LUNG PHYSIOLOGY & AIRWAY INFLAMMATION IN
COPD PATIENTS WITH PERSISTENT SPUTUM PRODUCTION

A thesis submitted to the University of Manchester
for the degree of Doctorate of Medicine
in the Faculty of Medical and Human Sciences

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Respiratory Medicine
Lung physiology and airway inflammation in COPD patients with persistent sputum production

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<tbody>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>β</td>
<td>beta, Regression coefficient</td>
</tr>
<tr>
<td>ATII</td>
<td>Alveolar type II</td>
</tr>
<tr>
<td>ATS</td>
<td>American Thoracic Society</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BTS</td>
<td>British Thoracic Society</td>
</tr>
<tr>
<td>CAIX</td>
<td>Carbonic anhydrase IX</td>
</tr>
<tr>
<td>CAT</td>
<td>COPD assessment tool</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CV</td>
<td>Closing volume</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DCC</td>
<td>Differential cell count</td>
</tr>
<tr>
<td>DLco</td>
<td>Carbon monoxide diffusing capacity</td>
</tr>
<tr>
<td>DMOG</td>
<td>Dimethyloxalylglycine</td>
</tr>
<tr>
<td>DPX</td>
<td>Di-n-butylPhthalate in Xylene</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECLIPSE</td>
<td>Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetracetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked sandwich immunoassay</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
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</table>
Lung physiology and airway inflammation in COPD patients with persistent sputum production

EGFR  Epidermal Growth Factor Receptor
ERK   Extracellular regulated kinase
FEV₁  Forced expiratory volume in 1 second
FFPE  Formalin-fixed paraffin-embedded
Fig   Figure
FRC   Functional residual capacity
FVC   Forced vital capacity
GM-CSF Granulocyte-macrophage colony-stimulating factor
GOLD  Global initiative for chronic obstructive lung disease
GSK   GlaxoSmithKline
H&E   Haematoxylin and Eosin
HBE   Human bronchial epithelial
HO-1  Heme oxygenase-1
HIER  Heat induced epitope retrieval
HIF   Hypoxia inducible factor
HRE   Hypoxia response element
HRP   Horse radish peroxidase
HNS   Healthy non-smoker
IHC   Immunohistochemistry
IC    Inspiratory capacity
ICC   Intraclass correlation coefficient
ICS   Inhaled corticosteroid
IFN   Interferon
Ig    Immunoglobulin
IHD   Ischaemic heart disease
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>IL</td>
<td>Interleukin (e.g. IL-8, IL-6 etc.)</td>
</tr>
<tr>
<td>IP-10</td>
<td>IFN-γ activated protein-10</td>
</tr>
<tr>
<td>IVC</td>
<td>Inspiratory vital capacity</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus activated kinase</td>
</tr>
<tr>
<td>KCO</td>
<td>CO-diffusion capacity</td>
</tr>
<tr>
<td>L/sec</td>
<td>Litres per second</td>
</tr>
<tr>
<td>LABA</td>
<td>Long acting β2 agonist</td>
</tr>
<tr>
<td>LAMA</td>
<td>Long acting muscarinic antagonist</td>
</tr>
<tr>
<td>LOS</td>
<td>Lipooligosaccharide</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LT</td>
<td>Leukotriene</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen activated protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemoattractant protein</td>
</tr>
<tr>
<td>MDC</td>
<td>Macrophage derived chemokine</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase (eg. MMP-8, MMP-9 etc.)</td>
</tr>
<tr>
<td>MMRC</td>
<td>Modified Medical Research Council</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MSD</td>
<td>Meso Scale Discovery</td>
</tr>
<tr>
<td>MUC</td>
<td>Mucin</td>
</tr>
<tr>
<td>n</td>
<td>number</td>
</tr>
<tr>
<td>nM</td>
<td>nanomoles</td>
</tr>
<tr>
<td>NE</td>
<td>Neutrophil elastase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>NK cells</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non small cell lung carcinoma</td>
</tr>
<tr>
<td>p</td>
<td>probability value</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid Schiff’s</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PHD</td>
<td>Prolyl hydroylase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>QOL</td>
<td>Quality of life</td>
</tr>
<tr>
<td>R²</td>
<td>Coefficient of determination</td>
</tr>
<tr>
<td>Raw</td>
<td>Airways resistance</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation normal T-cell expressed and secreted</td>
</tr>
<tr>
<td>Ri</td>
<td>Intraclass correlation coefficient</td>
</tr>
<tr>
<td>RPMI</td>
<td>Rosewell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>RV</td>
<td>Residual volume</td>
</tr>
<tr>
<td>6MWT</td>
<td>Six minute walk test</td>
</tr>
<tr>
<td>sGAW</td>
<td>Specific conductance</td>
</tr>
<tr>
<td>SABA</td>
<td>Short acting β₂ agonist</td>
</tr>
<tr>
<td>SAMA</td>
<td>Short acting muscarinic antagonist</td>
</tr>
<tr>
<td>SCLC</td>
<td>Small cell lung cancer</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SGRQ</td>
<td>St George’s respiratory questionnaire</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>sGaw</td>
<td>Specific airway conductance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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</tr>
<tr>
<td>Tc1</td>
<td>Type-1 cytotoxic T-cells</td>
</tr>
<tr>
<td>Th1</td>
<td>Type-1 helper T-cells</td>
</tr>
<tr>
<td>TARC</td>
<td>Thymus and activation regulated chemokine</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TCC</td>
<td>Total cell count</td>
</tr>
<tr>
<td>TCC/g</td>
<td>Total cell count per gram</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor (e.g. TGF-α and TGF-β)</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of matrix metalloproteinase</td>
</tr>
<tr>
<td>TLC</td>
<td>Total lung capacity</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor (e.g. TNF-α and TNF-β)</td>
</tr>
<tr>
<td>µ</td>
<td>Micro</td>
</tr>
<tr>
<td>VA</td>
<td>Alveolar volume</td>
</tr>
<tr>
<td>VC</td>
<td>Vital capacity</td>
</tr>
<tr>
<td>VHL</td>
<td>Von Hippel-Lindau</td>
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ABSTRACT

A Thesis submitted by Shruti Khurana for the degree of Doctorate of Medicine in the Faculty of Medicine and Human Sciences

LUNG PHYSIOLOGY AND AIRWAY INFLAMMATION IN COPD PATIENTS WITH PERSISTENT SPUTUM PRODUCTION

(Month and year of submission: December 2013)

Background The clinical and pathological presentation of COPD is heterogeneous. ‘Chronic bronchitis’ is a phenotype of COPD, which is a clinical diagnosis of a productive cough of ≥ 3 months for ≥ 2 consecutive years. Chronic bronchitis is associated with worse lung function, frequent exacerbations, recurrent hospitalisations and premature death in patients with COPD. Chronic bronchitis sufferers can be further subphenotyped into those who produce sputum during exacerbation or during winter months only and those who are ‘persistent sputum producers,’ who experience mucus hypersecretion throughout the year. An improved understanding of persistent sputum producers is the object of this thesis.

Aims 1) To compare the clinical characteristics and airway inflammatory biomarker profile of COPD persistent sputum producers to that of COPD sputum non-producers 2) To investigate the short term repeatability of sputum parameters in COPD persistent sputum producers 3) To study the expression and relationship of mucins, hypoxia inducible factor (HIF-1α) and carbonic anhydrase IX (CAIX) in COPD persistent sputum producers.

Methods 1) Lung physiology, health status, sputum inflammatory biomarkers and sputum culture results were compared between COPD persistent sputum producers and sputum non-producers 2) Repeatability of spontaneous and induced sputum parameters at 8 weeks was assessed in COPD persistent sputum producers 3) Immunohistochemistry was performed on bronchial biopsies of COPD persistent sputum producers and control groups (COPD sputum non-producers, smokers with normal lung function and lifelong healthy non-smokers with normal lung function) to study the expression of MUC5AC, MUC5B, HIF-1α and CAIX 4) The association between HIF-1α and MUC5B expression was investigated in vitro.

Results and Conclusions The findings suggest that 1) COPD persistent sputum producers have clinically more severe disease, increased airway inflammation, increased impact on health status, increased rate of bacterial colonization and higher number of exacerbations compared to COPD sputum non-producers 2) Induced sputum is repeatable over short term in COPD persistent sputum producers 3) Expression of MUC5B, HIF-1α and CAIX is increased in COPD persistent sputum producers compared to COPD sputum non-producers, smokers with normal lung function and healthy non-smokers 4) HIF-1α can potentially cause increased MUC5B expression. This work reveals potential targets for the development of novel therapies to limit mucus hypersecretion in COPD.
DECLARATION

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PREFACE

The experiments in this thesis were developed by me, under the supervision of Professor Singh.

To recruit the patients for these studies, a search was performed on the Medicines Evaluation Unit volunteer database to identify COPD patients that could potentially be used in the study. The patients were then contacted by phone or post to explain to about the studies. I screened the majority of the patients involved in these studies which included performing lung function measurements and the collection and processing of all biological samples. However, I did receive help from my colleagues at the Medicines Evaluation Unit and the Education Research Centre in collecting some of this data and sputum cytokine analysis was performed by GSK.

Finally, I was fully responsible for the statistical analysis and the interpretation of all the data from the experimental chapters.
ACKNOWLEDGEMENTS

Without the help and support of the following people, this thesis would not have been possible. I would like to thank

Professor S Dave Singh (supervisor) for giving me the encouragement, teaching and patience in completing this thesis. I would also like to thank him for supporting me to attend various research related meetings I have had the privilege of participating in over the last two years.

Professor Jorgen Vestbo (co-supervisor), Dr Stephen Fowler (advisor) and Dr. Jonathan Plumb for their advice and support.

My colleagues at the Medicines Evaluation Unit and the Education Research Centre and in particular my dear friends James Pearson, Kate Brown, Arjun Ravi and Paul Hitchen for their help and support.

My parents, Mr. P.N Seth and Mrs. V.M. Seth who have been my pillars of strength, my husband Rahul and my son Sid for lots of encouragement and support.
LIST OF ABSTRACTS AND POTENTIAL PUBLICATIONS

Poster discussion - Clinical characteristics and airway inflammation profile of COPD Persistent Sputum Producers (American Thoracic Society, San Francisco, May 12)

Clinical characteristics and airway inflammation profile of COPD Persistent Sputum Producers (Khurana S, Ravi A, Sutula J, Milone R, Williamson R, Plumb J, Vestbo J, Singh D) - in submission to CHEST.

MUC5B and Carbonic Anhydrase IX (CAIX) expression is increased in the bronchial surface epithelium of COPD Persistent Sputum Producers (Khurana S, Brown K, Vestbo J, Plumb J, Singh D) - in submission to Respiratory Research.
CHAPTER 1: INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is a major cause of morbidity and mortality (Murray and Lopez 1997), with poorly reversible airflow obstruction and pulmonary inflammation being hallmark features (Hogg et al. 2004). The aetiology of COPD is related to the inhalation of noxious gases and fumes, predominantly, to the inhalation of cigarette smoke. A study in 1977 estimated that 15% of smokers develop COPD, a figure still widely quoted (Fletcher and Peto 1977). However, a recent study of a large cohort of smokers followed up for 25 years suggest that this figure is higher at 25% (Lokke et al. 2006). Exposure to indoor pollution or use of biomass cooking fuels can also cause COPD and living in low socio-economic conditions is a contributing factor to the development of COPD (Prescott and Vestbo 1999; Kurmi et al. 2010). A number of genetic susceptibilities have been identified in COPD (Qiu et al. 2011). Less than one percent of COPD sufferers have α1-antitrypsin genetic deficiency; however, 80% of those with the worse form of deficiency will develop COPD (Sorheim et al. 2010; Barnes 2000).

It is now recognized that COPD is a heterogeneous disease comprising subgroups of patients with distinct clinical or pathological characteristics and with different prognosis or response to treatment. Such subgroups are often referred to as COPD phenotypes (Han et al. 2010). This thesis will focus on the phenotype of COPD patients with persistent sputum production.

1.1 Effect of smoking on the lungs

Smoking and the Immune Response in the Lungs

The lung is continuously exposed to inhaled particles and antigens. The defense against these antigens can be divided into two general types of reactions: reactions of innate immunity and reactions of adaptive immunity. The innate immune system is characterized by a non-specific response combined with a lack of immunological memory. Two types of phagocytic cells – neutrophils and macrophages are the cornerstones of this response. In addition to phagocytosis, neutrophils secrete proteases that break down the extracellular matrix. The adaptive immune system is called into action against pathogens that are able to evade or overcome innate immune defenses. There are two types of adaptive immune
response: humoral immunity, mediated by antibodies produced by B lymphocytes, and cell-mediated immunity, mediated by T lymphocytes.

The inhalation of toxins from cigarette smoke can cause the lungs to respond in a similar manner to an infection, initially causing activation of the innate immune defence system (Tetley 2005). However, after prolonged cigarette smoke exposure, there is evidence of altered adaptive immunity, characterized by increased numbers of lymphocytes in the lungs.

Cigarette smoke damages the bronchi in a number of ways including direct toxicity to the epithelium, oxidative damage and increased epithelial permeability (Thorley and Tetley 2007; Borrill et al. 2008). Cigarette smoking can cause inflammation in the tracheobronchial tree in the absence of airflow limitation (Saetta et al. 2001; Jeffery 1998). There is an increase in the number of lymphocytes and macrophages within the airway wall (Saetta et al. 2001) and increased numbers of neutrophils in the lumen and bronchial secretions (Saetta et al. 2001).

At this stage, before COPD is established, early ‘structural’ changes may occur in peripheral airways, causing increased resistance (Hogg et al. 1968; Niewoehner et al. 1974). However, repeated cycles of injury and repair of the airway walls causes structural remodelling and lumen narrowing and leads to development of COPD in some individuals (Leopold and Gough 1957; Lean 1958).

1.2 Pathophysiology of COPD

The hallmark feature of COPD is airway inflammation leading to pathological and structural changes such as small airway fibrosis, smooth muscle hypertrophy and progressive destruction of alveolar walls resulting in airflow limitation (Saetta et al. 2000). There is a reduction in lung elasticity and loss of radial traction on the airways during expiration, in turn leading to airway collapse, gas trapping and hyperinflation of the lungs. There is also epithelial metaplasia and mucociliary dysfunction, leading to increased mucus secretion and accumulation. The occlusion of small airways by mucus plugs also leads to expiratory airflow limitation and decrease in FEV₁.
Inflammation

Structural changes

Mucus accumulation

Airway limitation

Figure 1.1: Factors leading to airflow limitation in COPD

1.2.1 Inflammatory cells in COPD

Neutrophils

Cigarette smoke and other irritants activate macrophages and airway epithelial cells in the respiratory tract, which release neutrophil chemotactic factors, including interleukin-8 (IL-8) and leukotriene B4 (LTB4) (Moretto et al. 2009). Neutrophils and macrophages release proteases that break down connective tissue in the lung parenchyma, potentially resulting in emphysema. Neutrophils and their products also play several key roles in epidermal growth factor (EGFR)-dependent mucus hypersecretion. Neutrophils secrete tumor necrosis factor (TNF)-α, neutrophil elastase and reactive oxygen species; all of these induce EGFR activation in airway epithelial cells (Takeyama et al. 2000; Kim and Nadel 2004). Neutrophil elastase also causes impairment of ciliary function (Amitani et al. 1991), stimulation of mucin release (Kim et al. 1987) and secretory cell metaplasia and hyperplasia (Christensen et al. 1987). Sputum neutrophil percentage is increased in COPD patients compared to controls (O'Donnell et al. 2004a; Keatings et al. 1996).

Macrophages

Macrophages are derived from monocytes and are the most frequent cells in induced sputum and bronchoalveolar lavage (BAL) of healthy non-smokers. Macrophages play an important role in the pathogenesis of COPD (Shapiro 1999) and their numbers are markedly increased in the airways and lung parenchyma of smokers and COPD patients. Macrophages are involved in initiation, maintenance, and termination of inflammatory reactions by infiltrating the affected tissue and releasing pro- and anti-inflammatory cytokines and mediators (Lohmann-Matthes et al. 1994). Whether pro- or anti-inflammatory actions prevail depends on the activation state of macrophages; Classical
activation of macrophages promotes the release of proinflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α as well as elastolytic enzymes, including matrix-metalloproteinase (MMP)-2, MMP-9, MMP-12, cathepsins K, L, and S, and neutrophil elastase taken up from neutrophils (Russell et al. 2002b). In contrast, alternative activation leads to production of anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist (Hamilton et al. 1999).

Alveolar macrophages from COPD patients phagocytose less *Haemophilus influenzae* compared with smokers without COPD (Berenson et al. 2006). One reason for this might be the chronic activation of macrophages resulting in reduced phagocytic ability (Culpitt et al. 2003; Russell et al. 2002a). Another theory is that the lack of pathogen removal is an inherent defect in circulating monocytes from COPD patients that unmask during maturation into macrophages (Taylor et al. 2010). Also, reduced levels of the toll-like receptor TLR2 present on macrophages which helps in recognizing gram-positive bacteria have been reported in macrophages from COPD patients and cigarette smokers (Droemann et al. 2005). This defect in phagocytosis of bacteria that most frequently cause acute exacerbations of COPD is likely to be an important factor leading to colonization of the lower airways and the tendency for bacterial exacerbations in COPD (Taylor et al. 2010).

COPD responds poorly to corticosteroid therapy. The reasons for the lack of response are not entirely known, but one theory is that cigarette smoking reduces the response to corticosteroids by decreasing histone deacetylase activity, a nuclear enzyme that switches off inflammatory genes activated through the transcription factor nuclear factor (NF)-κB in key inflammatory cells such as alveolar macrophages (Ito et al. 2001; Barnes et al. 2004; Kent et al. 2009). Another theory is that in COPD there is increased production of steroid insensitive cytokines produced by macrophages such as granulocyte macrophage-colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), and IL-8. Production of these steroid insensitive cytokines is also a feature of healthy macrophages but an increase in the number of macrophages in COPD leads to an increase in the cytokine production including these steroid insensitive mediators (Armstrong et al. 2009).
Eosinophils

Eosinophilic airway inflammation is a classic feature of asthma (Bousquet et al. 1990), but an increase in the number of eosinophils is also noted in the airways in 20-40% of steroid naive stable COPD patients (Saha and Brightling 2006). Studies have shown that the presence of eosinophils in the airways of COPD patients is a predictor of good response to treatment with corticosteroids (Brightling et al. 2000; Papi et al. 2000). During an exacerbation of COPD there is an increase in eosinophils compared to stable state which returns to baseline upon successful treatment with corticosteroids (Saetta et al. 1994; Bathoorn et al. 2008). Sputum eosinophils may therefore be a biomarker of corticosteroid responsiveness in COPD.

Lymphocytes

Cytotoxic T cells (CD8+) have been proposed to play a role in the progression of inflammation in COPD, even after the cessation of smoking (Willemse et al. 2005). There is an increase in CD8+ T cells observed in the lung parenchyma and peripheral and central airways of patients with COPD, with greater tendency of CD8+ infiltration rather than CD4+ cells (Fournier et al. 1989). These cytotoxic cells have the capacity to cause apoptosis of alveolar epithelial cells and alveolar wall destruction through the release of perforins, granzyme B and TNF-α (Hashimoto et al. 2000). An inverse correlation between CD8+ cell numbers in the airway wall and FEV1 has been shown (O'Shaughnessy et al. 1997). Figure 1.2 illustrates the inflammatory mechanisms in COPD.
Figure 1.2: Inflammatory mechanisms in COPD: Cigarette smoke (and other irritants) activate macrophages in the lungs. Macrophages and epithelial cells release chemokines that cause neutrophil recruitment. Macrophages and neutrophils release proteases that break down the connective tissue in the lung parenchyma, resulting in emphysema. Cytotoxic T cells (CD8+) may be recruited and involved in alveolar wall destruction. Proteases also stimulate mucin hypersecretion.
1.2.2 Cytokines in COPD

Cytokines are extracellular signal proteins produced by structural cells such as epithelial cells, endothelial cells, smooth muscle cells and fibroblasts, as well as by inflammatory cells. They recruit, activate, and promote the survival of multiple inflammatory cells in the respiratory tract. They play an important role in airway inflammation and mucus hypersecretion.

Cytokines can be classified by their roles. Pro-inflammatory cytokines are cytokines that amplify and prolong inflammation. Lymphokines are cytokines secreted by T cells and regulate immune responses. Growth factors are cytokines that increase cell survival and alter airway structure. Chemokines attract inflammatory cells. Anti-inflammatory cytokines suppress inflammation (Barnes 2008a). The measurement of these cytokines allows an estimation of the levels of these mediators in health and disease and has provided a potential for anticytokine therapy to suppress inflammation in COPD (Fulton et al. 1997; Leng et al. 2008).

Elevated levels of cytokines have been found in serum, induced sputum, BAL fluid and/or bronchoscopic biopsy specimens of COPD patients (Ollerenshaw and Woolcock 1992b; Bhowmik et al. 2000; Martin et al. 1985; Wedzicha et al. 2000) and these cytokines play an important role in the pathogenesis of COPD in different ways. Table 1.1 lists the cytokines implicated in COPD and their functions.

Importantly, some of these cytokines such as IL-1β, IL-4, IL-5, IL-9, IL-6, IL-10, IL-13, IL-17, MCP-1 and TNF-α have been shown to cause mucin gene upregulation and thus may contribute to airflow obstruction in COPD by increased mucus secretion (Table 1.1). Of these the levels of IL-4, IL-13, MCP-1 and TNF-α have been found to be elevated in the central airways of chronic bronchitis patients (Capelli et al. 1999; Miotto et al. 2003a; Ma et al. 1999). Other cytokines found elevated in the central airways of chronic bronchitis patients are IL-8 and MIP-1β which are chemoattractants for neutrophils and eosinophils respectively (Capelli et al. 1999; Chanez et al. 1996).

Induced sputum is a safe and non-invasive method of studying airway cytokines (Pizzichini et al. 1996). Increased levels of several cytokines such as GM-CSF, IL-1β, IL-6, IL-8, IL-17, IFN-γ, MCP-1 and TNF-α have been observed in induced sputum from patients with stable COPD (Chung 2001; Doe et al. 2010; Moermans et al. 2011; Saha et
al. 2009). It has been shown that IL-8 remain elevated over time (Beeh et al. 2003; Brightling et al. 2001; Aaron et al. 2010).

Levels of IL-6, IL-8 and TNF-α in induced sputum have been found to be higher in patients with severe or very severe COPD than in patients with mild or moderate COPD and also in those with a longer duration of disease (Hacievliyagil et al. 2006). Patients with more frequent exacerbations have higher baseline sputum levels of IL-6 and IL-8, which may predict the frequency of future exacerbations (Hacievliyagil et al. 2006; Bhowmik et al. 2000).

