors and those taken from healthy donors. This is counter to some previous work that has suggested significantly reduced levels of HO1 (163) in COPD donors, while some of the literature reports elevated levels of these antioxidant genes in COPD donors (127). So far, our findings have suggested that culture of donor cells at ALI may induce a return to a

homeostatic epithelium, due to the absence of long-term stressors such as CS, potentially erasing some of the differences highlighted between donor groups when looking at cells *in vivo*. Therefore, due to no long-term stressor acting on the epithelium, the COPD donor cells have no reason to express any antioxidant genes differently to the healthy donor cells.

The addition of TNFα to the epithelium had no effect on the expression of antioxidant proteins HO1 or NRF2, in either the healthy or the COPD donors. Despite the epithelial layers being incubated for 24 hours in the presence of TNFα, the expression levels of these antioxidant genes did no vary from baseline or between the two donor groups. It may be that to obtain a measurable antioxidant response, cells would need incubation with TNFα for a longer period. There was a significant difference in the expression of GCLM when stimulated with TNFα, between the COPD and healthy donors, with the healthy donors exhibiting a higher expression. While statistically significant, the spread of expression values for the healthy donors is very broad, with some of the donors exhibiting a decrease in expression when the epithelium is stimulated. Due to this, any significance may be due down to inter donor variability as opposed to a general response exhibited by all healthy donor epithelium.

4.3.2 Comparisons between cultures grown with RA and those grown without RA

The baseline expression of TSLP of cultures grown without RA does not significantly differ from that of the cultures grown with RA, despite a slight decrease in both donor groups. The addition of TNFα does not induce a significant difference in expression profiles. This absence of alteration in TSLP expression may not be that unexpected. Due to its involvement in the activation of dendritic cells (160), the absence of these cells from the ALI culture model may result in the lack of a feedback loop, resulting in downregulation of expression. Similarly, expression may have changed but returned to baseline by 24 hours, with a potential change in expression level not being detected by qPCR. Similar to TSLP, IL-6 has no alteration of baseline expression for those cultures grown without RA, with the addition of TNFα failing to alter expression. While this

suggests attenuation of the proinflammatory response in areas of SM epithelium, this does not hold true with all of the proteins. Base line expression of IL-8 is lower in both donor groups grown without RA when compared to those grown with RA, but the addition of TNFα still results in an increase in expression of IL-8, proving significant for the COPD donors. The expression profile of RANTES exhibits the same pattern, with a trend for lower expression at baseline while still maintaining an increase in expression when stimulated. This shows that for some of the proinflammatory cytokines, there is slight attenuation with no effect on the induction of genes, while for some, SM results in null expression when stimulated with TNFα.

Removal of RA from the culture medium has no effect on the expression levels of NRF2. Basal expression without RA is the same as those cultures grown with RA and the addition of TNFα has no significant effect. The lack of a response to NRF2 tells us little about the SM in relation to normally differentiated epithelium, due to the similar expression levels. Despite there being no alteration in the expression of NRF2, GCLM expression significantly decreases in the COPD cultures grown without RA, with a trend for the same decrease for the healthy donors. This decrease in expression remains with the addition of TNFα, with no further change in expression levels occurring. This difference in GCLM expression suggests that the regulation of the gene may not be fully dependant on the NRF2 pathway, and may have alternative methods of regulation. However, while the expression of GCLM is dependent on the presence of RA in the culture media, the healthy donors exhibit a trend for higher expression in both culture conditions. Research into squamous cell carcinomas has shown an increased expression of GCLM (164), an increase which is not seen in our model inducing the development of squamous metaplasia. This suggests that the expression of GCLM may be dependant on more than stimulation with TNFα.

In stark contrast to this, removal of RA resulted in a significant increase in baseline expression of HO1, when compared to cultures grown in the presence of RA, which is not influenced by the addition of TNFα. The expression is also different between the two donor populations, with a significant increase in expression occurring in those cells grown from healthy donors. This shows us that

the expression of HO1 is primarily dependant on the presence of SM in the epithelium, inducing higher levels of expression, and secondarily by the donor source, with significant differences between the two donor groups only occurring in the cultures grown without RA.