Previous studies have used quantitative enzyme-linked sandwich immunoassays (ELISA) to measure a limited number of cytokines in sputum (Keatings et al. 1996). Newer multiplex techniques such as Luminex and Meso Scale Discovery (MSD) allow measurement of a large panel of cytokines from small volume sputum or serum samples, reducing time, cost and effort. Each of these two platforms has its own merits with the Luminex having better precision and the MSD better sensitivity and accuracy (Chowdhury et al. 2009). The advent of these new techniques has resulted in a rapid expansion of cytokine research in recent times.
### Table 1.1: Summary of cytokines in COPD

<table>
<thead>
<tr>
<th>Inflammatory mediator</th>
<th>Effect in COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proinflammatory cytokines</strong></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>Released by macrophages, neutrophils, T cells, epithelial cells and airway smooth muscle cells. Induces IL-1, IL-8 and MCP-4 in airway cells (Kwon et al. 1994). Can cause mucin gene upregulation (Song et al. 2003), induce airway mucus cell metaplasia and hypersecretion (Lora et al. 2005). Increased in sputum and BAL in COPD, especially during exacerbations (Aaron et al. 2001). Levels increased in chronic bronchitis (Ma et al. 1999; Huang et al. 1997).</td>
</tr>
<tr>
<td>IL-6</td>
<td>Increased in sputum in COPD, particularly during exacerbations. Releases CRP from liver increasing systemic inflammation (Barnes 2008a).</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Along with TNF-α stimulates macrophages to produce MMP-9 and bronchial epithelial cells to produce tenascin (Culpitt et al. 2003). Sputum in COPD contains increased levels of MMP-9, -8 and -1 and tissue inhibitor of metalloproteases (TIMP-1). MMP-9 and TIMP-1 concentrations are increased in chronic bronchitis (Higashimoto et al. 2005).</td>
</tr>
<tr>
<td>IL-18</td>
<td>Released by activated macrophages. Induces emphysema and airway and vascular remodelling via IFN-γ, IL-17A and IL-13 (Kang et al. 2012). Strongly expressed in CD8+ T-cells, both the bronchiolar &amp; alveolar epithelia of COPD patients. Levels significantly greater in the sera of patients with GOLD stage III and IV COPD (Imaoka et al. 2008).</td>
</tr>
<tr>
<td>IL-32</td>
<td>Induced by IFN-γ. Increased expression in epithelial cells, macrophages, and CD8+ cells of patients with COPD, and is correlated with disease severity (Calabrese et al. 2008). Results in secretion of TNF-α and IL-1β through the activation of NF-κB and p38 mitogen-activated protein kinase (MAPK).</td>
</tr>
<tr>
<td><strong>Lymphokines</strong></td>
<td></td>
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<tr>
<td>Th1 cytokines gff</td>
<td></td>
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<tr>
<td>IFN-γ</td>
<td>Main cytokine produced by T lymphocytes and induces chemokine release from alveolar macrophages and epithelial cells (Szabo et al. 2002). In COPD there are an increased number of T cells in the airway secreting IFN-γ, as found in sputum, blood and BAL (Hodge et al. 2007; Moermans et al. 2011).</td>
</tr>
<tr>
<td>IL-2</td>
<td>Important for proliferation of T and B lymphocytes. IL-2 receptor positive cells (CD25 positive cells) are increased in the bronchial mucosa in chronic bronchitis (Saetta et al. 1993).</td>
</tr>
</tbody>
</table>
**Lung physiology and airway inflammation in COPD patients with persistent sputum production**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-12</strong></td>
<td>Important for differentiation and activation of Th1 cells (Trinchieri et al. 2003). Acts via activation of STAT 4, which is phosphorylated in the airways and BAL fluid lymphocytes of COPD patients (Di Stefano et al. 2004). Biologically active form is called IL-12p70.</td>
</tr>
<tr>
<td><strong>Th2 cytokines</strong></td>
<td></td>
</tr>
<tr>
<td><strong>IL-4</strong></td>
<td>Induces differentiation of naive T cells to Th2 cells (Barnes 2008b). Increases production of IgE (Kroegel et al. 1996). Can induce mucin gene expression and goblet cell hyperplasia (Murata et al. 1999).</td>
</tr>
<tr>
<td><strong>IL-13</strong></td>
<td>Increases the production of IgE and induces airway remodelling (Kroegel et al. 1996; Kang et al. 2012). Can induce mucin gene expression and goblet cell hyperplasia. IL-4 and IL-13 levels are elevated in subjects with chronic bronchitis (Miotto et al. 2003a).</td>
</tr>
<tr>
<td><strong>IL-5</strong></td>
<td>Stimulates B cell growth and increases immunoglobulin secretion. Key mediator in eosinophil activation (Stirling et al. 2001).</td>
</tr>
<tr>
<td><strong>Th17 cytokines</strong></td>
<td></td>
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<tr>
<td><strong>IL-17</strong></td>
<td>IL-17A levels are increased in COPD (Eustace et al. 2011; Chang et al. 2011). Induces release of neutrophil chemoattractants: IL-1 and IL-8 (Dragon et al. 2007). Increases expression of MUC5AC and MUC5B in airway epithelial cells (Chen et al. 2003).</td>
</tr>
<tr>
<td><strong>Growth Factors</strong></td>
<td></td>
</tr>
<tr>
<td><strong>GM-CSF</strong></td>
<td>Released by alveolar macrophages. Important for increased survival of neutrophils and macrophages in the airways (Culpitt et al. 2003). Increased levels found in BAL in COPD, particularly during exacerbations (Balbi et al. 1997).</td>
</tr>
<tr>
<td><strong>TGF-β</strong></td>
<td>Induces proliferation of fibroblasts and smooth muscle cells, deposition of extracellular matrix and epithelial repair in the lung. Has immunoregulatory effect resulting in suppression of Th1 &amp; Th2 cells (Wan and Flavell 2007). Increased expression noted in chronic bronchitis (Chung 2001).</td>
</tr>
<tr>
<td><strong>EGF</strong></td>
<td>Activates EFGR tyrosine kinase and may play an important role in regulating mucus secretion in COPD (Barnes 2008a). Expression of EGFR and EGF is increased in airway epithelial cells in COPD and chronic bronchitis (Chung 2001).</td>
</tr>
<tr>
<td><strong>Chemokines</strong></td>
<td></td>
</tr>
<tr>
<td><strong>CCR2 agonists</strong></td>
<td></td>
</tr>
<tr>
<td><strong>MCP-1 (CCL2)</strong></td>
<td>Chemoattractant of monocytes, important in macrophage accumulation. Increased in sputum, BAL and lungs in COPD (de Boer et al. 2000). Induces MUC5AC &amp; MUC5B mRNA and protein expression in airway epithelium (Monzon et al. 2011).</td>
</tr>
</tbody>
</table>
Lung physiology and airway inflammation in COPD patients with persistent sputum production

| MCP-4 (CCL13) | Chemoattractant of monocytes, eosinophils, T lymphocytes and basophils. Can bind to CCR2, CCR3 and CCR5. Activity has been implicated in allergic reactions such as asthma. Can be induced by inflammatory cytokines, IL-1 and TNF-α (Garcia-Zepeda et al. 1996). |
| CCR3 agonists | Eotaxin (CCL11) | Binds to CCR3 on eosinophils, causes eosinophil chemotaxis and degranulation. May be important in differentiation of eosinophils and release from bone marrow. Produced by TNF-α & IL-4 in bronchial epithelial cells (Matsukura et al. 1999). |
| | Eotaxin 3 (CCL26) | Similar to eotaxin, activates eosinophils and fibroblasts (Kitaura et al. 1999). |
| | RANTES (CCL5) | Eosinophil chemoattractant. Eotaxin, RANTES and CCR3 are upregulated in airways in exacerbations of chronic bronchitis, may account for the sputum eosinophilia in these patients (Bocchino et al. 2002; Smyth et al. 2008). |
| CCR4 agonists | MDC (Macrophage derived chemokine) | Recently identified, binds to CCR4 present on Th2 cells. Chemoattractant for dendritic cells, natural killer (NK cells) and Th2 cells (Godiska et al. 1997). T cells attracted by MDC mainly produce Th2-type cytokines eg. IL-4 and IL-5 (Imai et al. 1999). |
| | TARC (CCL17) (Thymus & activation regulated chemokine) | Specifically binds to and induces chemotaxis in T cells (Hieshima et al. 1997). |
| CCR5 agonists | MIP-1α (CCL3) | CCR5 is expressed on Th1 and Tc1 cells and is involved in the recruitment of these cells in the airways in COPD. CCR5 is activated by MIP-1α which is elevated in the lungs in COPD (Barnes 2008a). |
| | MIP-1β (CCL4) | Expressed primarily by B cells, T cells and monocytes after antigen stimulation (Schrum et al. 1996). Levels of MCP-1 and MIP-1β are raised in BAL fluid in chronic bronchitis (Capelli et al. 1999). May have a role as chemoattractant for eosinophils in chronic bronchitis (Barczyk et al. 2001). |
| CXCR2 agonists | IL-8 (CXCL8) | IL-8 activates CXCR1 and 2, important in neutrophil chemotaxis. Cigarette smoke increases production of IL-8 (Kent et al. 2010). Levels are significantly increased in sputum in COPD and correlate with the increased proportion of neutrophils (Borrill et al. 2008). Levels increased in those with a history of chronic bronchitis compared to COPD patients who do not report cough and phlegm (Chanez et al. 1996). |
| CXCR3 agonists | IP-10 (CXCL10) (IFN- γ induced protein) | Secreted by monocytes, endothelial cells and fibroblasts. Several roles – chemoattractant for monocytes/macrophages, T cells, NK cells |
and dendritic cells; promotes T cell adhesion to endothelial cells; antitumor activity; inhibits bone marrow colony formation and angiogenesis (Dufour et al. 2002; Angiolillo et al. 1995).
Other CXCR3 agonists are IL-9 and IL-10, which are also induced by IFN-γ and increased in sputum in COPD, correlate with disease severity (Takanashi et al. 1999).

<table>
<thead>
<tr>
<th>Anti-inflammatory cytokines</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-10</strong></td>
<td>Inhibits the synthesis of inflammatory proteins such as TNF-α, GM-CSF, IL-5 &amp; MMP-9. Levels reduced in sputum of COPD patients (Takanashi et al. 1999).</td>
</tr>
</tbody>
</table>
1.2.3 Systemic inflammation in COPD

Elevated markers of systemic inflammation are found in some but not all COPD patients (Agusti et al. 2012). Although not closely associated with specific features of COPD, systemic inflammation is associated with risk of exacerbations (Dahl et al. 2001; Thomsen et al. 2013) as well as incidence of comorbidities (Thomsen et al. 2012). Some of the factors that may contribute to systemic inflammation in COPD are reduced pulmonary gas exchange leading to systemic hypoxia, absorption of inhaled toxins from cigarette smoke and genetic predisposition to systemic inflammation (Yanbaeva et al. 2009). Several comorbidities of COPD have been associated with a systemic inflammatory response including ischaemic heart disease, heart failure, osteoporosis, normocytic anaemia, lung cancer, depression, diabetes, metabolic syndrome, skeletal muscle wasting and weakness (Chatila et al. 2008).

A systemic inflammatory response is characterized by mobilization and activation of inflammatory cells into the circulation, as well as the production of acute phase proteins and circulating pro-inflammatory mediators (Sinden and Stockley 2010). C-reactive protein (CRP) is a marker of acute phase inflammatory response and its levels are elevated in patients with COPD and associated with airflow obstruction (Sin et al. 2004). Elevated levels of CRP in COPD are associated with increased cardiovascular disease risk and is a marker of impaired health status (Garrod 2005). Patients with COPD also have increased circulating levels of fibrinogen (Jousilahti et al. 1999; Wedzicha et al. 2000), another acute phase protein, as well as increasing levels of pro-inflammatory mediators including TNF-α and IL-6 (Di Francia et al. 1994). TNF-α and IL-6 are associated with atherosclerosis (Gan et al. 2004). Elevated TNF-α may also contribute to muscle wasting and cachexia (Agusti et al. 2002). Recent studies suggest that elevated levels of IL-6 may be associated with an increased prevalence of insulin resistance in COPD (Bolton et al. 2007).

It is to be noted that the high frequency of comorbid disease in patients with COPD may also be partly experienced by the fact that the majority of patients with COPD are of an older age and thus more likely to suffer from poor health in general as well as their exposure to cigarette smoke, which is a major risk factor for COPD and many other chronic diseases (Corsonello et al. 2011).
1.2.4 Assessment of airway inflammation in COPD

Airway inflammation can be assessed in a number of different ways. A safe and non-invasive method of measuring airway inflammation is spontaneous and induced sputum. Sputum induction is simple to perform with the inhalation of nebulised saline and allows repeated measurements without causing patient discomfort. The technique of sputum induction is performed in a standardized way and measures are used to prevent adverse reactions (Pizzichini et al. 2002). The most common measure of inflammation in sputum is the sputum differential cell count expressed either as absolute count or percentage count. Soluble mediators such as cytokines are also easily measured in sputum supernatant.

Sputum processing methods commonly use Dithiothreitol (DTT) - a sputolysin which is used to break down sputum plugs. This has been shown not to affect cell counts and high repeatability of cell counts have been demonstrated (Brightling et al. 2001). However, DTT can affect the three-dimensional structure of proteins and interfere with immunoassays (Woolhouse et al. 2002). Therefore, the use of DTT may influence the detection of mediators in sputum supernatant (Kolsum 2010; Aaron et al. 2010). This problem can be overcome by using a two-step processing procedure; initial processing with phosphate buffered saline (PBS) to obtain supernatant for immunoassays, followed by DTT processing for cellular analysis (Badafahel et al. 2012).

In the paper by Bafadhel et al., comparison and validation of the effects of the additional PBS step with that of currently used sputum processing for cytospin preparation for the sputum total cell count and differential cell count was performed in 20 subjects with COPD and asthma (Bafadhel et al. 2012). MSD and Luminex platforms were used to measure cytokines, chemokines and matrix metalloproteinase levels. There were no significant differences in the cell differential counts between the methods with or without the additional PBS wash step. Analytes sensitive to DTT that had increased recovery in the two-step sputum process were IL-1β, 4, 5, 10, 13, IFN-γ, GM-CSF, CCL-2, 3, 4, 5, 13 and 17. This technique of sputum processing has also been shown to improve the recovery of previously difficult to measure Th2 cytokines such as IL-5 (Bafadhel et al. 2009).
1.2.5  Repeatability of sputum inflammatory biomarkers

The repeatability of induced sputum measurements such as differential cell count and IL-8 levels ranging over short term (1 day to 3 month intervals) in COPD has been established in various studies. Table 1.2 summarizes some of these studies. These studies demonstrate moderate to strong repeatability of percentage sputum neutrophils in COPD. This is something that may be expected as prominent neutrophilic inflammation is characteristic of COPD airway pathology (Hogg et al. 2004). A recent study by Singh et al. has also shown good repeatability of sputum neutrophil percentage over a longer interval of one year (Singh et al. 2010). The mean change over 1 year in neutrophil percentage was an increase of 3.5% in this study. Most variability was observed in samples with a low neutrophil percentage, which suggests that a low neutrophil percentage is often a transient phenomenon, and that repeated measurements "regress to the mean" - which is a higher value.
Table 1.2: Example of studies on repeatability of airway inflammatory biomarkers over short term

<table>
<thead>
<tr>
<th>Author</th>
<th>COPD patients</th>
<th>Interval between repeated induction</th>
<th>Interclass correlation coefficient (Ri)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peleman</strong></td>
<td>n = 21</td>
<td>10 days</td>
<td>Neutrophil %= 0.84</td>
</tr>
<tr>
<td>(Peleman et al. 1999)</td>
<td>ICS naive n=2</td>
<td></td>
<td>Eosinophil %= 0.90</td>
</tr>
<tr>
<td></td>
<td>FEV$_1 &lt; 54%$</td>
<td></td>
<td>Macrophage %= 0.67</td>
</tr>
<tr>
<td><strong>Balzano</strong></td>
<td>n = 10 (3: COPD, 2: Asthma, 2: HNS, 2: HS)</td>
<td>7 days</td>
<td>Total cell count= 0.33</td>
</tr>
<tr>
<td>(Balzano et al. 1999)</td>
<td>ICS naive &gt; 2 months</td>
<td></td>
<td>Neutrophil %= 0.95</td>
</tr>
<tr>
<td></td>
<td>FEV$_1$ = not stated</td>
<td></td>
<td>Eosinophil %= 0.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Macrophage %= 0.96</td>
</tr>
<tr>
<td><strong>Brightling</strong></td>
<td>n = 61</td>
<td>2 weeks</td>
<td>Total cell count= 0.54</td>
</tr>
<tr>
<td>(Brightling et al. 2001)</td>
<td>ICS naive ≥ 1 month</td>
<td></td>
<td>Neutrophil %= 0.57</td>
</tr>
<tr>
<td></td>
<td>FEV$_1$ = 41.6%</td>
<td></td>
<td>Eosinophil %= 0.63</td>
</tr>
<tr>
<td><strong>Beeh</strong></td>
<td>n = 12</td>
<td>4 weeks</td>
<td>Total cell count= 0.07</td>
</tr>
<tr>
<td>(Beeh et al. 2003)</td>
<td>ICS naive ≥ 3 months</td>
<td></td>
<td>Neutrophil %= 0.66</td>
</tr>
<tr>
<td></td>
<td>FEV$_1$ = 58 %</td>
<td></td>
<td>Eosinophil %= 0.49</td>
</tr>
<tr>
<td><strong>Boorsma</strong></td>
<td>n = 21</td>
<td>1 week</td>
<td>Total cell count= 0.52</td>
</tr>
<tr>
<td>(Boorsma et al. 2007)</td>
<td>ICS naive = 3 weeks</td>
<td></td>
<td>Neutrophil %= 0.61</td>
</tr>
<tr>
<td></td>
<td>FEV$_1$ = 65.2%</td>
<td></td>
<td>Eosinophil %= 0.72</td>
</tr>
<tr>
<td><strong>Sapey</strong></td>
<td>n = 14</td>
<td>1 day for 5 days</td>
<td>Neutrophil cell count = 0.77</td>
</tr>
<tr>
<td>(Sapey et al. 2008)</td>
<td>GOLD stage III</td>
<td></td>
<td>Macrophage cell count = 0.74</td>
</tr>
</tbody>
</table>
1.3 COPD Exacerbations

An exacerbation of COPD is currently defined as: “A sustained worsening of the patient's condition, from the stable state and beyond normal day-to-day variations, that is acute in onset and necessitates a change in regular medication in a patient with underlying COPD” (COPD NICE clinical guideline 2010). Exacerbations are triggered by respiratory bacteria and viruses and remain the commonest precipitant of death in COPD. Even after an exacerbation resolves, respiratory, physical, social and emotional impairment may persist for a prolonged time (Anzueto 2010).

COPD exacerbations are associated with increased airway and systemic inflammation and physiological changes, especially the development of hyperinflation (Donaldson et al. 2005a). They cluster in time implying that second exacerbations occur sooner rather than later (Hurst et al. 2009). Some patients are particularly susceptible to exacerbations, and show worse health status and faster disease progression than those who have infrequent exacerbations (Donaldson et al. 2005b; Seemungal et al. 1998; Miravitlles et al. 2000; Hurst et al. 2010).

Bacteria are isolated from sputum in 40-60% of acute exacerbations patients of acute exacerbations of COPD (Sethi 2004; Bari et al. 2010). The three most common bacteria isolated from sputum during COPD exacerbations are nontypable *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis* (Tager and Speizer 1975; Sethi 2004). Other infrequently isolated potential pathogens are *Haemophilus parainfluenzae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and members of the family Enterobacteriaceae. Gram-negative bacilli, including *Pseudomonas aeruginosa*, *Escherichia coli* and *Proteus mirabilis* are isolated most often in the setting of severe exacerbations in patients with advanced COPD (Soler et al. 1998). Viral infections also play a role in exacerbations of COPD and pathogens such as rhinovirus, influenza virus, parainfluenza virus and coronavirus are often detected in patients with COPD during an exacerbation (Sethi 2000).

Most of the bacteria isolated from sputum during exacerbations can be found in the lower airways of patients with COPD during stable phases of their disease (Wilson 2001; Gump et al. 1976). Studies that have used bronchoscopy to sample the lower airways directly
have shown that approximately 25% of patients with stable COPD have lower airway bacterial colonization with a potential pathogenic species, and the proportion increases to approximately 50% during an exacerbation (Monso et al. 1995; Monso et al. 1999; Zalacain et al. 1999; Pela et al. 1998). These bacteria have been cultured in lower airway secretions despite the presence of antibodies in serum and sputum against the bacterium (Groeneveld et al. 1990a) and despite courses of appropriate oral antibiotics (Groeneveld et al. 1990b), which suggests that when established, colonization is difficult to eradicate. Furthermore, the bacterial species isolated in these studies represent the same spectrum of pathogens commonly isolated from sputum cultures of patients with acute exacerbation. The study done by Monso et al. is especially informative, as it included a control group of 29 patients with stable COPD (Monso et al. 1995). They demonstrated that exacerbation was associated twice as often with distal airway infection at $\geq 10^3$ CFU of pathogenic bacteria per ml and four times as often with $\geq 10^4$ CFU/ml. Furthermore, Sethi et al. have shown that acquisition of a new strain of bacteria is a major predisposing factor to an exacerbation (Sethi et al. 2002).

Studies using sputum to detect bacterial infection have generally found higher rates of bacterial isolation than the bronchoscopy studies with bacteria found in up to half of stable COPD patients. This is likely due to higher rates of contamination with upper respiratory organisms in sputum (Patel et al. 2002; Wilkinson et al. 2003; Hurst et al. 2005). Another problem of sputum culture-based techniques is that they underdiagnose bronchial colonisation at loads below the detection limit of the sputum culture, and potential pathogenic micro-organisms have been identified in one-tenth of culture-negative sputum samples (Murphy et al. 2004). The use of molecular methods like PCR amplification of the 16S rRNA gene followed by cloning and traditional Sanger sequencing in bronchial secretion samples has allowed the identification of bacterial species previously undetected by the selective cultures used for identification of potential pathogenic micro-organisms (Charlson et al. 2011; Erb-Downward et al. 2011; Hilty et al. 2010). These culture-independent microbiological techniques have demonstrated that the lungs are not sterile during healthy state and have suggested that the bronchial microbiome may be heterogeneous in COPD, with significant differences between bronchial sections (Erb-Downward et al. 2011; Armougom et al. 2009). The bronchial microbiome in COPD includes genera present in healthy subjects, with an additional increased presence of
various genera of *Proteobacteria* that are unusual in the normal population and well known potential pathogenic micro-organisms such as *Haemophilus* and *Moraxella* (Cabrera-Rubio et al. 2012). However, at present, sputum samples remain the most common lung samples taken because they are readily obtained with non-invasive techniques.

Risk factors for colonization include the degree of airways obstruction (Monso et al. 1999) and current smoking (Zalacain et al. 1999). The presence of bacteria in the lower airways of patients with COPD impairs the host defence mechanisms which sets up a vicious cycle of epithelial cell damage, impaired mucociliary clearance, mucus hypersecretion and inflammatory cell infiltration, thereby causing further impairment of host defences and bacterial adherence and growth (Murphy and Sethi 1992). A study by Adler et al. showed that seven of 28 (25%) strains of nontypeable *H. influenzae*, 10 of 26 (34%) strains of *S. pneumoniae*, and 12 of 18 (66%) strains of *P. aeruginosa* stimulated mucin secretion (Adler et al. 1986). Bacterial colonization also contribute directly to epithelial cell injury and airway inflammation (Soler et al. 1999; Bresser et al. 2000; Hill et al. 2000) and it has been shown that pathogen positive acute exacerbations have substantially increased measures of airway inflammation in expectorated sputum compared to pathogen-negative bacteria (Sethi et al. 2000). Bacterial colonization is also a risk factor for viral colonization. *Haemophilus influenzae* has been shown to increase the expression of intracellular adhesion molecule -1 and Toll-like receptor-3 and augments binding of rhinovirus to cultured human airway epithelial cells (Sajjan et al. 2006). Rising airway bacterial load and species change is associated with an accelerated decline in FEV₁ (Wilkinson et al. 2003). Figure 1.3 illustrates these phenomena. Patients with frequent exacerbations also have higher induced sputum levels of pro-inflammatory cytokines such as IL-6 and IL-8 in the stable state than patients with infrequent exacerbations (Bhowmik et al. 2000).

All this is in keeping with the finding that bacterial colonization in the stable state increases the frequency of COPD exacerbations (Patel et al. 2002) and Sethi et al. have shown that isolation of a bacterial pathogen by culture is associated with a significant increase in the incidence of exacerbations (Sethi et al. 2002). Patients with chronic bronchitis have higher rate of bacterial colonization (Hill et al. 1999) and generally experience more exacerbations (Burgel et al. 2009; Miravitlles et al. 2000). Exacerbation
frequency also relates to subsequent decline in lung function (Donaldson et al. 2002) and health status (Seemungal et al. 1998) suggesting that colonization may be important in disease progression, although it does not directly relate to decline in FEV₁ (Monso et al. 1999).

![Diagram of bacterial colonization and exacerbations in COPD]

**Figure 1.3: Vicious cycle of bacterial colonization and exacerbations in COPD:** Bacterial colonization causes airway inflammation and exacerbations. Exacerbations further increase airway inflammation as well as result in mucus hypersecretion, both of which cause airway obstruction. Mucus hypersecretion also creates an environment favourable for bacterial colonization.
1.4 Phenotyping of COPD

FEV\textsubscript{1} is often used as an indicator of disease in COPD as it predicts morbidity and mortality. However, there are several limitations associated with the sole use of FEV\textsubscript{1}. COPD is a multisystem disease with characteristics and physiological abnormalities that are not fully described by FEV\textsubscript{1}. The results of a recent large international study, the Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points (ECLIPSE) study, showed that within GOLD stages 2-4, symptoms, exercise tolerance, number of reported exacerbations and the prevalence of co-morbidities varied widely between patients, and that even in patients with severe airflow obstruction there were a substantial proportion of patients who did not complain of symptoms, report exacerbations or show impaired exercise tolerance (Agusti et al. 2010). FEV\textsubscript{1} also correlates weakly with clinical outcome measures e.g. dyspnoea.

The latest GOLD COPD strategy document addresses this limitation to some extent by grouping COPD patients after combining symptomatic assessment with spirometric classification and risk of exacerbations (Vestbo et al. 2012) (Table 1.3).

Table 1.3: ABCD criterion for classification of COPD

<table>
<thead>
<tr>
<th>Patient category</th>
<th>Character</th>
<th>Spirometric classification</th>
<th>Exacerbations per year</th>
<th>MMRC</th>
<th>CAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Low risk, fewer symptoms</td>
<td>GOLD 1-2</td>
<td>≤ 1</td>
<td>0-1</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>B</td>
<td>Low risk, more symptoms</td>
<td>GOLD 1-2</td>
<td>≤ 1</td>
<td>≥ 2</td>
<td>≥ 10</td>
</tr>
<tr>
<td>C</td>
<td>High risk, fewer symptoms</td>
<td>GOLD 3-4</td>
<td>≥ 2</td>
<td>0-1</td>
<td>&lt;10</td>
</tr>
<tr>
<td>D</td>
<td>High risk, more symptoms</td>
<td>GOLD 3-4</td>
<td>≥ 2</td>
<td>≥ 2</td>
<td>≥ 10</td>
</tr>
</tbody>
</table>

When assessing risk, the highest risk is chosen according to GOLD grade or exacerbation history. Abbreviation: MMRC – Modified Medical Research Council score, CAT – COPD Assessment Tool.

The term COPD phenotype has been defined as "a single or combination of disease attributes that describe differences between individuals with COPD as they relate to clinically meaningful outcomes (symptoms, exacerbations, response to therapy, rate of disease progression or death)” (Han et al. 2010). Such phenotypic characteristics other than FEV\textsubscript{1} may predict a) prognosis b) health status and c) therapeutic response. In their paper, Han et al. have discussed COPD phenotypes based on clinical and physiological.
manifestations, radiologic characterization, frequent exacerbations and presence of systemic inflammation and comorbidities as potential phenotypes of COPD with different clinically meaningful outcomes. Several other studies have supported this concept. For example Celli et al. showed that the body mass index (BMI), airflow obstruction, dyspnoea, and exercise capacity (BODE) index, is better than FEV₁ at predicting the risk of death in patients with COPD (Celli 2006). Biomarkers of the systemic manifestations of COPD including BMI and CRP have also been shown to predict mortality in COPD (Dahl et al. 2007).

Furthermore, age, past medical history, smoking history, sex, and ethnicity may all significantly impact disease presentation and progression. For example, lung function declines with increasing age (Fletcher and Peto 1977). Female sex appears to be associated with worse quality of life (QOL) and higher rates of depression and anxiety (Han et al. 2007). Women may also differ from men in the extent and distribution of airway abnormality and emphysema (Han et al. 2007). Similarly, depression and anxiety have been independently associated with an increased frequency of hospital admissions and COPD exacerbations (Jennings et al. 2009; Dahlen and Janson 2002).

There is thus a need to further phenotype and characterize the disease as not all COPD patients may benefit from every treatment. Also, currently there are no reliable, validated, and easily accessible biomarkers that reflect the inflammatory state of the airways (Cazzola and Novelli 2010; Barnes et al. 2006). The ECLIPSE study recently identified patients with frequent exacerbations and those with persistent systemic inflammation as distinct phenotypes of COPD, the latter associated with a poor clinical outcome (Agusti et al. 2012; Hurst et al. 2010). The identification of phenotyping biomarkers may greatly aid in the determination of disease progression and health outcomes (Agusti et al. 2010).
1.4.1 Chronic bronchitis – Why is this important?

Chronic bronchitis is a disorder characterized by hypersecretion of bronchial mucus and defined as cough productive of sputum on most days for a minimum of three months per year for at least two consecutive years, which cannot be attributed to other pulmonary or cardiac causes (MRC 1965). The disease is characterised with periods of stability, interspersed with episodes of worsening symptoms (exacerbations).

Chronic bronchitis is seen in 3.4 to 22.0% of adults (Harmsen et al. 2010; de Marco et al. 2007; Cerveri et al. 2001). This wide range of prevalence estimates may be due to varying definitions of chronic bronchitis (i.e., chronic phlegm versus chronic cough and phlegm) used in epidemiological studies. Chronic bronchitis is usually defined by “cough and phlegm most days for > 3 months in two consecutive years”. The study by Montes et al. shows that use of another definition based on “phlegm on most days for at least 3 months per year for ≥ 2 years” almost doubled the prevalence of chronic bronchitis (de Oca et al. 2012). Other definitions have defined chronic bronchitis by using a definition based on “emission of > 30 ml of sputum daily at least 3 months a year, for > 1 year” (Miravitlles et al. 2000; Prescott et al. 1995). However, regardless of the definition, the prevalence of chronic bronchitis consistently increased with increasing airflow limitation (Agusti et al. 2010; Burgel et al. 2009; Lu et al. 2010; Vestbo and Lange 2002). Other reasons of differences in prevalence could be due to differences in the populations sampled as well as differences in smoking exposure.

Chronic bronchitis is associated with increased morbidity and mortality. It is an independent risk factor for all cause mortality both in COPD (Stavem et al. 2006), and in subjects with normal lung function, even when smoking has been accounted for (Frostad et al. 2006; Stavem et al. 2006; Ebi-Kryston et al. 1989). The overall ten year mortality following a diagnosis of chronic bronchitis is 50%, with respiratory failure following an acute exacerbation being the most frequent terminal event (Turato et al. 1995).

The primary risk factor for CB is smoking. Smokers have a higher incidence of chronic bronchitis than non-smokers (Lange et al. 1989). There is a six-fold rise in prevalence from 6.3% in non-smokers to 40% in heavy smokers (Thurlbeck 1976a), with a linear
relationship between cigarette smoke exposure and chronic bronchitis (Tager and Speizer 1976). Other potential risk factors include inhalational exposures to biomass fuels, dusts, and chemical fumes (Trupin et al. 2003; Matheson et al. 2005). Another risk factor for chronic bronchitis is the presence of gastroesophageal reflux, possibly by pulmonary aspiration of refluxed gastric contents producing acid-induced injury and infection or neurally mediated reflex bronchoconstriction secondary to irritation of oesophageal mucosa (Barish et al. 1985).

The presence of chronic bronchitis is a marker of increased risk of developing COPD (Pelkonen et al. 2006). On average, 34% of COPD patients suffer from chronic cough and sputum expectoration (Kanner et al. 2001; Agusti et al. 2010). COPD patients with chronic bronchitis have worse clinical characteristics such as more severe airflow obstruction and breathlessness (de Oca et al. 2012; Corhay et al. 2013), an increased risk of exacerbations (Seemungal et al. 1998; Burgel et al. 2009; Miravitlles et al. 2000) and higher mortality rates (Lange et al. 1990; Pelkonen et al. 2006; Ekberg-Aronsson et al. 2005). However, the recent ECLIPSE study failed to reproduce these findings for breathlessness, airflow obstruction and exacerbations (Agusti et al. 2010). The reasons for differences between studies are unclear. A possible explanation is that chronic bronchitis encompasses a range of symptom severity; some COPD patients fulfill the definition of chronic bronchitis by producing phlegm for only three months per year. In contrast, other COPD patients have more severe symptoms and have a persistent cough for most or every day of the year. It is possible that the negative findings may be due to over-representation of patients with milder symptoms of chronic bronchitis.

Chronic mucus hypersecretion is also associated with an excessive decline in FEV\textsubscript{1} (Vestbo et al. 1996; Nishimura et al. 2012), although the latter is not a uniform finding (Vestbo et al. 2011). In the latter study by Vestbo et al., patients with chronic bronchitis did not have a more rapid rate of decline in FEV\textsubscript{1}, but they did have a lower mean FEV\textsubscript{1} (43 ± 20 ml per year) at baseline than patients without chronic bronchitis. Again, the reason for this may be the inclusion of patients with varying spectrum and duration of symptoms of chronic bronchitis in the different studies. Another theory that has been postulated is that in the latter study by Vestbo et al., patients were included from specialist
sites and in some of these, their COPD may have “burnt out” or at least stabilized during the three years of observation.

Subjects with chronic bronchitis and COPD have been shown to be phenotypically different from subjects with COPD without chronic bronchitis. In general, they are younger, more commonly men, more likely to be current smokers with a greater pack year smoking history, more symptomatic and more frequently have co-morbidities (Kim et al. 2011). Lower socioeconomic status and occupational exposure have also been associated with the presence of chronic bronchitis (Ferre et al. 2011).

Pathological studies have suggested that symptoms of chronic cough and sputum production are mostly associated with changes in the large (proximal) airways (Mullen et al. 1985; Saetta et al. 1997), where mucus-producing cells; both epithelial goblet cells and mucus glands are increased (Innes et al. 2006; O’Donnell et al. 2004b). Mucus accumulation is also identified in the epithelium and lumen of small conducting airway, and the extent of mucus exudates within the airway lumen was closely associated with the severity of airflow limitation in a study on a large group of patients with COPD (Hogg et al. 2004) and a subsequent study by the same group showed that sputum plugging in the small airways is a predictor of mortality in COPD (Hogg et al. 2007). However, the majority of pathological studies on mucus production are limited to large airways, whereas small airways are not easily assessed as they require lung resection specimens. Progress in imaging techniques in the future may allow non-invasive assessment of mucus exudates obstructing small airways (Burgel and Martin 2010).

It is important to characterize and quantify inflammatory changes in the assessment of subjects with chronic bronchitis as a number of studies have suggested a pathogenic role for airway inflammation in the induction of chronic sputum production (Vestbo and Rennard 2010). An improved understanding of how inflammation and mucus relate to pulmonary function is also needed.

Many COPD patients experience a productive cough during particular times of the year, such as the winter months, or during infective exacerbations; some of these patients fall into the category of ‘chronic bronchitis’ due to a productive cough for a proportion of the year. In contrast, some COPD patients have a productive cough for most or every day of
the year. The term ‘chronic bronchitis’ does not distinguish between patients with persistent sputum production, and those with more variable sputum production. Persistent sputum producers are therefore a subgroup of chronic bronchitis patients, being at the more severe end of the spectrum of symptoms.

1.4.2 Frequent exacerbator

There are large differences in yearly exacerbation rates between patients of similar COPD severity giving rise to the concept of two distinct phenotypes; frequent and infrequent exacerbators. Some studies have used the median of recorded exacerbations per year as a cut-off point to define the concept of “frequent COPD exacerbator” (Seemungal et al. 1998; Spencer et al. 2004; Hurst et al. 2009). For example, Seemungal et al. observed more than 2.96 episodes, Spencer et al. more than 1.65, and more recently Hurst et al. found 1.97 events yearly. Differences in these thresholds are likely related to differences in the characteristics of the populations investigated and also to the different definitions used for COPD exacerbation. While some studies have used an event-driven definition (Spencer et al. 2004), others used a symptom-based definition (Hurst et al. 2009; Seemungal et al. 1998). The latter detects a higher number of exacerbations because it also takes into account unreported events.

Exacerbations become more frequent and severe as the severity of COPD increases. This was demonstrated in the ECLIPSE study: exacerbation rates in the first year of follow up were 0.85 per person in stage II COPD, 1.34 in stage III and 2.00 in stage IV. Overall, 22% patients with GOLD stage II, 33% with GOLD stage III and 47% with GOLD stage IV were found to be frequent exacerbators, defined as 2 or more exacerbations per year. The most reliable predictor of exacerbations in an individual patient across all GOLD stages was a history of exacerbations. In addition to its association with more severe disease and prior exacerbations, the frequent exacerbator phenotype was independently associated with a history of gastroesophageal reflux or heartburn, poorer quality of life, and elevated white-cell count (Hurst et al. 2010). However, of note is that ECLIPSE did not include patients with GOLD stage I disease, as it mainly recruited from secondary and tertiary centres, therefore the study is likely not representative of all COPD patients.
1.4.3 Rapid decliner

Rapid decline in FEV\textsubscript{1} is not only a predictor of morbidity, mortality and hospital rates (Wise 2006) but has also been linked to distinct plasma biomarkers (Devanarayan et al. 2010). The rate of change in FEV\textsubscript{1} among patients with COPD is highly variable, with increased rates of decline among current smokers, patients with bronchodilator reversibility, and patients with emphysema (Vestbo et al. 2011). Research from the Lung Health Study found that patients who quit smoking have a significant reduction in lung function decline compared to those who continue to smoke. In this study, patients who remained smoke-free had a rate of decline in FEV\textsubscript{1} of 31 mls/year; half that of the continuing smokers group (62 mls/year). At 11 years, 38% of those who continued to smoke had an FEV\textsubscript{1} < 60% of the predicted normal value compared to 10% of the sustained quitters (Anthonisen et al. 1994). Other factors that have been associated with rapid FEV\textsubscript{1} decline are frequent lower respiratory tract illnesses and a genetic predisposition towards it (Kanner et al. 2001; Molfino 2007). Recently Nishimura et al. defined rapid decliners as those with an FEV\textsubscript{1} loss of ≥ 63 mls/year. The study showed that the severity of emphysema and a low BMI were independently associated with a rapid decline in FEV\textsubscript{1} in COPD (Nishimura et al. 2012). An increased rate of decline of FEV\textsubscript{1} is associated with mortality in COPD (Mannino et al. 2006).

Rapid physiologic progression as indicated by change in FEV\textsubscript{1} may therefore indicate a distinct phenotype of COPD (Han et al. 2010). These observations on rapid decliners indicate the need for specific or more aggressive approaches to the management of this phenotype of COPD.

1.4.4 Systemic inflammation

A systemic inflammatory response is characterized by mobilization and activation of inflammatory cells into the circulation, as well as the production of acute phase proteins and circulating pro-inflammatory mediators (Sinden and Stockley 2010). Its presence in COPD is considered to be a key mechanism responsible for the increased rate of co-morbidities, including cardiovascular complications (Sin and Man 2003; Thomsen et al. 2012).
The presence of systemic inflammation may represent a unique COPD phenotype. Elevated biomarkers including CRP, serum amyloid A and proinflammatory cytokines IL-6, IL-8, TNF-α appear to be present only in a subgroup of COPD patients. Evidence of systemic inflammation can also be detected in patients who appear to be otherwise clinically stable (Agusti 2008) but with evidence of further increase during exacerbations (Hurst et al. 2006). COPD patients with persistent systemic inflammation have been shown to have an increased all-cause mortality and exacerbation frequency per year compared to non-inflamed patients (Agusti et al. 2012). In the study by Agusti et al, six inflammatory biomarkers in peripheral blood (white blood cells (WBC) count and CRP, IL-6, IL-8, fibrinogen and TNF-α levels) were quantified in a large cohort of COPD patients and control groups and followed up for three years. It was found that at baseline 30% of COPD patients did not show evidence of systemic inflammation whereas 16% had persistent systemic inflammation. Even though pulmonary abnormalities were similar in these two groups, persistently inflamed patients during follow-up had significantly increased all-cause mortality (13% vs. 2%, p < 0.001) and exacerbation frequency [1.5 (1.5) vs. 0.9 (1.1)] per year, p < 0.001) compared to non-inflamed ones.
1.5 Pathophysiology of chronic bronchitis

Mucus overproduction in response to inflammatory signals is the pathologic foundation for chronic bronchitis. In chronic bronchitis there is inflammation in the epithelium of the central airways and in the submucosal glands (Hogg 2004). The inflammatory cells release serine proteases that are potent secretagogues for mucus (Sommerhoff et al. 1990). Oxidants derived from cigarette smoke and released from inflammatory leukocytes may also be involved in the overproduction of mucin (Shao et al. 2004).

There is also submucosal gland hypertrophy and hyperplasia and goblet cell metaplasia in the bronchial epithelium resulting in the production of increased mucus which is more acidic due to post-translational sulphation of airway mucins (Degroote et al. 2003). Goblet cells are prolific in the central airways becoming sporadic in distal lung. However, goblet cell hyperplasia seen in chronic bronchitis may extend into more peripheral airways compared to goblet cells observed in normal tissue (Saetta et al. 2000). Therefore, the smaller (< 400 micron in diameter) airways, which normally hold only a sparse population of mucus cells, become important contributors to excess mucus in this disease. The submucosal glands in chronic bronchitis are characterized by a higher mucus/serous cell ratio resulting in a more gel like, viscous mucus lacking in lysozymes and antiproteases, which normally provide protection against both infection and proteolytic injury.

There is impairment of mucociliary clearance due to inhibition of ciliary activity by proteinases such as neutrophil elastase released from neutrophils recruited to the lungs (Smallman et al. 1984), the presence of bacterial products (Wilson et al. 1987) and epithelial damage. The increased mucus production along with impaired mucociliary clearance results in partial or complete occlusion of small (< 2mm) airways with mucus plugs and the replacement of the surfactant lining the small airways with mucus results in increased surface tension at the air-liquid interface, further predisposing to airway collapse. This results in airflow limitation, mucus accumulation, increased bacterial colonization and a tendency towards increased respiratory tract infections (Thurlbeck 1976b). These infections lead to further inflammatory cell recruitment and this results in a vicious cycle that damages the lungs (Figure 1.4).
When mucociliary clearance fails, cough becomes the secondary mechanism of clearance of the excess and accumulated secretions which are expectorated in the form of sputum, which is a mixture of bronchial secretions, cells, cellular debris, cleared organisms and saliva. Thus a productive cough is the key presenting symptom of chronic bronchitis.

**Figure 1.4: Mechanism of lung damage in chronic bronchitis:** Structural changes in the lung along with mucus accumulation allows organisms to colonize the lungs resulting in subclinical infection. The low level infection leads to inflammatory cell recruitment that further damages the lungs.
1.5.1 Airway inflammation in chronic bronchitis

Previous studies have used different compartments of the lung to assess airway inflammation in chronic bronchitis and a different inflammatory profile is obtained depending on the compartment examined; i.e., the lumen of the central airways using sputum analysis, the airway wall using bronchial biopsies, and the peripheral airways using bronchoalveolar lavage (BAL).

Central airway inflammation

COPD patients with chronic bronchitis have a higher percentage of sputum eosinophils (and lower eosinophil counts in biopsies) than other COPD sufferers (Snoeck-Stroband et al. 2008). Increased numbers of eosinophils in sputum that have migrated through the epithelial layer may contribute to mucus hypersecretion through the action of transforming growth factor (TGF)-α (Burgel et al. 2001) or by stimulating degranulation of mucus-producing cells through the release of inflammatory mediators, including cysteinyl leukotrienes (Shimizu et al. 2003a). This is further supported by other studies showing increased sputum eosinophils during COPD exacerbations (Saetta et al. 1994). Furthermore, it has been shown that the expression of T-helper 2 cytokines, IL-4 and IL-13 is higher in patients with chronic bronchitis (Miotto et al. 2003b), and these cytokines are involved in the regulation of both eosinophil influx (Bettiol et al. 2002) and mucin production (Atherton et al. 2003).

The study by Snoeck-Stroband et al. also showed that chronic bronchitis patients had a higher percentage of macrophages and lower percentage of neutrophils in sputum (Snoeck-Stroband et al. 2008). However, this difference was not significant after adjustment for smoking and sex. No difference in any other sputum parameters was found between the two groups. Compared to BAL, the percentage of neutrophils in sputum has been found to be significantly higher in sputum in patients with chronic bronchitis (Maestrelli et al. 1996). Other sputum cytokines elevated in patients with chronic bronchitis are listed in Table 1.1.
Inflammation in airway wall

Studies on the epithelial and submucosal compartments have provided evidence of an inflammatory process, consisting predominantly of mononuclear cells in the large airways of patients with chronic bronchitis (Ollerenshaw and Woolcock 1992a; Saetta et al. 1993). An increased number of neutrophils, macrophages and CD8+ cells are also noted in the bronchial glands of patients with chronic bronchitis, as compared with asymptomatic COPD patients (Saetta et al. 1997). Conversely, there is a decrease in the number of eosinophils in the airway wall, especially around the glands which may contribute to mucus hypersecretion. Eosinophils are a major cellular source of transforming growth factor (TGF)-β (Zagai et al. 2007) and Baraldo et al. showed that impaired TGF-β signalling is associated with bronchial gland enlargement. Therefore, a lower number of eosinophils around the bronchial glands may lead to bronchial gland enlargement and a rise in mucus in the airway lumen, due to decreased local TGF-β availability (Baraldo et al. 2005).

Peripheral airway inflammation

The bronchial lavage fluid from patients with chronic bronchitis tends to contain more cells and a higher percentage of neutrophils compared with asymptomatic smokers and non-smokers. Furthermore, those with higher bronchial sample neutrophils have been shown to have significantly more sputum production and airflow obstruction compared to patients with lower bronchial sample neutrophils (Thompson et al. 1989b). This observation is likely because neutrophils cause mucus hypersecretion (Kohri et al. 2002; Takeyama et al. 2000) and mucus hypersecretion and plugging is an important cause of airflow obstruction in these patients.
1.5.2 Airway mucins

Mucus is a complex aqueous solution consisting of a variety of lipids, proteins and electrolytes which protects the underlying epithelium from dehydration, pathogens, and chemical and particulate irritants. The production of airway mucus is an essential homeostatic function of the lung epithelium that, under normal circumstances, provides protection against inhaled irritants including noxious gases, particulates and bacteria. Airway mucus exists as a lower sol (aqueous) phase and an upper gel phase, with the sol phase bathing the base of the ciliated cells and the cilia, and the gel phase being transported along the mucociliary escalator to the throat where mucus is either coughed out or swallowed and delivered to the gastrointestinal tract for degradation (Knowles and Boucher 2002). Thus, any inhaled particles trapped in the sticky mucus layer are removed from the airways by mucociliary clearance. Recently, Button et al. have proposed a gel-on-brush model in which the periciliary layer is occupied by membranous mucins and mucopolysaccharides densely tethered to the airway surface. This brush prevents mucus penetration into the periciliary space and causes mucus to form a distinct layer (Button et al. 2012).

The viscous consistency of mucus is a crucial property required for its function and is provided by the presence of a particular group of glycoproteins called mucins. Mucin proteins are large glycosylated proteins assembled from disulphide linkage of monomers and, in normal individuals, make up approximately 2% of airway mucus. These macromolecules are complex glycoconjugates, consisting of a protein backbone (apomucin), encoded by MUC genes, to which hundreds of carbohydrate chains are added by post-translational N- and O-linked glycosylation (Perez-Vilar and Hill 1999). The protein backbone is made up of a variable numbers of tandemly-repeated serine- and/or threonine rich regions (mucin domains), interspersed with regions rich in cysteine residues (termed cys domains). The N- and C- terminals contain von Willebrand factor (vWF)-like domains (D domain, B domain, C domain and CK domain). The sequence and size of the amino acids and the N- and C- terminal domains is unique for each different mucin species (Thornton et al. 2008)(Figure 1.5)
Figure 1.5: Generic representation of a polymeric mucin polypeptide. The N- and C-terminal von Willebrand factor (Vwf)-like domains (D domains, the B domain, the C domain, and the CK domain) and the central region containing two mucin domains (MD) and one cys domain are shown. Dense oligosaccharide side chains are attached to the mucin domains. The mucin peptides are linked together by disulphide bonds. Adapted and modified from ‘Structure and Function of the polymeric mucins in airways mucus’ (Thornton et al. 2008).

More than 20 mucin genes have been identified, and 12 of these have been shown to be expressed in the respiratory epithelium (Curran and Cohn 2010). The airway mucins exist as two structurally different classes; monomeric, membrane-bound mucins (example MUC1 and MUC4) and oligomeric, secreted mucins (MUC5AC, MUC5B and MUC2). Of the oligomeric, secreted mucins, MUC2 is only a very minor component and expressed at low levels (Thornton et al. 2008). Membrane-bound mucins participate in events such as cellular adhesion, pathogen-binding and signal transduction, though they may also play a role in mucociliary clearance as recently proposed by Button et al. (Button et al. 2012).