The extrapolation of these results suggests that while there is very little difference in the response to stimulation between the two donor groups, the development of SM has a key impact on the regulation and expression of certain proteins. The development of SM results in an altered expression of antioxidant proteins, increasing the expression of HO1 while decreasing the expression of GCLM. While the cause for this different expression is unclear, the fact that both are independent of the addition of TNFα tells us that the expression has been altered by the development of SM.

As previously discussed in chapter 3, the qPCR process quantifies the levels of mRNA in terms of fold induction when compared to a baseline value. Further work would need to analyse the protein level of these particular genes, using both western blots and ELISAs to determine the levels of intracellular and secreted protein. There is also the possibility that changes are occurring at the mRNA level that are not being detected due to the single 24-hour time point. An expansion of this study could include a wider range of time points to elicit a more detailed understanding of whether or not changes in mRNA are occurring earlier than 24 hours.

4.4 Conclusion

Overall, the data suggests that there is very little difference between the two donor groups when both are grown in the presence of RA. Baseline expression and the effects of TNFα are similar between the two donor groups, with only GCLM being significantly different between the two.

The removal of RA and induction of SM alters the expression profile of both the antioxidant genes and the proinflammatory genes. There appears to be stronger baseline expression for the antioxidant HO1 in the SM epithelium when compared to normally differentiated epithelium, and the pro inflammatory response shows evidence of attenuation.

5 Final Discussion and Future Work

5.1 Novel Findings

The work in this report contributes to the study of COPD, in particular the development of SM of the respiratory epithelium. The removal of RA from ALI cultures results in the terminal differentiation of the epithelium into SM, presenting as a thickening of the epithelial layer and a significant increase in the TEER reading across the epithelial barrier (Chapter 3). Immunofluorescent staining also showed a loss of cilia on the apical surface. The expression of structural and secretory genes also differed, with lower expression of CBE1, TEKT1, MUC5B and CK7, but higher expression of CK13 and Involucrin. SM occurs naturally in the body in areas of high abrasion (81), suggesting that the occurrence within the airways is a protective mechanism, due to exposure to environmental insults. Epithelial samples from both healthy donors and those with COPD could have SM induced by the removal of RA from the culture medium. The only real significant difference between the two donor groups was the consistent reduction of SCGB1A1 expression in the COPD donors, suggesting lower levels of CCSP secretion in the bronchial epithelium.

The use of TNFα as a stimulatory cytokine invokes a pro-inflammatory response that did not differ significantly between donor groups, when cells were grown in the presence of RA (Chapter 4). However, the addition of TNFα had very little effect on the induction of anti-inflammatory genes HO1, NRF2 and GCLM. The removal of RA resulted in the attenuation of the pro-inflammatory response, with the loss of IL-6 induction. Both IL-8 and RANTES expression were still induced by the addition of TNFα, but the baseline expression and expression post stimulation for both of these genes was lower when compared to those cultures grown with RA. The removal of RA from the culture medium resulted in a

significant increase in HO1 expression, and a reduction in GCLM expression, significant for the COPD donors, when compared to cultures grown with RA. These finding suggests that the presence of SM *in vivo* may have an effect on the epithelial response to stimulation, potentially trying to limit further damage by reducing the pro-inflammatory response while maintaining some antioxidant response.

5.2 Critical Overview

The ALI culture method relies upon the presence of RA in the culture medium to induce differentiation of the epithelial cells into a columnar epithelial layer, with the production of mucus and cilia. As has been previously discussed, ALI cultures have a similar transcriptional profile similar to that of cells *in vivo* (127, 165). Using this methodology, the purpose of this research was to make direct comparisons between primary epithelial cells taken from healthy donors, and those taken from patients diagnosed with COPD. Any differences between the two cell populations may give an insight into how epithelial cells *in vivo* differ in individuals with COPD, with both physical form and gene expression of proteins potentially having an effect on the establishment of COPD as a disease.