In contrast to membrane-bound mucins, secreted mucins contribute heavily to the viscoelastic properties of extracellular mucus layers. Most secreted mucins are much larger than membrane-bound mucins (> 1000 kD) and contain cysteine-rich domains located at both the amino and carboxyl termini (Sheehan et al. 2004). The predominant gel-forming mucins secreted into airway mucus are MUC5B (which is itself found in a number of glycoforms), MUC5AC and, to a lesser extent, MUC2 (Thornton et al. 1997; Hovenberg et al. 1996b). However, new gel-forming secretory mucins such as MUC19 have recently been identified and our understanding of airway mucin secretions may alter in the future (Chen et al. 2004).
The main source of MUC5AC are goblet cells in the surface epithelium (Hovenberg et al. 1996a) and the main source of MUC5B are mucus cells in the submucosal glands (Finkbeiner 1999). It has been shown that MUC5AC production can also occur in glandular mucus cells, while some MUC5B and MUC2 synthesis can be detected in surface goblet cells (Groneberg et al. 2002). Whether these mucins can be co-expressed in the same cell or arise from different cells has not yet been determined. Activated transcription factors such as HIF-1α upregulate expression of MUC genes in the nucleus of these cells (Zhou et al. 2012). New MUC transcripts are translated to MUC proteins on ribosomes and inserted into the endoplasmic reticulum. Glycosylation of the MUC protein backbone is initiated post-translationally in the Golgi apparatus. Mature (fully glycosylated) mucins are packaged and stored in secretory granules until a mucin secretagogue triggers mucin secretion at the apical surface of the cell (Rose and Voynow 2006) (Fig 1.6). The production and secretion of mucins from both goblet cells and submucosal glands is a heavily regulated process influenced by many chemical mediators and neuronal activity as described below (Rogers 2001).
Figure 1.6: Overview of mucin biosynthesis in epithelial goblet cells. The major steps in polymeric mucin secretion are upregulation of mucin gene expression in the cell nucleus resulting in production of mucin proteins, their insertion in the endoplasmic reticulum, post-translational glycosylation in the golgi apparatus, storage inside secretory granules, secretion and formation of a mucus gel.
1.5.3 Cell signalling pathways involved in mucin expression

Mucin production can be stimulated by oxidants in cigarette smoke, proteases such as neutrophil elastase, cytokines like TNF-α, IL-1β and platelet activating factor (PAF), bacterial products like lipopolysaccharide (LPS) and cytoplasmic proteins, growth factors like epidermal growth factor (EGF) and TGF-α, viruses such as respiratory syncytial virus and rhinovirus and certain chemical agents for example, Phorbol (Thai et al. 2008).

Several cell signalling pathways have been demonstrated to be involved in increased mucin gene expression, most frequently ERK-MAP kinase pathway, but also the cytokine-JAK-STAT and TGF-β-SMAD pathways. Cytokines bind to specific receptors to activate associated tyrosine kinases that initiate downstream signalling. The most common effect of cytokines is activation of the JAK/STAT pathway, in which the tyrosine kinase JAK phosphorylates the transcription factor STAT to induce its dimerization. The STAT dimer then migrates to the nucleus to regulate gene transcription (Theodoropoulos and Carraway 2007).

A number of cytokines have been shown to induce mucin gene expression. TNF-α and IL-1β induce MUC2 and MUC5AC expression in the lung via the ERK or p38 MAPK pathway (Song et al. 2003). TNF-α may also induce MUC gene expression through nuclear factor NF-κB and Sp1 (Perrais et al. 2002). MCP-1 through its receptor CCR2 induces chemotaxis and activates MAPK, a kinase known to play a key role in mucin regulation in the bronchial epithelium (Monzon et al. 2011). IL-6 has also been shown to induce MUC5B gene expression via ERK, and IL-17 stimulates mucin mRNA expression partly through IL-6, and possibly through JAK 2 (Chen et al. 2003). IL-4, IL-13 and IL-9 bind to their specific receptors, which, in turn, heteromerize with gamma c chains or glycoprotein 130 molecules to activate intracellular signalling, via the JAK/STAT pathway (Murata et al. 1999; Mueller et al. 2002). Other cytokines such as IL-5 and IL-10 have also been shown to increase mucus expression (Lee et al. 2002; Lee et al. 1997).

Goblet cell hyperplasia has also been linked to several intracellular signalling pathways. The most well known of these is a Th2 lymphocyte-mediated mechanism involving IL-13 and STAT6 (Tanabe et al. 2011). Other studies have indicated EGFR tyrosine kinases in the induction of goblet cell hyperplasia via a MAPK/ERK extracellular signal-regulated
kinase cascade. Activation of PI3K and AKT may also be involved in goblet cell hyperplasia. More recently, hypoxia induced signalling has been shown to influence the development of goblet cell hyperplasia and HIF-1α nuclear expression has been detected in airway epithelial cells in areas of goblet cell hyperplasia and upregulation of MUC5AC expression in COPD (Polosukhin et al. 2011).

1.5.4 Mucin expression in COPD

A series of studies have shown that the gel-forming mucins – MUC5AC and MUC5B can be changed in amount, type, and size in airways disease (Thornton et al. 1990; Thornton et al. 1997; Thornton et al. 1991). Another mucin, MUC2 has also been demonstrated to be a large gel-forming type however significant amounts of this mucin have not been found in respiratory tract secretions (Thornton et al. 1996).

The overproduction of MUC5AC and MUC5B in the airways of patients with chronic airway diseases, greatly contributes to airway obstruction, morbidity and mortality in these patients. The sustained mucin secretion requires increased biosynthesis of mucins, and the upregulation of MUC genes and goblet cell hyperplasia are fundamental processes that contribute to mucin overproduction in chronic airway diseases. A key observation from work done by Kirkham et al. is that compared with secretions from normal subjects and individuals with asthma, there is more MUC5B in cystic fibrosis and COPD sputum, and there is a significant increase in the amount of the low charge form of the MUC5B mucin in the diseased, possibly infected sputa (Kirkham et al. 2002). This might suggest a link between infection/inflammation and MUC5B production.

Studies on central airways have shown that MUC5AC expression is increased in the bronchial surface epithelium in COPD and smokers with normal lung function compared with control non-smokers (Innes et al. 2006; Caramori et al. 2009; O'Donnell et al. 2004b). Caramori et al. also found increase in MUC5AC expression in the submucosal glands of COPD patients compared with smokers with normal lung function and non-smokers. The expression of MUC5B in both bronchial epithelium and submucosal glands was not significantly different between groups in this study (Caramori et al. 2009). This is in contrast to the study by Innes et al. which found decreased expression of MUC5B in bronchial epithelium of smokers compared with non-smoking subjects (Innes et al. 2006).
This may be due to the absence of an age-matched control group in the study performed by Innes and/or to difference in the immunohistochemistry methodology used in the studies. Interestingly, increased MUC5B expression in the bronchial surface epithelium has been reported in other inflammatory lung diseases such as diffuse panbronchiolitis which is also associated with mucus hypersecretion (Chen et al. 2001; Kamio et al. 2005).

A study by Caramori et al. on peripheral airways has shown an increase in MUC5AC expression in COPD patients compared with smokers and non-smokers with normal lung function (Caramori et al. 2004). MUC5AC expression was higher in current smokers compared with ex-smokers and control non-smokers, indicating that active smoking might be a stimulus of increased expression of MUC5AC. Conversely, the presence of chronic bronchitis had no influence on mucin expression in peripheral airways. This is also replicated in the study by Laperre on central airways which showed no difference in the total mucin content of the surface epithelium of COPD patients with and without chronic bronchitis (Lapperre et al. 2007a). Table 1.4 provides a summary of these studies on mucin expression in COPD. One reason for the lack of difference of mucin expression in patients with and without chronic bronchitis may be because these studies have focused on mucin expression and not secretion. Secondly, most of these studies did not examine mucin expression from the submucosal glands, which are the main site of MUC5B production. Finally, chronic bronchitis encompasses a broad spectrum of patients with persistent sputum production on one end of the spectrum to those who produce phlegm only during an exacerbation, at the other end, thus using a mixed population of patients may mask potential differences in mucin expression between groups.

As previously mentioned, studies on sputum have shown that lower-charged glycoform of MUC5B is the major mucin in the gel phase of sputum in COPD (Kirkham et al. 2008; Kirkham et al. 2002). This change in mucin type might have an important role to play in the properties of the mucus gel in COPD airways and secretions. Increasing amounts of MUC5B in sputum correlate with the degree of airflow obstruction and FEV₁. This data is at odds with the mucin gene expression studies discussed previously which show a predominant increase in MUC5AC in COPD airways but again most of these studies did not analyze mucin production by submucosal glands which have been shown to undergo hypertrophy in COPD. Clinicopathological studies performed in COPD patients have
found a correlation between the presence of chronic bronchitis and submucosal gland hypertrophy in the central airways of smokers (Jamal et al. 1984; Barnes 2000). However, in comparison to control group of healthy non-smokers, the study by Kirkham et al. did find an increase in MUC5AC in sputum in COPD and smokers with normal lung function (Kirkham et al. 2008).
Table 1.4: (a) Summary of studies on mucin expression in large airways in COPD

<table>
<thead>
<tr>
<th>Author</th>
<th>Subjects</th>
<th>Sample of airway studied</th>
<th>Method of quantifying MUC5AC and MUC5B</th>
<th>Mucin expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Innes (Innes et al. 2006)</td>
<td>15 COPD 9 S 19 HNS</td>
<td>Bronchial biopsies and epithelial brushings</td>
<td>Volume of epithelial mucin /surface area of basal lamina &amp; /volume of epithelium</td>
<td>Goblet cell size and number higher in S (with/without COPD) compared to HNS (p = 0.001). MUC5AC expression in surface epithelium higher in COPD compared to S (p = 0.029) and HNS (p&lt;0.001). MUC5B expression in surface epithelium lower in S than HNS (p &lt; 0.0001).</td>
</tr>
<tr>
<td>Ma (Ma et al. 2005)</td>
<td>18 COPD 20 S</td>
<td>Lung resections from solitary peripheral carcinoma</td>
<td>Percentage of total epithelial area</td>
<td>Increased goblet cell hyperplasia in COPD compared to S (p &lt; 0.05). Increased MUC5AC expression in COPD (0.27% ± 0.09%) compared to S (0.20 ± 0.10%; p &lt; 0.05).</td>
</tr>
<tr>
<td>Cararmori (Caramori et al. 2009)</td>
<td>20 COPD 10 S 10 HNS</td>
<td>Bronchial rings from lobar or segmental bronchus of lung resection specimens.</td>
<td>Percentage positive cells</td>
<td>Area occupied by MUC5AC+ cells in surface epithelium higher in S (with/without COPD); 73.5% (25-92%) compared to HNS 15% (2.7-32%), p&lt;0.01. Area occupied by MUC5AC+ cells in submucosal glands higher in COPD [20% (5.5-31.7%)] compared with S [9.5% (2.5-17.5%); p&lt;0.05] and HNS [2% (0.4-6.2%), p&lt;0.01]. Gland size, PAS, Alcian blue staining and MUC5B expression not significant among groups.</td>
</tr>
<tr>
<td>O'donnell (O'Donnell et al. 2004b)</td>
<td>40 COPD 11 S 10 HNS</td>
<td>Bronchial biopsy specimens</td>
<td>Percentage of total epithelial area</td>
<td>Goblet cells numbers (PAS staining) higher in S compared to HNS (p &lt; 0.01). Increase in goblet cell staining for MUC5AC in S compared to HNS (p &lt; 0.001). No difference in PAS staining or MUC5AC expression between COPD and S. No difference in MUC5B expression between groups. No difference in mucin staining in S due to presence of chronic bronchitis.</td>
</tr>
<tr>
<td>Kirkham (Kirkham et al. 2008)</td>
<td>15 COPD 17 S</td>
<td>Lung resection surgery specimens</td>
<td>Percentage of total epithelial area</td>
<td>MUC5AC expression seen mainly in epithelial cells. MUC5B expression mainly in submucosal glands, also in some epithelial cells in COPD.</td>
</tr>
<tr>
<td>Lapperre (Lapperre et al. 2007a)</td>
<td>114 COPD</td>
<td>Bronchial biopsy specimens</td>
<td>Goblet cell (PAS/Alcian blue+) area (%)</td>
<td>Trend towards higher goblet cell area in current smokers compared to ex- smokers (p = 0.081). No relation between chronic bronchitis and PAS+ area.</td>
</tr>
</tbody>
</table>
Table 1.4(b): Mucin expression in small airways in COPD

<table>
<thead>
<tr>
<th>Author</th>
<th>Subjects</th>
<th>Sample of airway studied</th>
<th>Method of quantifying MUC5AC and MUC5B</th>
<th>Mucin expression</th>
</tr>
</thead>
</table>
| Caramori (Caramori et al. 2004) | 9 COPD 11 S 6 HNS | Peripheral lung sections | Semi-quantitative assessment using localization & extension of bronchiolar epithelial cell staining. Also presence or absence of luminal mucus. | MUC5AC expression higher in bronchiolar epithelium in COPD compared to S and HNS (p < 0.05).  
MUC5B expression in luminal mucus higher in COPD compared to S and HNS (p < 0.05).  
Intraluminal PAS higher in COPD compared to S and HNS (p < 0.05).  
No difference in PAS & Alcian blue epithelial staining between groups.  
No difference due to presence of chronic bronchitis. |
1.5.5 Current therapeutic treatments for airway mucus hypersecretion

There is an unmet need in the market for treatments aimed at relieving mucus hypersecretion in COPD.

Mucoactive drugs (for example N-acetylcysteine and carbocysteine) have shown efficacy in reducing exacerbations in COPD patients, but there is no evidence that the effects of these drugs is mediated through effect on mucus hypersecretion (Decramer and Janssens 2010; Poole and Black 2006). Glucocorticoids can inhibit mucin secretion indirectly by suppression of inflammatory mediators such as leukotrienes and cytokines and directly by repressing MUC gene expression (Chen et al. 2006; Kai et al. 1996). However, the mucus hypersecretory phenotype in COPD patients is poorly treated by the use of glucocorticoids due to the resistance COPD shows towards the anti-inflammatory effects of glucocorticoids (Plumb et al. 2012; Barnes et al. 2004; Armstrong et al. 2009; Kent et al. 2010). Inhaled and oral steroids may also have no or limited effect on mucus production in COPD (Hogg et al. 2007; Burgel et al. 2004).

The inefficacy of long acting bronchodilators is shown in patients who continue to have mucus obstruction in small airways despite treatment with these drugs (Hogg et al. 2007). Cholinergic activation of airway mucus output is via muscarinic M3 receptors on the secretory cells and anticholinergics with selectivity for M3 receptors may have some therapeutic effects on mucus hypersecretion (Eglen et al. 2001).

Other drugs such as cysteinyl leukotriene receptor antagonists have been shown to inhibit mucus output in sensitized guinea pigs (Liu et al. 1998). However, the large number of mediators involved in the pathophysiology of COPD suggests that no single receptor antagonist will significantly effect mucus hypersecretion. Erythromycin is a macrolide antibiotic that inhibits mucus secretion in experimental preparations. Erythromycin reduces excessive mucus secretion in children (Suez and Szefler 1986) and the elderly (Marom and Goswami 1991) but its mechanism of action is unclear (Wales and Woodhead 1999). Other drugs such as proteinase inhibitors, cytokine antagonists and EGFR tyrosine kinase inhibitors are all in experimental stages and none has so far been shown to definitely reduce mucin secretion (Hansel 2012).
Therefore drugs that limit mucus hypersecretion in COPD without affecting normal secretion and mucociliary clearance are needed. This requires identification of the predominant airway mucins in COPD patients with persistent sputum production.
1.6 Role of Hypoxia inducible factor (HIF-1α) in COPD

Airflow obstruction can lead to tissue hypoxia in COPD (Hogg 2012). Krick et al. showed that COPD patients who showed signs of alveolar type II (ATII) epithelial cell hyperplasia are more likely to have regions which are hypoxic (Krick et al. 2005). HIF-1α is a transcription factor that accumulates rapidly when hypoxia occurs (Yu et al. 1998). The HIF-1α protein binds to hypoxia response elements and enhances the transcription of several genes encoding vascular endothelial growth factor, inducible nitric oxide synthetase, erythropoietin and glycolytic enzymes, which increase oxygen availability and allow adaption to reduced oxygen conditions. Activation of HIF-1 is a protective mechanism in the maintenance of airway epithelial barrier integrity under conditions of oxidative stress during inflammation (Olson et al. 2011). There is also growing evidence that HIF-1α is involved in the inflammatory process by regulating angiogenesis and functions of inflammatory cells (Zhou and Brune 2006; Dery et al. 2005a).

HIF is a heterodimer made up of an α subunit, with three alternatives α1, α2 or α3 and a β subunit. The α subunit is stable under hypoxic conditions and binds to β subunit, this heterodimer recognizes hypoxia response elements in the promoter regions of hypoxia responsive genes. When conditions are normoxic the α subunit interacts with Von Hippel-Lindau (VHL) protein and is ubiquinated and rapidly degraded (Groulx and Lee 2002). For VHL to recognise HIF two proline residues, 402 and 564, must be hydroxylated. Hydroxylation is carried out by prolyl hydroxylases. In hypoxic conditions HIF is not hydroxylated and rapidly accumulates. This leads to heterodimer formation and the production of hypoxia gene products (Figure 1.7). The activity of HIF can therefore be increased through stabilization by either true hypoxic conditions (Maxwell et al. 1997), by inhibition of prolyl hydroxylase enzymes (Asikainen et al. 2005) or by inactivation of the VHL tumor suppressor that targets the HIF-1α subunit for oxygen dependent proteolysis (Maxwell et al. 1999).
Lung physiology and airway inflammation in COPD patients with persistent sputum production

**Figure 1.7: Regulation of HIF-1α by oxygen concentration:** Under normoxic conditions, HIF-1α is hydroxylated on proline residues by prolyl hydroxylase enzymes (PHD), leading to binding of Von Hippel Lindau protein (VHL), ubiquitination and degradation of the protein. Under hypoxic conditions, HIF-1α binds to HIF-1β to form a heterodimer that attaches to Hypoxia Response Element (HRE) on target genes resulting in their activation.
In addition to hypoxia, HIF-1α can also be stabilized and activated in response to growth factors and pro-inflammatory cytokines, such as EGF, TNF-α and IL-1β (Jiang et al. 2010; Dery et al. 2005b), vascular hormones (Richard et al. 2000) and viral proteins (Wakisaka et al. 2004). Varying amounts of HIF-1α have been detected even under normoxic conditions (Stroka et al. 2001; Zhong et al. 1998). Therefore during inflammation, low oxygen alone may not fully explain the activation of HIF-1α and the combination of hypoxia with cytokines may be important. Whereas tissue hypoxia is associated with protein stabilization and increase in half-life, normoxic activation of HIF-1α occurs via increased protein synthesis or its modification. A number of studies have found that the predominant mechanism of induction of HIF-1α in normoxic conditions is an increase in HIF-1α protein translation via the PI3K pathway and MAPK pathways (Maynard and Ohh 2007).

Another potential hypoxia marker is carbonic anhydrase IX (CAIX). Carbonic anhydrase 9 is a HIF-1 target gene whose product CAIX, a transmembrane protein, normalizes intracellular pH under hypoxic conditions, this is achieved by catalysing the reversible hydration of carbon dioxide to carbonic acid. CAIX correlates well with lowered O₂ in vivo (Loncaster et al. 2001) and in vitro (Wykoff et al. 2000) and is expressed even under mild hypoxic conditions (Kaluz et al. 2002). The CAIX protein is highly stable (Rafajova et al. 2004b) and is under a tight transcriptional control via the hypoxia response element in the CA 9 promoter region (Kaluz et al. 2002). These factors make CAIX one of the best intrinsic markers of cellular hypoxia (Loncaster et al. 2001; Olive et al. 2001). CAIX can be used as a tool for monitoring HIF-1α activity (Wykoff et al. 2000). Also, as CAIX has a relatively long half-life (Salceda and Caro 1997; Rafajova et al. 2004b), areas that have recently become normoxic are likely not to express HIF but may express both CAIX and the normoxia marker VHL.
1.6.1 Hypoxia inducible factor and airway mucins

Recent studies have suggested a role of hypoxia inducible signalling in mucus production in COPD. HIF-1α plays a key role in the activation of the transcription of MUC5AC (Young et al. 2007; Zhou et al. 2012). HIF-1α drives goblet cell hyperplasia and causes increased expression of MUC5AC via increased ERK1/2 signalling in COPD patients (Polosukhin et al. 2011). HIF-1α may also up-regulate other genes important for goblet cell differentiation and function, such as the trefoil factor family (Hernandez et al. 2009). Furthermore, Goven et al. showed that HIF-1α induces heme oxygenase-1 (HO-1) expression in patients who smoke and have a primary spontaneous pneumothorax (Goven et al. 2010). Previous studies indicate that STAT6 and HO-1 play important roles in MUC5AC production in COPD (Almolki et al. 2008; Kuperman et al. 1998). Cigarette smoke has also been shown to increase HIF-1α production, HIF-1α activity and MUC5AC expression via EGFR mediated pathways (Yu et al. 2012a). Hypoxia-inducible signalling may thus have an important effect on mucus hypersecretion. It has not been reported whether HIF-1α can affect MUC5B expression.

The microenvironments of bronchial epithelial cells are characterized by hypoxia as well as inflammatory conditions during the pathogenesis of inflammatory airway diseases such as COPD (Polosukhin et al. 2007b). As previously discussed, inflammatory cytokines cause both mucin and HIF-1α upregulation. Furthermore, HIF-1α can cause mucin secretion through the above stated mechanisms. Mucus hypersecretion and plugging lead to bacterial colonization and inflammation that result in the release of more inflammatory cytokines (Figure 1.8). It is likely that the co-operative regulation of airway mucins by HIF-1α and inflammatory cytokines as well as the regulation of HIF-1α by inflammatory cytokines and hypoxia are important mechanisms of mucus hypersecretion in COPD and further studies on this can provide new insights and identify novel therapeutic targets to treat mucus hypersecretion.
Figure 1.8: Role of HIF-1α and mucins in mucus hypersecretion: Cigarette smoke and other irritants cause epithelial remodelling and the release of inflammatory mediators. These mediators and the creation of hypoxic conditions due to airway inflammation causes activation of HIF-1. HIF-1α causes mucus hypersecretion via goblet cell hyperplasia and other mechanisms. Inflammatory cytokines can also cause mucin gene upregulation and mucus secretion. Mucus accumulation causes bacterial colonization and this further results in airway inflammation and the activation of HIF-1α and mucus secretion.
CHAPTER 2. HYPOTHESIS, AIMS AND OBJECTIVES

The hypothesis of this thesis was that COPD patients with persistent sputum production have more severe disease characteristics, increased airway inflammation and increased expression of airway mucins and hypoxia inducible factor-1α compared to COPD sputum non-producers.

The aims of the thesis were:

1. To compare the clinical characteristics and airway inflammatory biomarker profile between COPD persistent sputum producers and COPD sputum non-producers.
2. To investigate the short term repeatability of spontaneous and induced sputum in persistent sputum producers.
3. To study the expression of MUC5B and MUC5AC in persistent sputum producers.
4. To study the expression of HIF-1α and CAIX in persistent sputum producers.
5. To investigate a potential link between HIF-1α and MUC5B expression.

In order to achieve these aims, experiments were designed with the following objectives:

1. To measure spirometry, body plethysmography, gas transfer, single breath nitrogen washout, six minute walk test distance, spontaneous and induced sputum indices (differential cell count, cytokines and culture), health status and risk of exacerbations in COPD persistent sputum producers and compare it to sputum non-producers.
2. To measure sputum indices in a cohort of persistent sputum producers at two visits separated by an eight week interval to assess their repeatability.
3. To study the expression of MUC5AC and MUC5B in bronchial biopsy specimens of COPD persistent sputum producers, COPD non-producers and control groups (smokers with normal lung function and lifelong healthy non-smokers with normal lung function).
4. To study the expression of HIF-1α and CAIX in bronchial biopsy specimens of COPD persistent sputum producers, COPD non-producers and control groups (smokers with normal lung function and healthy non-smokers).

5. To investigate if HIF-1α can increase MUC5B expression in vitro.
CHAPTER 3: METHODS

3.1 Subjects

Subjects were recruited from the clinical research database held at the Medicines Evaluation Unit, University Hospital of South Manchester Foundation Trust. COPD was diagnosed according to the GOLD criteria (GOLD 2011), based on a smoking history of at least 10 pack years with typical symptoms (one of more of productive cough, breathlessness and wheeze) and evidence of airflow obstruction. An ex-smoker was defined as one having no cigarettes for a period of more than one year, whilst a non-smoker was defined by a smoking history of less than one pack year. Patients with a history of asthma, known α-1 antitrypsin deficiency, an exacerbation or any change in their COPD therapy within 6 weeks of the study, or a history of lung cancer were excluded. The studies were approved by the local ethics committee and all subjects gave written informed consent prior to their participation in the studies. All procedures were carried out in accordance with recommendations found in the Declaration of Helsinki (Recommendations guiding physicians in biomedical research involving human subjects. World Medical Association Declaration of Helsinki 1994). Height was measured to the nearest cm and weight to the nearest 100 grams. BMI was calculated as weight in kg divided by squared height in meters.