The work for this report could be expanded by repeating all of the experiments using more COPD and healthy donors. For some of the data sets, statistical conclusions are being made on data which has a very wide spread, meaning that tests are heavily influenced by potential outliers. The spread of the data may result in an increased chance of type II errors. By increasing the number of data points, statistical tests become more reliable and differences between data sets may become more apparent, potentially altering the statistical significance. For experiments that use primary tissue, having a high number of donors is important due to inter-donor variability, with some donor epithelium responding differently when compared to other donors. Again, to counter this phenomenon, a high donor number would mean that any patterns seen would likely be genuine, because it would be occurring in multiple donors.

As previously discussed, more data could be gathered in the form of protein work, including ELISAs and Western blots. This, along with the mRNA data, would allow generation of a more detailed picture of the changes that are occurring within the epithelium between different donor groups, and between those grown either in the presence or the absence of RA. In this study, ALI cultures were stained with Toluidine blue to visualise the cell membranes and β-tubulin to visualise the cilia. This staining could be expanded to include proteins from the genes of interest that have been monitored via qPCR, especially those that were highlighted as showing significant differences, such as CCSP. This would allow for not only confirmation of whether or not the mRNA is translated into protein, but would also allow for the localisation of particular proteins to be determined. This would be another layer of analysis and would allow for comparisons in cell architecture between donor groups and culture conditions, to determine if there are any significant alterations.

5.3 Differentiation between Healthy and COPD Donors

Analysis of the two epithelial populations showed that there was very little difference between the two donor groups, with both exhibiting the ability to fully differentiate into pseudo stratified epithelium. While gene expression suggests that the epithelial layers were producing mucus, fluorescent staining for β-tubulin showed the presence of cilia on the apical surface of epithelium from both the Healthy and COPD donors, despite a slightly higher level CBE1 expression in the Healthy donors. The main difference between the two donor groups was a much higher expression of the gene SCGB1A1, encoding for the CCSP protein. This difference has been highlighted before, with lower circulating CCSP in COPD patients (123, 124) and fewer CCSP positive cells in tissue samples (125). Further analysis of cytokeratin expression revealed no differences between the two groups, indicative of structural similarities. Upon stimulation with TNFα, both donor groups responded in a similar manner, exhibiting a very similar pattern of gene expression for those genes involved with the regulation of pro-inflammatory and anti-oxidant responses. The only significant difference between the two donor

groups was the absence of an induction of GCLM in the COPD donors when stimulated with TNFα.

The similarities between these two cell populations is counter too much of the literature that suggests that epithelium from an individual with COPD is markedly different in its gene expression, with elevated levels of mucins (121) and anti-oxidant proteins such as GCLM (127). While the research presented here does suggest a difference in GCLM expression, the previous literature focuses on comparisons between unstimulated epithelium, from *in vivo* samples, as opposed to artificially stimulated epithelium. The Pierrou et al paper also segregates the data based on the stage of COPD, with the induction of genes varying with disease state. Due to the low number of donors used for this study (table 2.1), it is not possible to separate the donors based on disease stage, smoking status or age, three variables that may influence gene expression, and still maintain a significant sample size.

Previous research focussing on the pro-inflammatory response suggests that COPD donors should exhibit higher expression levels of IL-6 (147), IL-8 (152), Rantes (157) and TSLP (162) when compared to healthy controls. Despite this, none of the findings in this study were able to replicate this, possibly due to the low number of samples used, or the homeostatic nature of the epithelium. The removal of harmful and toxic components that may be present in the lungs of COPD patients, most commonly CS smoke, may allow the epithelium to return to a normalised state that is almost indistinguishable from epithelial cells sourced from a healthy donor. Therefore, over the course of a 21-day culture there may be very little difference between the two population groups, resulting in similar gene expression profiles even when stimulated with TNFα.