3.2 Skin prick test

Skin prick testing was performed using the following allergens - house dust mite (*Dermatophagoides pteronyssinus*), cat hair and grass pollen (Bayer, Elkhart, IN, USA). Normal saline and histamine were used as negative and positive controls respectively. Droplets of allergen were placed onto the volar aspect of the forearm. A sterile lancet was used to prick the skin through the allergen droplet, the skin was broken but blood not drawn. A separate lancet was used for each allergen. After 15 minutes the mean wheal diameter was measured. Sensitisation was defined as a wheal diameter ≥ 3 mm than the negative control (Brand et al. 1993).
3.3 Pulmonary function tests

3.3.1 Spirometry

Each subject had maximum expiratory flow volume measurements performed using a Vitalograph spirometer (Vitalograph, Buckinghamshire, UK). Calibration was performed daily after correction for ambient temperature and barometric pressure. Subjects performed the procedure in a seated position while wearing a nosepeg. They were asked to form a tight seal around a plastic mouthpiece and then forcibly exhale to residual volume (RV) for 12 seconds. FEV\textsubscript{1} and FVC readings were performed in triplicate. All values were related to the reference values of the European Community for Coal and Steel (Quanjer et al. 1993).

3.3.2 Body plethysmography

A constant volume plethysmograph (Sensormedics Vmax 6200, Bilthoven, Netherlands) was used to measure the following: Airways Resistance (Raw), Specific conductance (sGaw), Functional Residual Capacity (FRC), Vital capacity (VC) and Inspiratory capacity (IC).

Subjects sat inside the plethysmograph wearing a nose clip and formed a tight seal around the rubber mouthpiece while supporting their cheeks. Measurement commenced with tidal breathing until baseline FRC was established. Subjects then performed a series of ‘shallow breathing’ manoeuvres at a rate of approximately 60 per minute. At least three flow/box pressure loops were obtained, the shutter was then closed and at least three mouth pressure/box pressure loops were recorded. Subjects then returned to tidal breathing. Raw, sGaw and FRC were automatically calculated by the computer software. Measurements were performed in triplicate and if readings were not within 5% of each other, the measurement was repeated. Mean of the readings was taken for further analysis. VC and IC were measured during a separate manoeuvre. After a period of tidal breathing, subjects were instructed to inhale to maximum inspiratory capacity and then exhale non-forcefully to RV. The highest values were recorded.

Total Lung Capacity (TLC) and residual volume (RV) were calculated as follows:

\[ \text{TLC} = \text{FRC} + \text{highest IC} \quad \text{AND} \quad \text{RV} = \text{TLC} - \text{highest VC} \]
3.3.3 Gas Transfer (Single breath DLco)

Carbon Monoxide Diffusing Capacity (DLco) was measured using the Vmax 22 instrument (Viasys, California, USA). The procedure was carried out according to standard guidelines (Macintyre et al. 2005). Subjects wore a nose clip and formed a tight seal around the rubber mouth piece. They exhaled non-forcefully to RV. At RV, the subject’s mouthpiece was connected to the source of the test gas and the subject was asked to inhale rapidly to TLC. Subjects were asked to hold their breath for ten seconds to allow gas exchange to take place, then exhale to a volume of at least 1 L (the ‘washout’ phase). Two readings were taken four minutes apart and the highest value recorded. Carbon Monoxide Transfer Coefficient or diffusion capacity (KCO) was calculated by the computer software (DLCO/VA which is DLCO corrected for alveolar volume).

3.3.4 Single breath Nitrogen washout

Single breath Nitrogen washout test was done using the Vmax 22 instrument (Viasys, California, USA). The procedure was carried out according to standard guidelines (Wanger et al. 2005). Upon full exhalation following VC of 100% oxygen, four phases in the nitrogen concentration can be recognized. Phase I indicates gas expired from the respiratory dead space with no gas exchange, and therefore, the nitrogen concentration in this phase is mainly zero. This is followed by a sudden increase in the nitrogen concentration due to mixing of expired alveolar gas with the dead space gas, giving an S shape to Phase II. Eventually all the expired gas originates mainly from the alveoli and the curve forms a straight line (Phase III). Phase IV is caused by preferential emptying of the apex of the lung after the lower zone airways have closed. This is illustrated in Figure 3.1. Small airway disease can be assessed using the closing volume (point of start of phase IV of the curve) and slope of the curve (N2Δ/L).
Figure 3.1: Nitrogen washout curve

Phase I indicates gas expired from the respiratory dead space with no gas exchange, and therefore, the nitrogen concentration in this phase is mainly zero. This is followed by a sudden increase in the nitrogen concentration due to mixing of expired alveolar gas with the dead space gas, giving an S shape to Phase II. Eventually all the expired gas originates mainly from the alveoli and the curve forms a straight line (Phase III). Phase IV indicates the emptying of the poorly ventilated regions of the lung and thus has the highest nitrogen concentration [adapted from Ventilation/Blood Flow and Gas Exchange (West 1977)]

3.3.5 Six minute walk test (6MWT)

Subjects performed the test along a 20m long flat enclosed corridor as per standard guideline (ATS 2002). The length of the corridor was marked every five meters. Subjects were made to sit at rest in a chair for at least 10 minutes before the start of the test. Pulse oximetry was measured. Also assessment of baseline dyspnoea score and overall fatigue was done using the Borg Scale before the start of the test. Subjects were then asked to walk at their own pace for 6 minutes along the corridor. At the end of 6 minutes, the total distance walked was measured. Pulse oximetry, dyspnoea and fatigue scores were recorded again at the end of the test.
3.4 Health status questionnaires

Symptoms, activity and impact of disease on day to day life were assessed using the St. George's Respiratory Questionnaire (SGRQ), the modified Medical Research Council (mMRC) dyspnoea score and the COPD Assessment Tool (CAT).

3.4.1 St. George's Respiratory Questionnaire (SGRQ) - This is a self-reported health related quality of life tool that consists of fifty items divided into symptom, activity and psychosocial impact categories. Scores range from 0 (minimum disability) to 100 (maximum disability). The questionnaire has been shown to be reproducible, valid and responsive (Jones et al. 1992).

3.4.2 Modified Medical Research Council (MMRC) dyspnoea score - The MMRC dyspnoea score quantifies the disability associated with breathlessness. It comprises five statements that describe almost the entire range of respiratory disability from none (Grade 0) to complete incapacity (Grade 4). MMRC score can predict survival and is complementary to FEV₁ in describing disability in COPD (Mahler and Wells 1988).

3.4.3 COPD Assessment Tool (CAT) - The CAT is made up of 8 items, each scored on a numeric scale of 0 (no impact) to 5 (very severe impact). Each item is weighted equally for the final score, giving a range of CAT scores from 0–40. Scores for the individual items within the questionnaire provide insight into the relative influence that the different components of COPD have on a patient’s life and highlight areas that can be addressed through intervention (Mackay et al. 2012; Jones et al. 2009).

3.5 Sputum induction

Sputum induction was performed if the post bronchodilator FEV₁ was > 800mls, according to standard methods (Pizzichini et al. 1996). 3%, 4% and 5% saline was administered in sequence for five minutes each via an ultrasonic nebuliser (Flaem Nuova, Brescia, Italy). Subjects were asked to rinse their mouth and blow their nose to minimize salivary and post nasal drip contamination. Sputum was collected in a sterile container. After each period of inhalation, spirometry was performed for safety check with a Vitalograph spirometer (Vitalograph, Buckinghamshire, UK). The procedure was abandoned if FEV₁ fell by > 20% from baseline.
3.6 Sputum processing

Spontaneous and induced sputum samples were processed within two hours of expectoration using a two step procedure, using PBS processing first to obtain supernatant followed by DTT to obtain cells (Bafadhel et al. 2012). Briefly, sputum plugs were selected to separate sputum from saliva. The plugs were weighed and eight volumes of PBS (Sigma Aldrich, UK) added. The mixture was aspirated gently using a pasteur pipette, placed on a vortex for 15 seconds and then on a rocker for 15 minutes. The sample was centrifuged at 790g at 4°C for 10 minutes. Four volumes of supernatants (PBS) were removed and stored at -80°C. Freshly made 0.2% DTT (Sigma, Poole, UK) was added to the remaining PBS/sputum mixture in a 4:1 ratio to the original sputum weight. The suspension was aspirated gently using a pasteur pipette, vortexed for 15 seconds, then rolled for 15 minutes. The suspension was filtered using a pre-weighed 48µm nylon mesh (Sefar, Bury, UK). Sample viability and total leukocyte count was determined by trypan blue exclusion method using a modified Neubauer haemocytometer. Samples with a cell viability > 50% and squamous cell contamination < 30% were processed further. The sample was then centrifuged again at 790g at 4°C for 10 minutes and the cell pellet resuspended in PBS to a suspension of 0.5 x 10⁶ cells/ml PBS. Cytospins were prepared using 75µl for differential cell counts (DCC). The DCC slides were air dried, fixed with methanol and stained with Rapi-diff (Triangle, Skelmersdale, UK). Four hundred leukocytes were counted and results expressed as percentage of total leucocyte count and total cell count.

3.7 Quantitative sputum culture

This was performed by the microbiologist at the Medicines Evaluation Unit. Separated sputum plugs (minimum 0.15g) were homogenized with an equal volume of sterile 0.1% DTT and vortexed for 60 seconds. Samples were incubated at 37°C for 15 minutes to aid homogenization. Serial dilutions of homogenized sample were prepared in sterile PBS ranging from 10⁻¹ to 10⁻⁵. Dilutions 10⁻³, 10⁻⁴ and 10⁻⁵ were used to inoculate blood and chocolate agar plates. All plates were incubated at 37°C in 5% CO₂ enriched atmosphere. After 24 hours, the viable count of each bacterial species was obtained from plates yielding between 20 and 200 colonies. The number of colony forming units per g (cfu/g) was
calculated from the number of colonies obtained and the dilution of the sputum. Potential pathogenic species were subcultured for identification by standard tests (Barrow GI 1993).

### 3.8 Bronchial biopsy collection and slide preparation

For the immunohistochemistry studies, bronchial biopsies were obtained via fibreoptic bronchoscope. Bronchoscopy was conducted according to accepted BTS guidelines (Du Rand et al. 2013) on subjects with baseline oxygen saturations > 95% on air. Subjects fasted for six hours before the procedure. They were sedated with alfentanil and midazolam and nasal passage and upper airways anesthetised with 2% lignocaine prior to the procedure. Up to three endobronchial biopsies were taken from the right lobar bronchus and placed in 10% neutral buffered formalin. After dehydration through a graded alcohol series (50, 75, 90 and 100% vol/vol) and xylene, the biopsies were embedded in paraffin-wax by the pathology department of the University hospital of South Manchester. 4μm sections were cut using a rotary microtome (Leica 2235 Biocut, Solms, Germany). Cut sections were floated on water (40°C) and mounted onto poly-lysine coated glass slides (Surgipath, Peterborough, UK). One section was collected per slide. Slides were dried at 40°C overnight and stored at room temperature until required.

### 3.9 Tinctorial staining

Tinctorial staining included Haematoxylin & Eosin (H&E) and Periodic acid Schiff’s (PAS) and was performed on all the biopsies as below. All chemicals used along with supplier can be found in Appendix 1.

#### 3.9.1 Haematoxylin & Eosin (H&E)

Slides were dewaxed in xylene followed by graded alcohol, comprising of 100%, 90%, 75%, 50%, then running water (five, three, two, one and five minutes respectively). They were immersed in Modified Gill’s Haematoxylin for 15 minutes and subsequently washed in running water for 15 minutes and immersed in Borax solution for one minute. Slides were then immersed in Eosin Y (Leica) solution for one minute and washed in water. This was followed by dehydration through an ascending alcohol series ending in two changes of xylene, coverslipping and mounting with DPX.
3.9.2 Periodic acid Schiff’s (PAS)

Schiff’s reagent was allowed to equilibrate to room temperature for two hours before use. Slides were immersed in 1% periodic acid solution for five minutes and then washed in distilled water. Schiff’s reagent was added to the slides for ten minutes and the slides subsequently washed in tap water for ten minutes. They were stained in Gill’s Modified Haematoxylin for two minutes to give a nuclear counterstain. They were then washed in running water, immersed in an ascending alcohol series followed by xylene, coverslipped and mounted with DPX.

3.10 Immunohistochemistry (IHC)

The antibodies used in the immunohistochemistry studies were optimised in the Airway Pharmacology Groups Lab at the Educational Research Centre in the University Hospital of South Manchester.

3.10.1 Optimisation of MUC5AC and MUC5B antibody

Anti-MUC5AC and anti-MUC5B mouse monoclonal antibodies were purchased from Abcam (Cambridge, U.K) (anti-MUC5AC antibody, clone 1-13M1, code ab24070 and anti MUC5B antibody, clone 19.4E, code 77995).

Immunostaining was performed on formalin-fixed, paraffin-embedded (FFPE) bronchial biopsy sections. Briefly, sections from each subject were deparaffinised in xylene, taken through graded alcohols and then into distilled water. This comprised of 100%, 90%, 75% and 50% alcohols sequentially immersed for five, three, two and one minute respectively and finally running water for five minutes. 10mM DTT and 25mM iodoacetamide in Tris-HCL were used as reducing agent for disulfide bonds and alkylating agents respectively in the MUC5B protocol at this time for stabilisation of free sulfhydryls (cysteines) and reduction of disulfide bonds in peptides and proteins. The duration of each treatment was 30 minutes. Antigen retrieval was performed, the tissue sections were heated in a microwave oven (800 W for 20 minutes with 0.01 M trisodium citrate, pH 6.0 and Tris-EDTA, pH 9.0). They were then blocked with 1.5% (vol/vol) horse serum in Tris-buffered saline (TBS, blocking solution) for 30 minutes. This was followed by wash in TBS and incubation with the MUC5AC antibody (diluted 1:100, 1:200, 1:400, 1:800, 1:1000 and
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1:2000) and MUC5B antibody (diluted 1:100, 1:200, 1:400, 1:800, 1:1000 and 1:2000) overnight at 4°C. A section from a cell line or tissue known to express the protein of interest is used as a positive control. A bronchial biopsy block showing positive PAS staining in the bronchial epithelium, thus confirming the presence of mucins in the epithelium was used as a positive control. Omission of the primary antibody from the staining protocol and substitution of primary antibody with an isotype control antibody (Vector Labs) were used as negative controls.

The next day, endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 30 minutes. For detection of primary antibodies, the sections were washed in TBS, followed by incubation for 30 minutes with biotinylated horse anti-mouse IgG, diluted 1:200 in TBS (blocking solution), then treated with Elite Vector stain ABC kit for 30 minutes before microscopic detection with 3,3’-diaminobezidine (DAB). The slides were then counterstained for one minute using Gill’s modified haematoxylin, dehydrated and coverslipped with DPX.

It was concluded that most optimum staining for MUC5B was after treatment with 0.01M citric acid at pH 6 at an antibody dilution of 1:200 and the most optimum staining for MUC5AC was after treatment with EDTA at pH 9 at an antibody dilution of 1:2000 (Table 3.1).
### Table 3.1: Protocol for MUC5AC and MUC5B immunostaining

<table>
<thead>
<tr>
<th>Target</th>
<th>MUC5B protein</th>
<th>MUCAC protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample</strong></td>
<td>Biopsy</td>
<td>Biopsy</td>
</tr>
<tr>
<td><strong>Pre-treatment</strong></td>
<td>DTT (reducing agent), 30 minutes</td>
<td>EDTA pH 9 (20 minutes microwave)</td>
</tr>
<tr>
<td></td>
<td>Iodoacetamide (alkylating agent), 30 minutes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Citrate pH 6 (20 minutes, microwave)</td>
<td></td>
</tr>
<tr>
<td><strong>Serum block</strong></td>
<td>1.5% Normal horse serum in TBS (Vector, S 2000)</td>
<td>1.5% Normal horse serum in TBS (Vector, S 2000)</td>
</tr>
<tr>
<td><strong>Antibody Diluent</strong></td>
<td>1.5% Normal horse serum in TBS (Vector, S 2000)</td>
<td>1.5% Normal horse serum in TBS (Vector, S 2000)</td>
</tr>
<tr>
<td><strong>1º Antibody</strong></td>
<td>Mouse Anti-Human MUC5B antibody (19.4E), Abcam (77995)</td>
<td>Mouse Anti-Human MUC5AC antibody (1-13M1), Abcam (ab24070)</td>
</tr>
<tr>
<td><strong>Dilution Factor</strong></td>
<td>1:200 (5µg/ml)</td>
<td>1:2000 (0.05µg/ml)</td>
</tr>
<tr>
<td><strong>Incubation Time</strong></td>
<td>Overnight at 4ºC</td>
<td>Overnight at 4ºC</td>
</tr>
<tr>
<td><strong>Wash buffer</strong></td>
<td>TBS + 0.5% Tween</td>
<td>TBS + 0.5% Tween</td>
</tr>
<tr>
<td><strong>2º Antibody</strong></td>
<td>Horse anti-Mouse (biotinylated) (Vector, BA 2000), 30 mins.</td>
<td>Horse anti-Mouse (biotinylated) (Vector, BA 2000), 30 mins.</td>
</tr>
<tr>
<td><strong>Chromagen</strong></td>
<td>DAB (Vector, SK4100)</td>
<td>DAB (Vector, SK4100)</td>
</tr>
<tr>
<td></td>
<td>Incubation time - 3 minutes</td>
<td>Incubation time - 1 minute</td>
</tr>
</tbody>
</table>
3.10.2 Optimisation of HIF-1α immunostaining - Method development

3.10.2.1 Staining for HIF-1α

Successful staining of HIF-1α is defined as positive peri-nuclear staining with a concurrent isotype negative control, as opposed to a control omitting the primary antibody. Only a few papers have been found to achieve convincing HIF-1α staining along with a concurrent isotype control. Ito et al. achieved nuclear staining in formalin fixed paraffin embedded human gastric mucosa resections with ab1 (Abcam, Cambridge) (Ito et al. 2003).

Bronchial biopsies were subjected to the routine IHC approach as described for MUC5AC in section 3.10.1. Briefly biopsies were dewaxed and rehydrated through graded alcohols and placed under running water. Antigen retrieval was performed, the different antigen retrieval steps will be discussed later. Following blocking in normal serum, sections were incubated in HIF-1α antibody overnight at 4°C. Bound primary antibodies were detected using appropriate biotinylated secondary antibodies. Endogenous peroxidase was quenched using hydrogen peroxide in methanol or TBS, for 30 minutes at room temperature. Sections were incubated in ABC complex for 30 minutes at room temperature. Bound antibody was visualised using DAB. Sections were counterstained for 30 seconds using Gills' modified haematoxylin, then immersed in an ascending alcohol series ending in xylene, coverslipped and mounted with DPX.

3.10.2.2 Selection of positive control

A bronchial biopsy block representative of a central airway was initially chosen to optimise HIF-1α antibody. The same block was used as a positive control for MUC5B and MUC5AC, confirmed by positive PAS staining, as shown in Figure 3.2.
Gastric cancer tissue has been reported as a suitable positive control tissue for HIF-1α expression (Ito et al. 2003). Gastric cancer tissue was obtained from the pathology department of USHM after appropriate consent.

The protocol detailed in Ito et al. (2003) using HIF-1α staining (ab1, Abcam) was modified to incorporate it into our detection system. Sections from the human gastric cancer block and human upper airway were stained for HIF-1α and different antibody concentrations and pre-treatments attempted (Appendix 2a). The result is shown in Figure 3.3. No positive staining in the human gastric cancer tissue or upper airway section was observed. Also, no positive nuclear epithelial cell staining was observed with any of the pre-treatments compared to the isotype control. The only staining observed was non-specific, non-nuclear, background staining of the basement membrane and connective tissue.
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Figure 3.3: Representative photomicrographs showing no positive staining in the human gastric cancer tissue blocks (A) or upper airway section (B) stained with the HIF-1α antibody. Also, no staining observed in the upper airway section (C) compared to isotype control (D). Abbreviation: E - epithelium, S - submucosa. Scalebar: low magnification = 50μm, high magnification = 500μm.

3.10.2.3 Use of alternate antibody

An alternate antibody was sourced - HIF-1α (BD Biosciences, Oxford, 610958) and the method used by Griffiths et al. to stain HIF-1α using a Tyramide Signal Amplification (TSA) kit (Perkin Elmer) was used to stain the human gastric cancer block (Griffiths et al. 2007).

Alterations to the general IHC protocol previously described included: Quenching of endogenous peroxidase using 0.3% hydrogen peroxide in PBS (vol/vol); Sections blocked in TNB buffer prior to incubation in primary antibody; Incorporation of the TSA signal amplification kit. Antigen retrieval was carried out in low pH Citrate Buffer (pH 6, 10 mM Trisodium Citrate) and high pH Tris-EDTA buffer (pH 8/9, 1mM Tris-EDTA). Sections were blocked for 30 minutes in TNB buffer made up according to manufacturer’s
instructions (Appendix 1). Primary antibody was then added at a concentration of 2.5 µg/ml (1:100 dilution) in TNB and left overnight at 4°C in a humidified chamber.

There was good focal staining of tumour nuclei and also strong staining of some endothelial and stromal cells which can be seen in Figure 3.4A, 3.4B and 3.4C. No positive nuclear staining was observed in the isotype control as shown in Figure 3.4D.

Figure 3.4: Representative photomicrographs showing HIF-1α staining of gastric cancer sections using the TSA kit. A-C showing positive staining in tumour cell nuclei along with endothelial and stromal cell staining. D: isotype control showing no nuclear staining but some non-specific basement membrane and connective tissue staining. Abbreviation: E- epithelium, S – submucosa, SM - smooth muscle cells, ST - stromal cells. Scalebar = 50µm.

3.10.2.4 Methods to reduce non-specific background staining

Although good positive nuclear epithelial staining was observed, the background was deemed too intense to quantify. Adjustments to the concentration of hydrogen peroxide were made and an additional blocking step was introduced into the protocol. As previously described in section 3.10.2.1, 1.5% serum (v/v) corresponding to the host of the secondary antibody was initially applied for 30 minutes at room temperature. This showed no
significant difference to the original method used. It was decided to increase the concentration of serum and concentrations of 1.5%, 5% and 10% in TNB were used. The 10% serum in TNB visually appeared to reduce the background. Further attempts to reduce non-specific staining were attempted including a primary antibody dilution series. A summary of these pre-treatments is tabulated in Appendix 2(b).

The most optimum staining was observed using primary antibody dilution of 1:100 (2.5 µg/ml) with 10% serum block (in TNB) and 1% hydrogen peroxide in PBS (Table 3.2).

3.10.2.5 Staining of cohort

Bronchial biopsy section of a severe COPD patient with distinct signs of ATII hyperplasia (Figure 3.5) was stained for HIF-1α in parallel with the gastric cancer section using the protocol described. Strong nuclear staining was found in airway epithelium type II cells displaying signs of ATII hyperplasia. The protocol was taken forward to stain the bronchial biopsies of subjects used in the experimental chapter six, with this airway section and a gastric cancer section being used as positive controls.

![Figure 3.5: Representative photomicrographs showing: A. H&E of COPD patient showing ATII Hyperplasia; B. HIF-1α staining from the same patient. Scalebar = 500µm.](image-url)
Lung physiology and airway inflammation in COPD patients with persistent sputum production

Table 3.2: Protocol for HIF-1α and CAIX immunostaining

<table>
<thead>
<tr>
<th>Target</th>
<th>HIF-1α</th>
<th>CAIX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Biopsy</td>
<td>Biopsy</td>
</tr>
<tr>
<td>Pre-treatment</td>
<td>Citrate pH6 (20 minutes microwave)</td>
<td>Citrate pH6 (20 minutes microwave)</td>
</tr>
<tr>
<td>Block</td>
<td>TNB (Perkin-Elmer, Code NEL700A)</td>
<td>TNB (Perkin-Elmer, Code NEL700A)</td>
</tr>
<tr>
<td>Antibody Diluent</td>
<td>10% Normal horse serum in TNB (Vector, S 2000 &amp; Perkin Elmer, NEL700A)</td>
<td>10% Normal horse serum in TNB (Vector, S 2000 &amp; Perkin Elmer, NEL700A)</td>
</tr>
<tr>
<td>1st Antibody</td>
<td>Mouse Anti-Human HIF-1α BD (610958)</td>
<td>Rabbit Anti-Human CAIX Abcam (15086)</td>
</tr>
<tr>
<td>Dilution Factor</td>
<td>1:100 (2.50 µg/ml)</td>
<td>1:1000 (1.10 µg/ml)</td>
</tr>
<tr>
<td>Incubation Time</td>
<td>Overnight at 4ºC</td>
<td>Overnight at 4ºC</td>
</tr>
<tr>
<td>Wash buffer</td>
<td>TBS + 0.5%Tween</td>
<td>TBS + 0.5%Tween</td>
</tr>
<tr>
<td>Chromagen</td>
<td>DAB (Vector, SK4100) Incubation time -3 minutes</td>
<td>DAB (Vector, SK4100) Incubation time -3 minutes</td>
</tr>
</tbody>
</table>

3.10.3 Optimisation of CAIX antibody

Anti-Carbonic Anhydrase IX, rabbit monoclonal antibody was purchased from Abcam (code - ab15086) and immunostaining performed using different pre-treatments as outlined in Appendix 2(b). The final protocol is shown in Table 3.2.
3.11 Staining Quantification

Representative pictures from the slides were taken using a Nikon Eclipse 80i microscope (Nikon UK Ltd., Surrey, UK) equipped with a QImaging digital camera (Media Cybernetics, Marlow, UK).

3.11.1 H&E and PAS quantification

Pictures were interpreted blinded with no prior knowledge of the clinicopathological parameters. Histological staining (H&E and PAS) was assessed by two independent investigators, consensus scores were obtained where applicable. The area of bronchiolar epithelium to be studied was selected randomly. A five point scoring system developed in our laboratory was applied (Table 3.3). Example images of the grading criteria for H&E and PAS are shown in Appendix 3.

Table 3.3: Histological analysis of bronchial epithelium

<table>
<thead>
<tr>
<th>Score</th>
<th>H&amp;E</th>
<th>PAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal ciliated epithelium</td>
<td>Normal epithelium with occasional PAS+ cells</td>
</tr>
<tr>
<td>2</td>
<td>Hypertrophic epithelium</td>
<td>PAS+ cell hypertrophy</td>
</tr>
<tr>
<td>3</td>
<td>Hyperplastic epithelium</td>
<td>PAS+ cell hyperplasia</td>
</tr>
<tr>
<td>4</td>
<td>Metaplastic epithelium</td>
<td>Metaplasia with PAS+ cells throughout mucosa</td>
</tr>
<tr>
<td>5</td>
<td>Squamous cell metaplasia</td>
<td>Epithelial metaplasia/squamous plates with loss of PAS+ cells</td>
</tr>
</tbody>
</table>

Table showing the scoring system for H&E and PAS staining; increase in score is associated with an increase in epithelial alteration. Abbreviations: H&E – Haematoxylin and Eosin; PAS – Periodic acid Schiff’s.

3.11.2 Quantification of immunostaining

Staining quantification was performed using Image Pro-Plus software version 6.0 (Media Cybernetics). For each biopsy specimen, the entire intact epithelium was systematically assessed based on red, blue, green (RGB) colour balance. The digitised image of the sections was sampled interactively and the colour balance adjusted so that areas of positive staining were highlighted by the program. Images were thresholded against a staining
intensity determined by the positive control which was kept consistent for each stain. Any staining above the threshold was deemed positive staining for the antibody. The system selected all the pixels of the same RGB colour balance as the pre-determined positive staining reference range within the image. The area of epithelium was then delineated interactively and the percentage of positive staining within the epithelium determined. MUC5AC and MUC5B quantification was expressed as percentage of total area of bronchial surface epithelium positive for MUC5AC and MUC5B.

HIF-1α and CAIX staining was scored using a method modified from the literature (Ito et al. 2003; Ramsey et al. 2012; Kirkpatrick et al. 2008). The average extent of staining was initially evaluated using a scoring system for the percentage positive area of immunostaining. The scoring system for HIF-1α was as follows: 0, no nuclear staining; 1, < 2% nuclear staining; 2, 2-10% nuclear staining; 3, 10-20% nuclear staining; 4, >20% nuclear staining, The scoring system for CAIX was as follows: 1, 0-0.5% membranous staining; 2, 0.6-2.5% membranous staining; 3, 2.6-5% membranous staining and 4, >5% membranous staining. Staining intensity in the biopsy specimens was also scored as weak (1), moderate (2) or severe (3). Representative pictures of staining intensity gradation for HIF-1α and CAIX are shown in Appendix 3(c). Discordant results were handled by choosing the highest staining intensity. For the purpose of the analysis, the product of the intensity and the extent of immunostaining was used to calculate the immunohistochemical score (IHC) score of HIF-1α and CAIX for each subject.
3.12 Cell culture

Transformed human bronchial epithelial cells (16HBE cells) were purchased from the European Collection of Cell cultures.

Recovery from Frozen Stocks

16 HBE vials were removed from liquid nitrogen storage. Vials were held in a waterbath at 37°C with gentle agitation. When a small ice pellet remained, cells were added drop wise to 20 mls of growth media (RPMI 1640 containing 10% vol/vol FCS, 100U/ml penicillin, 100nM streptomycin and 2mM L-Glutamine) and spun at 400g for 10 minutes at 4°C. The cells were then resuspended in 20 mls of growth media and transferred to a 75cm² cell culture flask at appropriate density. Flasks were incubated for up to 7-10 days at 37°C and 5% CO₂. Growth media was changed every two days.

Subculturing of Proliferating Cell Cultures

On reaching 80-90 % confluence cells were subcultured by removing the growth media and washing twice with 5 mls PBS. They were then incubated at 37°C for two minutes in 0.05% trypsin-EDTA with occasional agitation by taping on the heel of the hand. Once 90-95% of cells had detached from the flask, cells were resuspended in growth media, and spun at 400g for 10 minutes at 4°C. The pellet was resuspended in a known volume of growth media and cells counted using trypan blue exclusion. The total cell solution was adjusted to give 1 x 10⁶ cells/ml by the addition of growth media. 150µl of this suspension was placed in each well of an eight well chamber slide. The chamber slides and the flask were then incubated at 37°C and 5% CO₂ to allow the cells to adhere, the attachment of cells was visualised with a microscope.

For experimental chapter six, cells in the chamber slides were incubated for a predetermined amount of time (1-24 hours) at 37°C in humidified 5% CO₂ in the presence or absence of 1mM dimethylloxalyglycine (DMOG), which is a prolyl hydroxylase (PHD) enzyme inhibitor and induces HIF-1α (Asikainen et al. 2005). At appropriate time points, cells were fixed in ice-cold acetone: methanol (1:1, vol: vol) for immunocytochemistry.
CHAPTER 4: Baseline characteristics of the COPD persistent sputum producers

4.1 Introduction

Chronic bronchitis is a phenotype of COPD defined by a productive cough for a minimum of three months for two consecutive years. A number of studies have shown that chronic bronchitis in COPD is associated with more severe airflow obstruction, worse health related quality of life, increased exacerbation rate and possibly increased mortality. However, a chronic bronchitis patient may be symptomatic of cough with phlegm for only a few months of the year and still fulfill its clinical definition. To better understand and stratify chronic bronchitis, a subphenotype of patients who have mucus hypersecretion throughout the year as their key presenting symptom needs to be identified and studied.

The repeatability of sputum in COPD is an important issue, as measurements that are more repeatable over time have greater applicability as biomarkers compared to those with naturally high variability. The repeatability of sputum indices needs to be extensively studied in the different phenotypes of COPD to ensure the validity of their use as accurate endpoints as well as to confirm drug effect in clinical trials.

The aim of this chapter was to compare the clinical characteristics and airway inflammatory biomarker profile between the persistent sputum producer phenotype of COPD and sputum non-producers. A secondary aim was to investigate the stability of spontaneous and induced sputum measurements over short term in COPD persistent sputum producers.

4.2 Methods

4.2.1 Subjects and study design

Fifty-two patients with COPD were recruited from the database of the Medicines Evaluation Unit, University Hospital of South Manchester and divided into persistent sputum producer and non-producer categories based on validated questionnaires from the American Thoracic Society (ATS 1969); Patients bringing up phlegm at least twice a day for four or more days of the week throughout the year were categorised as persistent sputum producers while those who produce phlegm only during an exacerbation were
categorised as sputum non-producers. Patients were excluded if they had experienced a respiratory tract infection or exacerbation of COPD in the preceding six weeks.

**Study design**

The following procedures were performed on a single study visit in order: skin prick test, pulmonary function tests (post-bronchodilator spirometry, body plethysmography, gas transfer, single breath nitrogen washout and six minute walk test) and sputum induction. For details of the methods please see section 3.3. Short and long acting bronchodilators were withheld for 6 and 12 hours respectively prior to the study visit. Smoking history, past medical history, treatment history and one year exacerbation history was recorded for each patient. Health status was measured using the SGRQ, MMRC and CAT questionnaires. The BODE index was calculated from BMI, post bronchodilator FEV₁, six minute walk test (6MWT) and MMRC score. Patients were also asked to provide a spontaneous sputum sample anytime in the visit up to the point of sputum induction. They were asked to rinse their mouth at the start of the visit to reduce buccal contamination in the sample. Patients in the persistent producer group had to be able to provide a spontaneous sputum sample in addition to an induced sputum sample to be eligible for the study. High resolution CT scan (GE Medical Systems, Light Speed L52002) had been performed on a subset of patients in the previous year.

**Sputum induction and processing**

This was performed according to methods stated previously (sections 3.5 and 3.6).

**Sputum cytokine analysis**

Cytokines and chemokine levels in sputum supernatants were assayed using Meso Scale Discovery (MSD) at GSK (Stevenage, UK). The lower limits of detection of these analytes in the assay were: eotaxin (34.80 pg/ml), eotaxin-3 (74.50 pg/ml), GM-CSF (0.14 pg/ml), IL-1β (0.57 pg/ml), IL-2 (0.41 pg/ml), IL-4 (0.41 pg/ml), IL-5 (0.50 pg/ml), IL-6 (1.21 pg/ml), IL-8 (0.12 pg/ml), IL-10 (0.47 pg/ml), IL-12p70 (0.84 pg/ml), IL-13 (0.36 pg/ml), IL-17 (0.26 pg/ml), IFN-γ (1.20 pg/ml), MCP-1 (0.81 pg/ml), MCP-4 (18.80 pg/ml), MDC (44.70 pg/ml), MIP-1β (1.09 pg/ml), TARC (16.40 pg/ml) and TNF-α (1.04 pg/ml).
Sputum repeatability

To assess sputum repeatability in the persistent sputum producer group, 12 COPD persistent sputum producers returned to the unit for a second visit, eight weeks after the first one. Spontaneous and induced samples were taken to assess for repeatability of sputum differential cell counts and percentages. All the patients were able to provide both the samples.

Sputum culture

To compare the rate of bacterial colonisation between COPD persistent sputum producers and non-producers, a further study at one year was conducted, where sputum culture was performed on 20 COPD persistent sputum producers and 13 non-producers, as described in section 3.7.

4.2.2 Statistical Analysis

Statistical analysis was performed using PRISM version 5 (Graph Pad Software, San Diego, California, USA) and SPSS version 20 (SPSS, Inc., Chicago, Ill., USA). Parametric and nonparametric data are presented as means (SD) and medians (range) unless otherwise stated. The Kolmogorov Smirnov test determined normality of the data. Unpaired t-tests were performed to compare differences between parametric data (lung function measurement, SGRQ and CAT scores and sputum data except eosinophils). Unpaired t-test was also used to compare the difference between spontaneous and induced sputum indices of sputum producers as the samples were not matched. Mann-Whitney U tests were used to compare non-parametric data (MMRC score, exacerbation history and sputum eosinophil data). Fisher’s exact test was used to compare the rates of bacterial colonization between groups.

The association between sputum cytokines and clinical parameters was examined using Pearson’s or Spearman’s rank correlation. Data with a univariate correlation significance of $p < 0.05$ were entered into a multiple regression model with age, sex, pack years and corticosteroid use as confounding variables. Since values for sputum differential cell count and percentage may incorrectly have an effect on each other, only one of these indices was used in the multivariate model if found significant on the univariate analysis.
To analyse results of sputum repeatability, the standard deviation of sputum neutrophil percentages between the baseline and eight week visits was used for power calculations that can be used in parallel group design study. Using the within subject standard deviation for percentage neutrophils (13%) it was estimated that a sample size of six patients will detect a 20% change with 80% power. The repeatability of sputum cell counts was assessed by determination of the intraclass correlation coefficient. The intraclass correlation coefficient (Ri) was calculated, with results interpreted as follows; 0 to 0.2 indicates slight repeatability, 0.21 to 0.40 indicates fair repeatability, 0.41 to 0.60 indicates moderate repeatability, 0.61 to 0.8 indicates good repeatability and 0.81 to 1.00 indicates very good repeatability.

4.3 Results

The population consisted of patients with GOLD stage I – III COPD. The majority of these patients were GOLD stage II-III. High resolution CT scan on 16 persistent sputum producers and 10 non-producers identified only two persistent sputum producers with minor bronchiectasis.

Seven patients were classified as being atopic following the results of skin prick testing (four persistent sputum producers and three non-producers). One patient was taking regular antihistamines and so did not have a positive skin prick reaction. For the purpose of this study, data obtained from this patient will be analyzed with skin prick test positive patients.

Fifty out of 52 patients were receiving inhaled bronchodilator therapy [(short acting β2 agonist (SABA), long-acting β2 agonist (LABA), short acting muscarinic antagonist (SAMA) and long acting muscarinic antagonist (LAMA)]. There were no patients on oral corticosteroids. The demographics of the patients are shown in Table 4.1.
Table 4.1: Patient demographics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Persistent sputum producer (n = 26)</th>
<th>Non producer (n = 26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>65.7 (± 6.91)</td>
<td>66.8 (± 6.46)</td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>12/14</td>
<td>14/12</td>
</tr>
<tr>
<td>Pack years *</td>
<td>35.3 (12.5 – 86)</td>
<td>32 (18.50 – 122.2)</td>
</tr>
<tr>
<td>Current smokers (n)</td>
<td>15/11</td>
<td>12/14</td>
</tr>
<tr>
<td>Atopics (n)</td>
<td>4/22</td>
<td>3/23</td>
</tr>
<tr>
<td>BMI (kg/m square)</td>
<td>28.5 (± 4.30)</td>
<td>27.6 (± 5.33)</td>
</tr>
<tr>
<td>Bronchiectasis</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>GOLD I (n)</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>GOLD II (n)</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>GOLD III (n)</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>GOLD IV (n)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SABA (n)</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>SAMA (n)</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>LABA (n)</td>
<td>17/9</td>
<td>20/6</td>
</tr>
<tr>
<td>LAMA (n)</td>
<td>12/14</td>
<td>11/15</td>
</tr>
<tr>
<td>ICS (n)</td>
<td>17/9</td>
<td>20/6</td>
</tr>
<tr>
<td>ICS dose (μg)</td>
<td>1267.9 (± 975.9)</td>
<td>1227.3 (± 869.14)</td>
</tr>
</tbody>
</table>

Characteristics of the COPD patients who participated in the study. Data presented as mean (± standard deviation). * Data represented as median (range). Abbreviations – SABA: short acting β agonist; SAMA: short acting muscarinic antagonist; LABA: long acting β agonist; LAMA: long acting muscarinic antagonist; ICS: inhaled corticosteroid
4.3.1 Lung function and exercise capacity

As shown in Table 4.2., persistent sputum producers had significantly lower FEV$_1$% predicted (p = 0.01) and diffusion capacity (KCO % predicted, p = 0.04), and a higher closing volume (p = 0.01) compared to sputum non-producers. Persistent sputum producers had numerically higher RV, but the difference between groups did not reach statistical significance (p = 0.13). Persistent sputum producers had a reduced exercise capacity measured by 6 minute walk test distance (p = 0.05) (Table 4.2).

Table 4.2: Physiological data from COPD patients

<table>
<thead>
<tr>
<th></th>
<th>Persistent sputum producer (n = 26)</th>
<th>Non producer (n = 26)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post bronchodilator FEV$_1$ % predicted</td>
<td>54.5 $(\pm$ 13.09)</td>
<td>65.1 $(\pm$ 16.25)</td>
<td>0.01*</td>
</tr>
<tr>
<td>TLC % pred.</td>
<td>105.7 $(\pm$ 16.75)</td>
<td>103.2 $(\pm$ 22.17)</td>
<td>0.55</td>
</tr>
<tr>
<td>RV</td>
<td>3.3 $(\pm$ 1.13)</td>
<td>2.6 $(\pm$ 0.88)</td>
<td>0.13</td>
</tr>
<tr>
<td>IC</td>
<td>2.0 $(\pm$ 0.66)</td>
<td>2.2 $(\pm$ 0.18)</td>
<td>0.26</td>
</tr>
<tr>
<td>IC/TLC</td>
<td>0.3 $(\pm$ 0.09)</td>
<td>0.4 $(\pm$ 0.09)</td>
<td>0.18</td>
</tr>
<tr>
<td>KCO % pred.</td>
<td>78.2 $(\pm$ 20.26)</td>
<td>93.5 $(\pm$ 21.73)</td>
<td>0.04*</td>
</tr>
<tr>
<td>Closing volume</td>
<td>1.0 $(\pm$ 0.41)</td>
<td>0.7 $(\pm$ 0.36)</td>
<td>0.01*</td>
</tr>
<tr>
<td>N$_2$Δ/L</td>
<td>6.5 $(\pm$ 2.54)</td>
<td>7.1 $(\pm$ 2.82)</td>
<td>0.77</td>
</tr>
<tr>
<td>6MWT (m)</td>
<td>350 $(\pm$ 85.40)</td>
<td>395.4 $(\pm$ 73.54)</td>
<td>0.05*</td>
</tr>
</tbody>
</table>

Pulmonary physiology consisted of post-bronchodilator spirometry, body plethysmography, transfer coefficient, single breath nitrogen washout and six minute walk test. Data represented as mean $(\pm$ SD). * indicates p value $\leq$ 0.05. Abbreviations – TLC: Total lung capacity; RV: Residual volume; IC: Inspiratory capacity; KCO: Diffusion capacity; N$_2$Δ/L: slope of nitrogen washout curve; 6MWT (m): six minute walk test distance (in metres);
**4.3.2 Symptoms, health status and exacerbations**

Persistent sputum producers had significantly higher mean SGRQ score \((p = 0.01)\) with higher symptom score \((p = 0.002)\) and impact score \((p = 0.01)\) and a trend towards significance for activity score \((p = 0.07)\). The CAT score \((p = 0.03)\) was also higher in persistent sputum producers compared to the non-producers, with a trend towards significance for MMRC score \((p = 0.07)\). The mean number of exacerbations in the previous year was higher in persistent sputum producers \((p = 0.03)\). The BODE index score was worse in persistent sputum producers \((p = 0.01)\) (Table 4.3).

**Table 4.3: Quality of Life questionnaire scores and exacerbation history**

<table>
<thead>
<tr>
<th></th>
<th>Persistent sputum producer ((n = 26))</th>
<th>Non producer ((n = 26))</th>
<th>(p) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGRQ score (Total score/100)</td>
<td>44.2 (± 20.34)</td>
<td>29.4 (± 17.21)</td>
<td>0.01*</td>
</tr>
<tr>
<td>SGRQ symptom score (Score/100)</td>
<td>52.9 (± 20.42)</td>
<td>31.7 (± 22.89)</td>
<td>0.002*</td>
</tr>
<tr>
<td>SGRQ impact score (Score/100)</td>
<td>56.3 (± 27.70)</td>
<td>43.5 (± 26.49)</td>
<td>0.01*</td>
</tr>
<tr>
<td>SGRQ activity score (Score/100)</td>
<td>34.5 (± 19.66)</td>
<td>21.0 (± 15.54)</td>
<td>0.07</td>
</tr>
<tr>
<td>MMRC score (0-4)</td>
<td>1.5 (1.0 – 4.0)</td>
<td>1.0 (0 – 3.0)</td>
<td>0.07</td>
</tr>
<tr>
<td>CAT score (Score/40)</td>
<td>20 (± 7.95)</td>
<td>14 (± 6.53)</td>
<td>0.03*</td>
</tr>
<tr>
<td>Exacerbations in last 12 months</td>
<td>1.1 (0 – 4.0)</td>
<td>0 (0 – 2.0)</td>
<td>0.03*</td>
</tr>
<tr>
<td>BODE Index</td>
<td>2.4 (0.67 – 4.14)</td>
<td>1.3 (0.02 – 2.74)</td>
<td>0.01 *</td>
</tr>
</tbody>
</table>

Subjects completed the St George’s Respiratory Questionnaire (SGRQ), Modified Medical Research Council Dyspnoea score (MMRC) and the COPD assessment test (CAT). BODE: Sum of Body mass index, \(\text{FEV}_1\)% predicted, MMRC score and 6MWT distance. Number of exacerbations in the last 12 months was noted for each patient. SGRQ and CAT scores are represented as mean (± SD); MMRC, BODE and number of exacerbations are expressed as median (range).\(^*\) indicates \(p\) value ≤ 0.05.
4.3.2 Sputum analysis

Sputum differential cell count

All the persistent sputum producers gave a spontaneous and an induced sample but in eight patients the spontaneous sample DCC slides were not countable due to high number of squamous cells (>10%). Two persistent sputum producers had non-viable induced samples. In the non-producers, sputum was obtained in 22 out of the 26 patients on induction. Differential cell counting was done by two skilled personnel, ensuring that all results were within an acceptable difference of 10%.

From induced sputum samples, the total cell count (TCC) was increased in persistent sputum producers, although this did not reach statistical significance (p = 0.09), while the total neutrophil count was significantly increased in persistent sputum producers (p = 0.02). Total eosinophil counts were increased in persistent sputum producers (p = 0.05). The cell differential percentage counts were similar in the two groups.

In the persistent sputum producer group, the mean viability of the spontaneous sample was less than that of the induced samples (p = 0.04), and the squamous cell percentage was higher in the spontaneous samples compared to the induced samples (p = 0.01). There were no other differences between spontaneous and induced samples. These results are tabulated in Table 4.4 and 4.5.
Table 4.4: Induced sputum cell counts in COPD persistent sputum producers & non-producers

<table>
<thead>
<tr>
<th></th>
<th>Persistent sputum Producer (n = 24)</th>
<th>Non-producer (n = 22)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell count x 10^6</td>
<td>3.5 (± 3.49)</td>
<td>2.2 (± 3.67)</td>
<td>0.09</td>
</tr>
<tr>
<td>Leucocyte viability %</td>
<td>74 (± 26.13)</td>
<td>71.1 (± 23.22)</td>
<td>0.66</td>
</tr>
<tr>
<td>Squamous cell %</td>
<td>1.6 (± 2.24)</td>
<td>1.1 (± 2.45)</td>
<td>0.34</td>
</tr>
<tr>
<td>Neutrophil %</td>
<td>73 (± 20.75)</td>
<td>60.9 (± 34.54)</td>
<td>0.61</td>
</tr>
<tr>
<td>Macrophage %</td>
<td>12.5 (± 13.09)</td>
<td>14.4 (± 14.83)</td>
<td>0.11</td>
</tr>
<tr>
<td>Eosinophil %</td>
<td>1.6 (0.50 - 3.69)</td>
<td>1 (0.19 - 2.31)</td>
<td>0.20</td>
</tr>
<tr>
<td>Neutrophil cell count x 10^6</td>
<td>2.1 (± 3.32)</td>
<td>1.6 (± 3.98)</td>
<td>0.02*</td>
</tr>
<tr>
<td>Macrophage cell count x 10^6</td>
<td>0.3 (± 0.42)</td>
<td>0.3 (± 0.55)</td>
<td>0.24</td>
</tr>
<tr>
<td>Eosinophil cell count x 10^6</td>
<td>0.1 (0.03 - 0.11)</td>
<td>0.02 (0.01 - 0.06)</td>
<td>0.05*</td>
</tr>
</tbody>
</table>

n = 24 in persistent sputum producer group as two patients gave induced sputum samples that were non-viable; n = 22 in the non-producer group as four patients did not produce sputum on induction. Data represented as mean (± SD). Eosinophil % and cell count expressed as median (± range). Abbreviation - TCC: Total cell count. * indicates p value ≤ 0.05.