5.4 The Development of Squamous Metaplasia

The second half of this report focused on development of a model of Squamous Metaplasia, exploring how the induction of this cellular response affects both the structural and immunological phenotype of epithelial cells.

The removal of RA from the culture medium resulted in a significant increase in the TEER readings when compared to those cultures grown with RA. This, combined with the GMA imaging shows that the epithelium is no longer differentiating into a pseudostratified epithelium, rather it is displaying signs of hyperplasia. While the expression levels of cilia markers are lower in the absence of RA, showing no increase over the 21 days of culture, there is a significant increase in the levels of involucrin expression. This data is in accordance with the literature, with elevated expression levels of involucrin and the development of keratin layer being found in naturally occurring SM (92) as well as in those cultures previously grown without RA (98, 129).

Baseline expression of inflammatory markers did not significantly differ in cultures grown without RA when compared to those that were. This evidence is counter to the literature, with some research showing elevated expression of inflammatory markers such as TNFα, IL-1β and IL-6 in SM samples (84). This same research shows that these markers can induce SM, suggesting that once SM is established, it could be self-propagating.

When stimulated with TNFα, the cultures grown in the absence of RA exhibited an attenuation of the pro-inflammatory response. There was an overall reduction in the expression of IL-8 and RANTES at baseline, when compared to cultures grown in the presence of RA, a reduction that was maintained despite TNFα stimulation resulting in an increase in expression. There was a complete loss of any induction of IL-6 post stimulation. The induction of SM results in a significant increase in the expression of HO1, regardless of donor group. However, this increase in expression occurs at baseline, with TNFα stimulation having no impact on the overall expression of HO1. Comparisons between the two donor groups, when grown without RA, revealed very little difference between the two. The only significant difference is in the expression CCSP, with COPD donors exhibiting a lower fold induction of mRNA expression over the 21 days of culture when compared to healthy donors. This is the same difference seen when cultures are grown with RA, suggesting that it is not a product of the removal of the RA, rather an inherent difference between the two cell populations.

Despite the discrepancies with the expression data found in the literature, the phenotypic differences seen in the cultures grown without RA are consistent with those found in SM *in vivo*.

5.5 Future Work

The development of a model of SM is important due to the implications that naturally occurring SM has on COPD disease progression. SM has been shown to increase with the severity of COPD (82), contributing to airway narrowing and increasing airway resistance (3). While the development of SM may be due to long-term damage by CS, it may also serve a function to protect against further environmental damage. More work is needed to fully determine the role SM plays in the immune response to the same insults. By studying how SM develops and differs from healthy differentiated epithelium, potential therapies can be developed that may help alleviate the prevalence of SM and delay the progression of COPD.

As well as contributing to the progression of COPD, there is some evidence that SM can lead to cancer. Rates of SM increase with smoking (83), as do the rates on lung cancer (166). There is some suggestion that the reversible cellular changes that develop into SM may be a prelude to dysplasia, which in turn can develop into neoplasia, resulting in aggressive lung cancers. If the study of developing SM can lead to therapies to prevent the initial presentation, or prevent the further development into dysplasia or neoplasia, then cancer rates could be lowered.

This body of work focussed on the use of TNFα as a pro-inflammatory stimulus. While this is a simplified model, release of TNFα does occur in the *in vivo* environment, often released from immune cells such as macrophages (167) and neutrophils (168), immune cells that are elevated in patients with COPD (57, 71). While the release of TNFα has been shown to correlate with COPD (84), it is

often released with other stimulatory factors. To investigate further, other stimulation that occurs in the lung could be used instead of TNFα.