Table 4.5: Induced and spontaneous sputum cell counts in COPD persistent sputum producers

<table>
<thead>
<tr>
<th></th>
<th>Persistent sputum Producer-Induced sample (n = 24)</th>
<th>Persistent sputum Producer-Spontaneous sample (n = 18)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell count x 10^6</td>
<td>3.5 (± 3.49)</td>
<td>3.1 (± 2.94)</td>
<td>0.18</td>
</tr>
<tr>
<td>Leucocyte viability %</td>
<td>74 (± 26.13)</td>
<td>44.2 (± 36.12)</td>
<td>0.04*</td>
</tr>
<tr>
<td>Squamous cell %</td>
<td>1.6 (± 2.24)</td>
<td>5.9 (± 3.20)</td>
<td>0.01*</td>
</tr>
<tr>
<td>Neutrophil %</td>
<td>73 (± 20.75)</td>
<td>68.1 (± 18.10)</td>
<td>0.31</td>
</tr>
<tr>
<td>Macrophage %</td>
<td>12.5 (± 13.09)</td>
<td>17.5 (± 14.11)</td>
<td>0.25</td>
</tr>
<tr>
<td>Eosinophil %</td>
<td>1.6 (0.50 - 3.69)</td>
<td>1.9 (0.20 – 3.67)</td>
<td>0.42</td>
</tr>
<tr>
<td>Neutrophil cell count x 10^6</td>
<td>2.1 (± 3.32)</td>
<td>2.6 (± 4.59)</td>
<td>0.24</td>
</tr>
<tr>
<td>Macrophage cell count x 10^6</td>
<td>0.3 (± 0.42)</td>
<td>0.6 (± 0.45)</td>
<td>0.76</td>
</tr>
<tr>
<td>Eosinophil cell count x 10^6</td>
<td>0.1 (0.03 - 0.11)</td>
<td>0.07 (0.04 – 0.17)</td>
<td>0.29</td>
</tr>
</tbody>
</table>

n = 24 in persistent sputum producer (induced) group as two patients gave induced sputum samples that were non-viable; n = 18 in persistent sputum producer (spontaneous) group as eight slides in this group were not countable due to high number of squamous cells (> 10%); Data represented as mean (± SD). Eosinophil % and cell count expressed as median (± range). Abbreviation - TCC: Total cell count. *indicates p value ≤ 0.05.
Supernatant cytokines

Persistent sputum producers had significantly higher levels of eotaxin ($p = 0.01$), GM-CSF ($p = 0.01$), MCP-1 ($p = 0.03$), MCP-4 ($p = 0.03$), TNF-$\alpha$ ($p = 0.04$) and IL-6 ($p = 0.05$), with a trend towards significance for IL-13 ($p = 0.07$) compared to non-producers (Fig 4.1). Twenty-five patients had GM-CSF levels below the lower limit of detection. There were no differences ($p > 0.05$) between eotaxin-3, IL-1$\beta$, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-17, IFN-$\gamma$, MIP-1$\beta$, MDC and TARC levels between the two groups. A graphical representation of the differences between the two groups of these cytokines and a table of all the cytokines with mean values is shown in Appendix 4.

Figure 4.1(a): Graphical representation of difference in sputum eotaxin, MCP-1, MCP-4, TNF-$\alpha$, IL-6 and IL-13 levels in COPD non-producers and persistent sputum producers. Columns represent mean values, error bars represent standard deviation (SD).

Figure 4.1(b): Dot-plot of difference in sputum GM-CSF levels in COPD non-producers and persistent sputum producers. Abbreviation – LOD: lower limit of detection.
Relationship between sputum cytokines and clinical parameters

Cytokines significantly different in the two groups were correlated with clinical parameters in both the groups using univariate and multivariate analysis (Section 4.2.2). In the persistent sputum producer group, there was a negative association between IL-6 and FEV$_1$% predicted ($r^2= 0.39$, $p = 0.01$). Conversely, there were positive associations between TNF-α and N$_2$Δ/L which denotes the slope of the single breath nitrogen washout curve ($r^2 = 0.33$, $p = 0.003$) and IL-4 and number of exacerbations in the previous year ($r^2 = 0.44$, $p = 0.03$). In the non-producer group, there was positive association between IL-6 and residual volume ($r^2 = 0.17$, $p = 0.02$). These results are summarized in Table 4.6 and 4.7.

Table 4.6: Linear Regression results of cytokine correlation to clinical parameters in the persistent sputum producer group.

<table>
<thead>
<tr>
<th>Clinical Parameter</th>
<th>Adjusted $r^2$</th>
<th>p value</th>
<th>β coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>FEV$_1$% predicted</td>
<td>0.39</td>
<td>0.01</td>
</tr>
<tr>
<td>TNF-α</td>
<td>N$_2$Δ/L (slope of nitrogen washout curve)</td>
<td>0.33</td>
<td>0.003</td>
</tr>
<tr>
<td>IL-4</td>
<td>Number of Exacerbations (in last the 12 months)</td>
<td>0.44</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Multiple regression analysis with the cytokines as outcome variables in the persistent sputum producer group. Abbreviation: $r^2$ - coefficient of determination; β - regression coefficient.

Table 4.7: Linear Regression results of cytokine correlation to clinical parameters in the non-producer group.

<table>
<thead>
<tr>
<th>Clinical Parameter</th>
<th>Adjusted $r^2$</th>
<th>p value</th>
<th>β coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>Residual volume</td>
<td>0.17</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Multiple regression analysis with the cytokines as outcome variables in the non-producer group. Abbreviation: $r^2$ - coefficient of determination; β - regression coefficient.
Sputum repeatability results

For induced sputum, there was fair agreement between the two visits on all the parameters - TCC (Ri = 0.6, p = 0.008), Neutrophil % (Ri = 0.6, p = 0.003), Macrophage % (Ri = 0.6, p = 0.004), Eosinophil % (Ri = 0.7, p = 0.001), Neutrophil cell count (Ri = 0.8, p = 0.003), Macrophage cell count (Ri = 0.7, p = 0.022) and Eosinophil cell count (Ri = 0.7, p = 0.026). The result is summarized in Table 4.8.

For spontaneous sputum, there was poor agreement between the two visits on most of the parameters - TCC (Ri = 0.4, p = 0.267), Neutrophil % (Ri = 0.1, p = 0.367), Macrophage % (Ri = 0.3, p = 0.146), Eosinophil % (Ri = -0.3, p = 0.513), Neutrophil cell count (Ri = 0.3, p = 0.156), Macrophage cell count (Ri = 0.3, p = 0.180) and Eosinophil cell count (Ri = -0.1, p = 0.515). The result is summarized in Table 4.9.
Table 4.8: Visit 1 (baseline) and Visit 2 (8 week) induced sputum measurements for sputum producers

<table>
<thead>
<tr>
<th>Sputum Indices</th>
<th>Visit 1 (Baseline) (n = 12)</th>
<th>Visit 2 (8 weeks) (n = 12)</th>
<th>Ri &amp; p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell count x10^6</td>
<td>3.5 (± 3.11)</td>
<td>4.4 (± 2.82)</td>
<td>0.6 (0.008)</td>
</tr>
<tr>
<td>Neutrophil %</td>
<td>73.4 (± 20.71)</td>
<td>76.1 (± 21.67)</td>
<td>0.6 (0.003)</td>
</tr>
<tr>
<td>Macrophage %</td>
<td>11.7 (± 22.64)</td>
<td>13.3 (± 19.84)</td>
<td>0.6 (0.004)</td>
</tr>
<tr>
<td>Eosinophil %</td>
<td>1.7 (0.54 – 2.94)</td>
<td>1.7 (0.59 – 2.98)</td>
<td>0.7 (0.001)</td>
</tr>
<tr>
<td>Neutrophil cell count, x 10^6</td>
<td>2.1 (± 3.96)</td>
<td>2.3 (± 4.14)</td>
<td>0.8 (0.003)</td>
</tr>
<tr>
<td>Macrophage cell count, x 10^6</td>
<td>0.3 (± 0.39)</td>
<td>0.4 (± 0.38)</td>
<td>0.7 (0.022)</td>
</tr>
<tr>
<td>Eosinophil cell count, x 10^6</td>
<td>0.08 (0.05 – 0.12)</td>
<td>0.09 (0.07 – 0.15)</td>
<td>0.7 (0.026)</td>
</tr>
</tbody>
</table>

Data represented as mean (± SD). Eosinophil % and cell count expressed as median (± range). Abbreviation - TCC: Total cell count. Ri and p value denote Intraclass correlation coefficient and its statistical significance.

Table 4.9: Visit 1 (baseline) and Visit 2 (8 week) spontaneous sputum measurements for sputum producers

<table>
<thead>
<tr>
<th>Sputum Indices</th>
<th>Visit 1 (Baseline) (n = 12)</th>
<th>Visit 2 (8 weeks) (n = 12)</th>
<th>Ri &amp; p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell count x10^6</td>
<td>3.3 (± 2.89)</td>
<td>2.5 (± 3.61)</td>
<td>0.4 (0.267)</td>
</tr>
<tr>
<td>Neutrophil %</td>
<td>68.7 (± 17.94)</td>
<td>78.4 (± 15.69)</td>
<td>0.1 (0.367)</td>
</tr>
<tr>
<td>Macrophage %</td>
<td>20 (± 14.44)</td>
<td>12.3 (± 12.97)</td>
<td>0.3 (0.146)</td>
</tr>
<tr>
<td>Eosinophil %</td>
<td>1.8 (0.61 – 2.92)</td>
<td>1.0 (0.45 – 1.79)</td>
<td>-0.3 (0.513)</td>
</tr>
<tr>
<td>Neutrophil cell count, x 10^6</td>
<td>2.6 (± 3.77)</td>
<td>3.2 (± 3.21)</td>
<td>0.3 (0.156)</td>
</tr>
<tr>
<td>Macrophage cell count, x 10^6</td>
<td>0.3 (± 0.27)</td>
<td>0.5 (± 0.45)</td>
<td>0.3 (0.180)</td>
</tr>
<tr>
<td>Eosinophil cell count, x 10^6</td>
<td>0.06 (0.02 – 0.10)</td>
<td>0.01 (0.00 – 0.04)</td>
<td>-0.1 (0.515)</td>
</tr>
</tbody>
</table>

Data represented as mean (± SD). Eosinophil % and cell count expressed as median (± range). Abbreviation - TCC: Total cell count. Ri and p value denote Intraclass correlation coefficient and its statistical significance.
Sputum culture analysis

The demographics of patients who had sputum culture performed are shown in Table 4.10.

Table 4.10: Demographics of patients who underwent sputum bacteriology

<table>
<thead>
<tr>
<th></th>
<th>Persistent sputum producer (n = 20)</th>
<th>Non producer (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>66.0 (± 6.76)</td>
<td>68.7 (± 4.70)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>11/8</td>
<td>7/6</td>
</tr>
<tr>
<td>Pack years *</td>
<td>39.2 (14 – 83)</td>
<td>47.5 (21.50 – 119.18)</td>
</tr>
<tr>
<td>Current smokers (n)</td>
<td>12/7</td>
<td>5/8</td>
</tr>
<tr>
<td>BMI (kg/m square)</td>
<td>26.4 (± 4.36)</td>
<td>28.5 (± 4.04)</td>
</tr>
<tr>
<td>GOLD I (n)</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>GOLD II (n)</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>GOLD III (n)</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>GOLD IV (n)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SABA (n)</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>SAMA (n)</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>LABA (n)</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>LAMA (n)</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>ICS (n)</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Post bronchodilator FEV₁ % predicted</td>
<td>59 (± 15.06)</td>
<td>71.5 (± 8.85)</td>
</tr>
</tbody>
</table>


Bacteria were detected at a significantly higher rate (p = 0.004) in persistent sputum producers (12 out of 20 patients; 60%) compared to non-producers (1 out of 13; 7.7%). The most frequent micro-organisms isolated in the producer group were *Haemophilus influenzae* [n = 7; mean count: 1.96 (SD 2.36) x 10⁸ cfu/g] and *Streptococcus pneumoniae* [n = 3; mean count: 4.39 (SD 4.91) x 10⁸ cfu/g]. Other species present were *Moraxella catarrhalis* (n = 1), *Staphylococcus aureus* (n = 1), *Haemophilus parainfluenzae* (n = 1) and *Pseudomonas aeruginosa* (n = 1). One persistent sputum producer grew both *H. influenzae* and *S. pneumoniae* and another grew both *H. influenzae* and *M. catarrhalis*. In the non-producer group only one sputum sample was positive for bacteria (*H. influenzae*).

There was no significant difference between the average total bacterial count obtained from persistent sputum producers [1.83 (SD 2.31) x 10⁸ cfu/g] compared to sputum non-producers [6.62 (SD 6.34) x 10⁷; p = 0.3].

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4.4 Discussion

This study demonstrates that COPD patients with persistent sputum production have more severe disease characteristics compared to COPD patients who do not produce sputum. Persistent sputum producers have worse airflow obstruction, evidence of greater hyperinflation and reduced exercise capacity. Furthermore, this group also has greater symptoms and more exacerbations. These clinical differences were accompanied by an increase in the levels of eotaxin, GM-CSF, IL-6, MCP-1, MCP-4 and TNF-α in induced sputum samples from persistent sputum producers. IL-6, MCP-1 and TNF-α are known to upregulate mucin gene transcription (Chen et al. 2003; Monzon et al. 2011; Song et al. 2003), and may be important mediators that drive mucus hypersecretion in patients with persistent sputum production. It is of note that approximately half the number of patients had GM-CSF levels below the lower limit of detection. This can be explained by the fact that in vivo and in vitro GM-CSF is rapidly internalised following receptor binding and therefore it is likely that the measurement of sputum GM-CSF is underestimated by the technique used in this study (Vlahos et al. 2006; Metcalf et al. 1999). Also, the concentration of free GM-CSF is under tight control, with measurable GM-CSF autoantibodies in healthy controls and those with disease (Uchida et al. 2009).

The persistent sputum producers had higher total sputum neutrophil counts compared to sputum non-producers. This is perhaps an expected result, as these persistent sputum producers should have a greater total number of inflammatory cells in the airways associated with an increased tendency to produce sputum. There was no difference in sputum neutrophil differential counts between groups. It is well recognised that the sputum neutrophil percentage is increased in COPD patients compared to controls, indicating a shift in the proportion of cell types within the airways as COPD develops. However, the largest published study of sputum neutrophil percentages within a group of COPD patients shows only very weak correlations between neutrophil percentage and clinical assessments of disease severity, indicating that the proportion of neutrophils in the airways in established COPD has little prognostic value (Singh et al. 2010). The current data also shows little value of neutrophil percentage in distinguishing COPD phenotypes with different prognosis.
Neutrophils and their products play several key roles in EGFR dependent mucus hypersecretion. Neutrophils secrete TNF-α, neutrophil elastase and reactive oxygen species; all of these induce EGFR activation in airway epithelial cells (Takeyama et al. 2000; Kim and Nadel 2004). Neutrophil elastase also causes impairment of ciliary function (Amitani et al. 1991), stimulation of mucin release (Kim et al. 1987) and secretory cell metaplasia and hyperplasia (Christensen et al. 1987). The increased absolute number of neutrophils within the airways of COPD sputum producers may thus be an important mechanism by which these pathophysiological events occur that contribute to the symptoms of increased sputum production.

An increased number of eosinophils was observed in persistent sputum producers; these cells may contribute to mucus hypersecretion through the action of TGF-α (Burgel et al. 2001) or by stimulating degranulation of mucus-producing cells through the release of inflammatory mediators, including cysteinyl leukotrienes (Shimizu et al. 2003b). A previous report has shown a preferential distribution of eosinophils towards the airway lumen in COPD patients with chronic bronchitis (Snoeck-Stroband et al. 2008), and there is also evidence that a subset of patients with chronic bronchitis have increased numbers of eosinophils in induced sputum (Pizzichini et al. 1998). Therefore, although COPD is thought to be a neutrophilic lung disease, the role of eosinophils should not be ignored.

Persistent sputum producers had significantly elevated levels of eotaxin, GM-CSF, IL-6, MCP-1, MCP-4, TNF-α and IL-13 compared to the non-producers. These inflammatory mediators all have biological roles that may be of importance in COPD and mucus hypersecretion; TNF-α is a potent neutrophil stimulant (Ferrante 1992) and GM-CSF prolongs neutrophil survival (Laan et al. 2003). IL-6, IL-13, MCP-1 and TNF-α all induce mucin gene upregulation (Chen et al. 2003; Zhu et al. 1999; Monzon et al. 2011; Song et al. 2003), and so may be responsible for the mucus hypersecretion seen in this phenotype. Previous studies on cytokines in chronic bronchitis have shown significantly higher levels of IL-4, IL-8, IL-13, MCP-1, MIP-1β and TNF-α in the central airways of subjects with chronic bronchitis (Capelli et al. 1999; Miotto et al. 2003a; Chanez et al. 1996; Ma et al. 1999). This study now shows that eotaxin and MCP-4 are raised in persistent sputum producers; these are eosinophil chemo-attractants (Garcia-Zepeda et al. 1996); further supporting a role for eosinophils in mucus hypersecretion.
Mucus hypersecretion can cause plugging of the small airways, predisposing to distal airway collapse. This may be one mechanism of increased closing volume and small airway disease, and lead to air trapping. Another possible mechanism of small airways disease may be the elevated levels of TNF-α found in this group. TNF-α is a pro-inflammatory cytokine and chronic inflammation causes small airway disease and parenchymal destruction, leading to airflow limitation (Cosio Piqueras and Cosio 2001). TNF-α levels correlated with the slope of the nitrogen washout curve which is suggestive of small airways disease. TNF-α is also a neutrophil activator and the slope of the nitrogen washout curve has been shown to correlate with the degree of neutrophilic inflammation in bronchial biopsies of patients with COPD (Lapperre et al. 2007b).

Persistent sputum producers also had a trend towards increased residual volume, suggestive of hyperinflation. There was a reduced KCO in this group, which is usually due to alveolar destruction and hence emphysema in COPD patients. It appears that the persistent sputum producers in this study had two possible causes of hyperinflation; emphysema due to alveolar destruction, and mucus plugging of the small airways.

The multiple regression analyses on sputum IL-6 are in accordance with previous literature that shows that COPD patients with high sputum IL-6 have a more rapid decline over time of FEV\(_1\)% predicted (Donaldson et al. 2005a). This is reflected in the fact that the persistent sputum producers had significantly higher IL-6 levels and lower FEV\(_1\)% predicted compared to the non-producers. Also, in this study, sputum IL-4 levels correlated with the number of exacerbations. The percentage of sputum CD8+IL4+ cells among lymphocytes is significantly increased at the onset of exacerbations in COPD (Makris et al. 2008).

It has previously been reported that mucus hypersecretion in COPD patients is a cause of increased exacerbation rates (Burgel et al. 2009; Miravitlles et al. 2000), and this finding has been observed in this study. A possible mechanism for this may be mucus impaction in the small airways leading to increased respiratory dysfunction. Another reason may be the higher rates of bacterial colonisation in the persistent sputum producers, as observed in this study. The sputum culture results are in keeping with previous literature which shows that *H. influenzae, S. pneumoniae* and *M. catarrhalis* are the most commonly found organisms.
in patients with chronic bronchitis (Tager and Speizer 1975). *P. aeruginosa* has also been isolated from the sputum of 4-15% of adults with COPD in many cross-sectional studies (Murphy et al. 2008).

The data on spontaneous and induced sputum is in line with previous data showing that induced sputum has a higher number of viable cells and less squamous cells compared to spontaneous sputum (Bhowmik et al. 1998). The reduced viability of sputum samples can create practical difficulties in cell identification and the detection of fluid phase proteins (Moretti 1999).

The sputum repeatability results demonstrate that clinical stability in mild to moderate COPD persistent sputum producers is also reflected by the ‘stability’ of surrogate markers in induced sputum. This has important implications for sequential sputum sampling, such as during clinical trials, as less variable methods have increased statistical power. Good repeatability in induced sputum was also observed for sputum lymphocytes, although their repeatability in induced sputum of selected patient populations has been judged to be poor by other authors (Brightling et al. 2001; Purokivi et al. 2000). ICC values for sputum percentages were lower compared to sputum cell counts. This is in keeping with the study by Boorsma showing that the repeatability of sputum differential percentage counts becomes lower over time from analysis performed at one week compared to three months (Boorsma et al. 2007). ICC values for neutrophil, macrophage and eosinophil percentages were also lower in this study compared to previous authors (Boorsma et al. 2007). Differences could be attributed to the different sputum processing techniques. Boorsma et al. (2007) used the whole sputum plug method while the current study used the selected sputum method.

This study however, had certain limitations. Individuals known to have bronchiectasis were included in the study and only a subset of patients had had a previous HRCT. However, the patients who had HRCT were confirmed to have only minimal amount of bronchiectasis likely to be secondary to COPD.

It would also have been useful to have control groups of smokers and non-smokers with normal lung function especially to compare sputum results. However, the sputum cell counts of patients in this study agreed with previous literature on sputum cell counts in
COPD patients (Ronchi et al. 1996), with a higher number of neutrophils when compared to sputum studies on smokers with normal lung function (Stanescu et al. 1996) and a higher number of neutrophils with reversal of the neutrophil/macrophage ratio when compared to sputum studies in healthy subjects (Belda et al. 2000).

The persistent sputum producer group had more current smokers than the non-producer group and this may have had an effect on the sputum inflammatory profile of the two groups. The mean age of the COPD patients in this study was 66. Therefore the ability of this study to explore differences between persistent sputum producers and non-producers in younger COPD patients is limited. Lastly, the study population consisted of individuals with GOLD stage I-III COPD, and no patients in GOLD stage IV. There is also limited representation of GOLD stages I and III, with 55% of the study population belonging to GOLD stage II category.

In conclusion, persistent sputum production in this study was associated with an increased burden of airway inflammation and a range of worse clinical characteristics such as reduced quality of life assessed by the SGRQ and CAT scores, reduced lung function, and reduced exercise capacity. The prognostic BODE index in persistent sputum producers accordingly gave a worse score in persistent sputum producers. It is possible that some of the effects of these markers of COPD severity are mediated through exacerbations. However, it seems plausible that persistent mucus hypersecretion per se affects health status measures and presence of mucus hypersecretion may indicate more persistent airway inflammation as previously proposed (de Oca et al. 2012). The proportion of persistent sputum producers with co-existent bronchiectasis was low. Bronchiectasis may account for persistent sputum production, but it appears that it is not the major cause of persistent sputum production in COPD patients. These results indicate a worse prognosis for COPD persistent sputum producers, which should probably be recognised as a severe phenotype that is a subgroup of patients with chronic bronchitis.
CHAPTER 5: Expression of MUC5B and MUC5AC in COPD persistent sputum producers

5.1 Introduction

The main feature of chronic bronchitis is mucus hypersecretion. Mucins are large glycosylated proteins that impart viscoelastic properties to mucus (Litt et al. 1974; Rose et al. 1979). The major mucin subtypes expressed in the airway epithelium of normal lung tissue are MUC5AC which is restricted to goblet cells and MUC5B which is expressed mainly in submucosal glands. It has been reported that there is an increase in MUC5AC expression in the epithelial cells of the proximal airways of COPD patients and smokers without airway obstruction compared to non-smokers, but no difference in MUC5B expression (Caramori et al. 2009; O'Donnell et al. 2004b). Increased MUC5B expression has been reported in other inflammatory lung diseases (Chen et al. 2001). Kamio et al. examined lung tissues of diffuse panbronchiolitis, a chronic inflammatory airway disease, affecting Asian populations and associated with mucus hypersecretion. This revealed that MUC5B is expressed not only in bronchial glands but also in increased numbers of goblet cells on the bronchial surface (Kamio et al. 2005). It is possible that the lack of difference between COPD patients and controls for MUC5B expression in goblet cells in previous studies is due to the heterogeneous nature of COPD patients, and that perhaps only a subgroup of COPD patients, such as those with severe chronic bronchitis, have an upregulation of MUC5B expression in goblet cells.

The hypothesis was that previous studies of MUC5AC and MUC5B expression in the bronchial epithelium conducted in heterogeneous COPD populations have overlooked the possibility that these mucins may have unique expression patterns in the subgroup of COPD patients with severe chronic bronchitis who have persistent sputum production on most days, and this study was undertaken to investigate the expression of these mucins in COPD persistent sputum producers and control groups (COPD sputum non-producers, smokers with normal lung function and lifelong healthy non-smokers with normal lung function).
5.2 Methods

5.2.1 Subjects and study design

Forty subjects were recruited from the database of the Medicines Evaluation Unit to undergo fibreoptic bronchoscopy according to approved BTS guidelines (Du Rand et al. 2013); 20 COPD patients, 10 asymptomatic smokers with normal lung function (S), and 10 lifelong healthy non-smokers with normal lung function (HNS). COPD was diagnosed according to current standards (GOLD 2011) and patients were divided into persistent sputum producer (n = 10) and non-producer categories (n = 10) based on validated American Thoracic Society questionnaire (ATS 1969); Patients bringing up phlegm at least twice a day for four or more days of the week were categorized as persistent sputum producers, while those who produce phlegm only during an exacerbation were categorized as non-producers. Subjects were excluded if they had experienced a respiratory tract infection or exacerbation of COPD in the preceding six weeks.