Cigarette smoke extract has been used by many people as a stimulatory factor for cell populations *in vitro* as it occurs in those people who smoke. Cigarette smoke can be collected in three ways. The first is to pass the smoke from a lit cigarette through a filter to collect the total particulate matter (TPM). The TPM can then be dissolved using solvent, allowing for direct application to cell cultures. The second method generates CSE by bubbling main stream cigarette smoke through a medium, usually PBS, assimilating components of the gas phase of the cigarette. The third, and less common method, is to isolate "whole" smoke from the cigarette and apply it directly to the cell cultures using specially designed chambers (169). As smoking is the most prominent risk factor for the development of COPD (3), the use of CSE in the study of the bronchial epithelium would yield results more representative of the *in vivo* environment. However, care would have to be taken as to which method of CS collection would be used. The use of TPM excludes the collection of gas and vapours while the use of CSE excludes the particulate matter found in TMP. Some research has shown that while both TPM and CSE can cause reactive oxygen species (ROS) in cells, some results differed between the two suggesting slightly different pathways of activity (170).

As discussed, very little difference occurred between ALI cultures derived from both healthy and COPD patients, potentially due to the homeostatic nature of the epithelium and the absence of a long-term stimulatory environment. Previous research has suggested that cultured epithelial cells from life-long non-smokers and those with COPD display different levels of IL-8 and TNFα expression, both at baseline and post CSE exposure (77, 171). As this research suggests that there should be a difference in baseline expression between the two donor groups, work could be performed in which ALIs are cultured in the presence of varying concentrations of CSE. The presence of CSE may be enough to maintain a more "COPD like" phenotype in the epithelial cells, highlighting any dissimilarities between the two donor groups. Likewise, long-term culture of epithelium in CSE may be more revealing in the effects of chronic CS exposure when compared to a

single exposure only lasting 24 hours. Further to this, the effects of CSE on the epithelium once SM has been established may demonstrate any adaptive processes that the epithelium may undergo to cope with the assault.

Our model shows that we can successfully grow fully differentiated epithelium from both healthy and COPD donors, and with the removal of RA, can induce the development of SM in the epithelial layers. Now this model has been established, it can be used to test other environmental factors that the epithelium comes into contact with in the natural environment. While the use of CSE exposure of the SM model has been discussed, other respiratory insults such as air pollution or isolated particulate matter could be used to test the cellular responses of an established SM culture. Research investigating the effect of diesel exhaust (DE) exposure to PBEC cultures showed an increase in IL-8 and GM-CSF, as well as a decrease in TEER (172, 173). While some research has used differentiated ALI cultures of COPD PBECs to investigate the effect of DE (174), the literature in this area could be expanded, including a focus on induced SM.

A lot of research has focussed on the effect that respiratory viruses have on PBECs, such as respiratory syncytial virus (RSV) (175, 176), Influenza (177) and Human Rhino Virus (HRV) (178, 179). Due to the association with childhood respiratory infection and the development of asthma (180), a considerable amount of work has focused on how infection influences the responses of PBECs taken from asthmatic donors in comparison to healthy donors (181). There is considerable literature regarding the prevalence of bacterial and viral colonisation of the lower airways in individuals with COPD (182-185). There is also strong evidence that exacerbations are highly influenced by this colonisation (148, 184, 186-188), with respiratory infection contributing to approximately 75% of exacerbations of COPD (189). These exacerbations can result in an increase in hospitalisations and increase the rate of decline of respiratory function (190). Most of the COPD pathogen work is *in vivo*, relying on second hand data such as hospital admissions and large-scale screening to try to draw conclusions about the prevalence of viral infection within those with COPD. The literature could be expanded, focussing on the introduction of pathogens to *in vitro* cultures of COPD PBECs, and those cultures with induced SM, to see if they differ from healthy donors.

5.6 Final Conclusions

The work in this report has found that the removal of RA from ALI cultures of primary epithelial cells, from both healthy and COPD donors, results in the development of SM, similar to that found *in vivo*. While the thickening of the epithelial barrier may result in protection from environmental insult on the airway, such as CS, the epithelium retains a slightly diminished inflammatory response and an altered anti-oxidant response, which may result in the further propagation of SM and further worsen the symptoms of COPD. Further work is required to determine the role that SM plays in the pathogenesis of COPD, generating a more detailed look at the transcriptome as well as the proteome. This model allows for the study of other respiratory ailments such as air pollution and respiratory infections, to determine how they may affect those with COPD.

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