Histology

Bronchial biopsy slides from each subject were stained with H&E and PAS (section 3.9) and staining analysis performed as described previously (section 3.11).

Immunohistochemistry

Sections from each biopsy were also stained against MUC5AC and MUC5B antigens (section 3.10) and staining analysis performed as described previously (section 3.11). Levels of MUC5AC and MUC5B immunoreactivity were recorded as percentage of total area of bronchial epithelium positive for MUC5AC and MUC5B.

5.2.2 Statistical Analysis

Normality of the data was determined using the Kolmogorov-Smirnov test. Normally distributed data are presented as means with standard deviations, and non-parametric data as median with range. When four groups were compared, one-way analysis of variance (ANOVA) was performed followed by the application of post-tests. To assess correlations, Pearson’s or Spearman’s test was used, depending upon the normality of the data. Statistical analysis was conducted using GraphPadInstat version 5 (GraphPad Software, Inc, San Diego, CA, USA).
5.3 Results

The demographics of the subjects are shown in Table 5.1. Subjects were relatively well-matched with regards to age and there was no significant difference in the smoking history between COPD persistent sputum producers, COPD non-producers and smokers with normal lung function (S). As expected from the selection criteria, smokers with COPD had a significantly lower FEV$_1$ % predicted and FEV$_1$/FVC ratio compared with S (p < 0.01) and healthy non-smokers (HNS) (p < 0.01). Also, COPD persistent sputum producers had significantly lower FEV1% predicted compared to COPD non-producers (p < 0.05).

Table 5.1: Subject demographics

<table>
<thead>
<tr>
<th></th>
<th>COPD Persistent sputum producer (n = 10)</th>
<th>COPD Non-producer (n = 10)</th>
<th>Smokers (S, n = 10)</th>
<th>Non-smokers (HNS, n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>59.4 (± 7.6)</td>
<td>62.3 (± 6.4)</td>
<td>51.1 (± 8.3)</td>
<td>47.7 (± 13.2)</td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>7/3</td>
<td>6/4</td>
<td>7/3</td>
<td>6/4</td>
</tr>
<tr>
<td>Current/Ex-smoker</td>
<td>6/4</td>
<td>5/5</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Pack years *</td>
<td>37.6 (14.6 – 63.2)</td>
<td>40.9 (19.2 – 55.6)</td>
<td>30.0 (18.2 – 41.1)</td>
<td>-</td>
</tr>
<tr>
<td>FEV$_1$/FVC %</td>
<td>45.0 (± 17.1)</td>
<td>50.2 (± 10.3)</td>
<td>78.8 (± 10.3)</td>
<td>79.5 (± 12.4)</td>
</tr>
<tr>
<td>FEV$_1$% predicted *</td>
<td>49.4 (± 8.7)</td>
<td>59.7 (± 11.2)</td>
<td>100.5 (± 7.7)</td>
<td>106.8 (± 11.0)</td>
</tr>
<tr>
<td>ICS usage</td>
<td>7</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ICS (µg/day)</td>
<td>1192 (± 976.)</td>
<td>1201 (± 844)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Data is represented as mean (± standard deviation). * data represented as median (range). * p < 0.05 between COPD persistent sputum producers and COPD non-producers; p < 0.01 in COPD (producers + non-producers) versus smokers and non-smokers. Abbreviation: FEV$_1$ - forced expiratory volume in first second; FVC - forced vital capacity; ICS - inhaled corticosteroid.
Histological analysis

H&E staining of bronchial biopsies showed significantly higher epithelial staining grades in both COPD groups and smokers with normal lung function (S), indicating increased bronchial epithelial remodelling compared to healthy non-smokers with normal lung function (HNS; p < 0.01). Epithelial staining grades in COPD persistent sputum producers, COPD non-producers and S were similar (Figure 5.1).

![Figure 5.1: Difference in H&E staining in the bronchial surface epithelium in COPD and control groups. A 5 point scoring system was adopted to grade the H&E staining (Appendix 3a). Horizontal bars represent means. Error bars represent ± 1SD.](image-url)
PAS staining showed significantly higher epithelial staining grades in the COPD persistent sputum producers compared to COPD non-producers ($p = 0.05$) and HNS ($p < 0.01$), with a trend towards significance for S ($p = 0.06$), indicating more goblet cell hyperplasia. PAS staining grades were significantly higher in COPD non-producers and S compared to HNS ($p = 0.01$ and $0.02$ respectively). These findings are illustrated in Figure 5.2 below.

**Figure 5.2: Difference in PAS staining in the bronchial surface epithelium in COPD and control groups.** A 5 point scoring system was adopted to grade the PAS staining (Appendix 3b). Horizontal bars represent geometric means. Error bars represent $\pm$ 1SD.
MUC5AC expression in the bronchial surface epithelium

MUC5AC expression was successfully demonstrated in all four cohorts. Representative images of the staining are shown in Figure 5.3 below.

Figure 5.3: Representative photomicrographs of bronchial surface epithelium immunostained for MUC5AC (brown). A. COPD persistent sputum producer; B. COPD non-producer; C. Healthy current smoker; D Healthy non-smoker; E. Control upper airway and F. PAS of upper airway. Abbreviation: C. Cartilage, SG. Submucosal Glands, AL. Airway Lumen, EP. Epithelium. Scalebar: low magnification = 50μm, high magnification = 500μm.
MUC5AC expression was numerically higher in COPD persistent sputum producers [15.17 ± 7.09 %] compared to COPD non-producers [10.67 ± 4.94 %], but this difference did not reach statistical significance (p = 0.07). There was a significant difference between COPD producers and S [9.49 ± 4.90 %] (p < 0.01) and COPD producers and HNS [4.23 ± 3.53 %] (p < 0.01). There was also a significant difference between COPD non-producers and HNS (p = 0.01) and S and HNS (p = 0.01). There was no significant difference between COPD non-producers and S (p = 0.54). This is illustrated in Figure 5.4.

Figure 5.4: MUC5AC expression in bronchial surface epithelium in COPD and control groups. Columns represent geometric means. Error bars represent ± 1SD.
**MUC5B expression in the bronchial surface epithelium**

MUC5B expression was successfully demonstrated in all four cohorts. Representative images of the staining are shown in Figure 5.5.

**Figure 5.5: Representative photomicrographs of bronchial surface epithelium immunostained for MUC5B (brown).** A. COPD persistent sputum producer; B. COPD non-producer; C. Healthy current smoker; D. Healthy non-smoker; E. MUC5B in submucosal glands of a COPD patient and F. negative control. Scalebar = 50μm.
MUC5B expression was significantly higher in the COPD persistent sputum producers [8.69 ± 6.77 %] compared to COPD non-producers [3.06 ± 2.45 %] (p = 0.04), S [1.39 ± 1.10 %] (p = 0.002) and HNS [1.65 ± 1.69 %] (p = 0.003). There were no differences in MUC5B expression between COPD non-producers, S and HNS. This is illustrated in Figure 5.6.

**Figure 5.6:** MUC5B expression in bronchial surface epithelium in COPD and control groups. Columns represent geometric means. Error bars represent ±1SD.
Correlation between mucin immunoreactivity and clinical parameters

Correlation between mucin immunoreactivity and clinical parameters was performed for all the subjects. An inverse relationship was found between MUC5AC expression in bronchial surface epithelium and FEV₁ % predicted (r = -0.44, p = 0.01). A direct correlation was found between pack years history and MUC5AC expression in bronchial surface epithelium (r = 0.32, p = 0.04). There was significantly higher MUC5AC expression in current vs. ex-smokers (p = 0.03).

An inverse relationship was found between MUC5B expression in bronchial surface epithelium and FEV₁% predicted (r = -0.45, p = 0.01). No correlation was found between MUC5B expression in the surface epithelium and pack year history (r = 0.22, p = 0.18). There was no significant difference in MUC5B expression between current and ex-smokers (p = 0.36). Figure 5.7 illustrates these results.
Figure 5.7: Correlation between mucin expression and clinical parameters. Figures showing (a) correlation between MUC5B and MUC5AC and FEV$_1$% predicted; (b): correlation between MUC5B and MUC5AC expression and pack years and (c): Effect of smoking status on MUC5B and MUC5AC expression.
5.4 Discussion

This study reports for the first time that increased MUC5B expression in the bronchial epithelium is a feature of the phenotype of COPD patients with persistent sputum production.

The hypothesis of this study was that previous studies of MUC5AC and MUC5B expression conducted in heterogeneous COPD populations have overlooked the unique expression patterns that exist in the phenotype of COPD patients with severe chronic bronchitis who have persistent sputum production. The results for MUC5AC and MUC5B certainly show unique expression profiles for these mucins, and signify the value of detailed clinical phenotyping of COPD patients when conducting studies using lung samples to understand disease mechanisms; “Lumping” all COPD patients into one group can hide potentially important biological signals, while “splitting” patients into discrete phenotypes can uncover mechanisms restricted to subgroups of patients.

For example, previous studies on large airways have shown no difference in MUC5B expression in the surface epithelium between COPD patients and smokers and healthy non-smokers (Caramori et al. 2009; O'Donnell et al. 2004b). Another study by Innes et al. found decreased expression of MUC5B in the surface bronchial epithelium of smokers compared with non-smoking subjects (Innes et al. 2006). This may be due to the absence of an age-matched control group in the study performed by Innes et al. and/or due to difference in the immunohistochemistry methodology used in this study. Furthermore, Lapperre et al. reported no difference in the total mucin content of the bronchial epithelium of the central airways of COPD patients with chronic bronchitis compared to those without chronic bronchitis (Lapperre et al. 2007a). The current study chose a different approach, by selecting patients who fitted a more severe definition of chronic bronchitis, and was able to uncover differences in mucin expression between different groups of COPD patients. There was higher MUC5B expression in COPD persistent sputum producers compared to control groups but no difference between COPD non-producers, healthy smokers and healthy non-smokers. It should therefore be noted that the positive findings relate specifically to COPD patients with persistent sputum production, and cannot be generalised to all patients with chronic bronchitis.
Clinicopathological studies performed in COPD patients have found a correlation between the presence of chronic bronchitis and submucosal gland hypertrophy in the central airways of smokers (Jamal et al. 1984; Barnes 2000). It is likely that the mucin output from the submucosal glands, which are the main site of MUC5B production, is also significantly higher in COPD persistent sputum producers versus COPD non-producers. However unfortunately, this study was not able to assess the expression of mucins in the submucosal glands because glands were either partially present, completely absent or distorted by crush artefact, in the vast majority of biopsies.

MUC5B expression correlated to FEV$_1$% predicted, an established measure of airflow obstruction. Increasing amounts of MUC5B in sputum have also been shown to correlate with FEV$_1$ (Kirkham et al. 2008).

Previous studies on large and small airways have shown that MUC5AC expression is increased in the epithelial cells in COPD patients compared to controls (Innes et al. 2006; Caramori et al. 2004; Ma et al. 2005). This study also found increased MUC5AC expression in the large airways of both COPD phenotypes compared to healthy non-smokers, and in COPD persistent sputum producers compared to healthy smokers. It is likely that MUC5AC expression was also increased in the peripheral airways of these COPD patients, especially COPD persistent sputum producers. Mucus plugging is an important cause of small airway obstruction and this could explain why COPD persistent sputum producers had more airflow obstruction than COPD non-producers. In contrast to the above, some other studies have shown no difference in MUC5AC expression between COPD and smokers with normal lung function (Caramori et al. 2009; O'Donnell et al. 2004b). Again, the reason for this may lie in a mixed population of patients with varying symptoms of chronic bronchitis being used in the COPD group in these studies.

A correlation was found between pack year history and MUC5AC expression, and current smokers had higher MUC5AC epithelial expression than ex-smokers. This data is in line with *in vitro* studies demonstrating that tobacco smoking (and its components such as acrolein and oxidants) increases the synthesis of MUC5AC in bronchial epithelial cells through the action of NF-κB (Rose and Voynow 2006). There was an inverse relation
between MUC5AC expression and FEV$_1$% predicted, as shown previously (Caramori et al. 2009).

H&E staining results, as expected suggest that smoking and COPD are associated with various degrees of bronchial epithelial changes (Auerbach et al. 1979; Jeffery 2000). PAS staining was increased in the COPD persistent sputum producers compared to control groups indicating more goblet cell hyperplasia in the sputum producers, in accordance with previous literature on chronic bronchitis (Saetta et al. 2000; Jeffery and Zhu 2002).

Compared to the study by Caramori (Caramori et al. 2009), this work found a much lower area of bronchial surface epithelium expressing MUC5AC as well as MUC5B in all the cohorts. This is probably related to the fact that Caramori et al. analysed the number of MUC5AC and MUC5B positive cells in the surface epithelium rather than the percentage positive area of MUC5AC and MUC5B staining. The technique used in this study of quantifying mucins using percentage positive area of immunostaining in the surface epithelium was adopted to minimise the level of subjectivity in the quantification of mucins. Furthermore, as mucins are secreted by cells they are not confined to the cellular compartment but can be found anywhere in the epithelium. Therefore, this technique of quantifying mucins is likely to provide a more accurate picture of mucin expression in the different phenotypes of COPD. Also, Caramori et al. used resected bronchus as the study sample so had more epithelium as well as glands in the sample. This may be one limitation of bronchoscopy samples which may provide only a small snapshot of the airway.

There could be a number of reasons for the altered mucin profile in COPD persistent sputum producers. One reason could be MUC5B gene polymorphisms in this phenotype of COPD, as reported in subjects with diffuse panbronchiolitis who have mucus hypersecretion and increased MUC5B expression in the surface epithelium (Kamio et al. 2005). Another may be the altered post-translational modification of MUC5B and MUC5AC genes in COPD persistent sputum producers as mucin protein backbones can be glycosylated differently in the same tissues (Kirkham et al. 2002; Davies et al. 1996). Glycosylation can stabilize mucins at the cell surface, by limiting their endocytosis (Altschuler et al. 2000; Engelmann et al. 2005) and by protecting the polypeptide chain from degradation by extracellular proteases (Loomes et al. 1999). Mucin glycans are known receptors for microorganisms and changes in glycosylation may also favour
bacterial colonization in the sputum producers, increasing the risk of infection (Sethi and Murphy 2001a). Furthermore, increased amounts of the low-charge density MUC5B might form a tighter gel that is less efficiently transported, and this could result in reduced lung function and increased incidence of infections, leading to exacerbations (Kirkham et al. 2008). MUC5B may also be less susceptible to proteolytic degradation than MUC5AC (Davies et al. 1999).

The previous study showed that persistent sputum production in COPD is associated with an increased burden of airway inflammation and it has been reported that the expression of MUC5B in surface goblet cells is increased in inflammatory lung diseases (Chen et al. 2001). This is in keeping with the finding of increased MUC5B expression in the bronchial surface epithelium of persistent sputum producers in this study. The elevated levels of pro-inflammatory mediators in the sputum producers may also regulate MUC5AC and MUC5B gene expression (Thai et al. 2008). Persistent sputum producers had higher levels of IL-6, IL-13, MCP-1 and TNF-α compared to the sputum non-producers. IL-6 and TNF-α are both pro-inflammatory cytokines and have previously been shown to cause mucin gene upregulation (Chen et al. 2003; Song et al. 2003). MCP-1 has also been shown to induce MUC5AC and MUC5B protein expression (Monzon et al. 2011). IL-13 causes goblet cell hyperplasia and can induce MUC5AC gene expression (Zhu et al. 1999). These cytokines may thus have an important role to play in the higher expression of mucins in the COPD persistent sputum producers and thus in mucus hypersecretion.

In conclusion, this study shows that MUC5B expression is higher in the COPD persistent sputum producers and this may account for the mucus hypersecretion seen in this phenotype of COPD.
CHAPTER 6: Expression of Hypoxia-inducible factor (HIF-1α) and Carbonic Anhydrase IX (CAIX) in COPD persistent sputum producers and association of HIF-1α with MUC5B expression

6.1 Introduction

In COPD large airway remodelling is accompanied by significant thickening and fibrosis of the subepithelial basement membrane, reduction of subepithelial microvasculature and perivascular fibrosis (Polosukhin 2001). These processes reduce oxygenation of the airway mucosa, leading to activation of HIF-1α in the lungs of COPD subjects (Polosukhin et al. 2007a). Cigarette smoke itself has also been shown to induce HIF-1α production and activation to upregulate MUC5AC synthesis via EGFR-mediated signalling pathways, including ERK1/2 and PI3K (Yu et al. 2012a).

Recent studies have suggested a role of hypoxia inducible signalling in mucus production in COPD. HIF-1α increases the transcription of MUC5AC (Zhou et al. 2012; Young et al. 2007) and drives goblet cell hyperplasia in COPD patients (Polosukhin et al. 2011). HIF-1α may also up-regulate other genes important for goblet cell differentiation and function, such as the trefoil factor family (Hernandez et al. 2009). Furthermore, Goven et al. showed that HIF-1α induces heme oxygenase-1 (HO-1) expression in patients who smoke and have a primary spontaneous pneumothorax (Goven et al. 2010). Previous studies indicate that STAT6 and HO-1 play important roles in MUC5AC production in COPD (Almolkii et al. 2008; Kuperman et al. 1998). This data indicates an important effect of hypoxia-inducible signalling in mucus hypersecretion in COPD. Based on this, it is also likely that HIF-1α expression is different in COPD persistent sputum producers compared to sputum non-producers. The previous work in this thesis has shown that COPD persistent sputum producers have increased expression of MUC5B in the bronchial surface epithelium. However, unlike MUC5AC, it is not been reported whether HIF-1α can affect MUC5B expression.

This study was therefore set up to investigate if 1) the expression of HIF-1α in the bronchial surface epithelium is different in COPD persistent sputum producers compared to control groups and 2) whether MUC5B expression is regulated by HIF-1α, by
investigating the effect of hypoxia induced HIF-1 upregulation on MUC5B expression in bronchial epithelial cells *in vitro*.

To investigate the association between HIF-1α and MUC5B expression, 16HBE cells were used. 16HBE cells are a continuously proliferating and dividing source of homogenous primary lung epithelial cells. The cells reach confluence after 7-10 days in culture, at which stage they need to be removed, divided and reseeded in fresh medium. 16HBE cells were utilised because of their use in similar experiments before (Yu et al. 2012b; Olson et al. 2011).

The expression of HIF is principally controlled at the posttranslational level by prolyl hydroxylase (PHD) enzymes which cause degradation of the oxygen-labile α subunit of HIF. Therefore, to upregulate HIF in the cell cultures, DMOG which is a prolyl hydroxylase enzyme inhibitor was used at a standard concentration of 1mM (Asikainen et al. 2005; Jaakkola et al. 2001).
6.2 Expression of HIF-1α and CAIX in bronchial biopsies of COPD and control groups

6.2.1 Methods

6.2.1.1 Subjects and study design

IHC was used to identify areas of HIF-1α and CAIX expression in the bronchial surface epithelium of the cohort of patients previously described; COPD persistent sputum producers (n = 10), COPD non-producers (n = 10), asymptomatic smokers with normal lung function (S; n = 10) and lifelong non-smokers with normal lung function (HNS; n = 10). Staining analysis was performed as previously documented (section 3.11).
Lung physiology and airway inflammation in COPD patients with persistent sputum production

6.2.1.2 Statistical Analysis

Normality of the data was determined using the Kolmogorov-Smirnov test. Normally distributed data are presented as means with standard deviations, and non-parametric data as median with range. When four groups were compared, one-way analysis of variance (ANOVA) was performed followed by the application of Tukey’s post-test. To assess correlations, Pearson’s or Spearman’s test was used, depending upon the normality of the data. Statistical analysis was conducted using GraphPadInstat version 5 (GraphPad Software, Inc, San Diego, CA, USA).

6.2.2 Results

The demographics of the patients are shown in Table 6.1.

Table 6.1: Subject demographics (n = 40)

<table>
<thead>
<tr>
<th></th>
<th>COPD Persistent sputum producer (n = 10)</th>
<th>COPD Non-producer (n = 10)</th>
<th>Smokers (S, n = 10)</th>
<th>Non-smokers (HNS, n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>59.4 (± 7.6)</td>
<td>62.3 (± 6.4)</td>
<td>51.1 (± 8.3)</td>
<td>47.7 (± 13.2)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>7/3</td>
<td>6/4</td>
<td>7/3</td>
<td>6/4</td>
</tr>
<tr>
<td>Current/Ex-smoker</td>
<td>6/4</td>
<td>5/5</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Pack years #</td>
<td>37.6 (14.6 – 63.2)</td>
<td>40.9 (19.2 – 55.6)</td>
<td>30.0 (18.2 – 41.1)</td>
<td>-</td>
</tr>
<tr>
<td>FEV₁/FVC %</td>
<td>45.0 (± 17.1)</td>
<td>50.2 (± 10.3)</td>
<td>78.8 (± 10.3)</td>
<td>79.5 (± 12.4)</td>
</tr>
<tr>
<td>FEV₁ % predicted *</td>
<td>49.4 (± 8.7)</td>
<td>59.7 (± 11.2)</td>
<td>100.5 (± 7.7)</td>
<td>106.8 (± 11.0)</td>
</tr>
<tr>
<td>ICS usage</td>
<td>7</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ICS (μg/day)</td>
<td>1192 (± 976.)</td>
<td>1201 (± 844)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Data is represented as mean (± standard deviation), # data represented as median (range). * p < 0.05 between COPD persistent sputum producers and COPD non-producers; p < 0.01 in COPD (producers + non-producers) versus smokers and non-smokers. Abbreviation: FEV₁ - forced expiratory volume in first second; FVC – forced vital capacity; ICS - inhaled corticosteroid.
**HIF-1α expression in the bronchial surface epithelium**

Representative images of staining for HIF-1α in the different cohorts are shown in Figure 6.1 below.

![Representative images of HIF-1α staining](image)

**Figure 6.1: Representative photomicrographs of bronchial surface epithelium immunostained for HIF-1α (brown).** A. COPD persistent sputum producer; B. COPD non-producer; C. Healthy current smoker; D. Healthy non-smoker; E. COPD persistent sputum producer to compare with F. Isotype control, same patient and area as E. Scalebar = 50μm.
HIF-1α expression

There was significant between individual variation of HIF-1α expression; it was noted that 21 patients who received oxygen during bronchoscopy had minimal HIF expression in the bronchial epithelium, which was lower compared to patients who had not received oxygen during bronchoscopy \((p = 0.02)\) (Table 6.2, Figure 6.2). It was therefore not possible to perform statistical analysis between groups for HIF-1α in the small number of patients who had not received oxygen.

Table 6.2: Expression of HIF-1α in subjects who received oxygen during bronchoscopy vs. subjects who did not receive oxygen during bronchoscopy

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean % positive area of HIF-1α in subjects with no oxygen during bronchoscopy</th>
<th>Mean % positive area of HIF-1α in subjects with oxygen during bronchoscopy</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COPD sputum producer</td>
<td>15.04 (± 5.31) ((n = 3))</td>
<td>0.76 (± 0.67) ((n = 7))</td>
<td>0.02*</td>
</tr>
<tr>
<td>COPD non-producer</td>
<td>5.07 (± 1.80) ((n = 3))</td>
<td>0.55 (± 0.48) ((n = 7))</td>
<td>0.02*</td>
</tr>
<tr>
<td>Healthy smoker (S)</td>
<td>4.71 (± 3.70) ((n = 6))</td>
<td>0.14 (± 0.18) ((n = 4))</td>
<td>0.01*</td>
</tr>
<tr>
<td>Healthy non-smoker (HNS)</td>
<td>1.06 (± 0.79) ((n = 7))</td>
<td>0.04 (± 0.05) ((n = 3))</td>
<td>0.02*</td>
</tr>
</tbody>
</table>

Data represented as mean (± SD). Abbreviation: SD – standard deviation. * denotes \(p \leq 0.05\)

Figure 6.2: Effect of oxygen on HIF-1α expression. Comparison between subjects who received oxygen during bronchoscopy \((n = 21)\) versus those who did not receive oxygen during bronchoscopy \((n = 19)\).
CAIX expression in the bronchial surface epithelium

Representative images of staining for CAIX in the different cohorts are shown in Figure 6.3 below.

Figure 6.3: Representative photomicrographs of bronchial surface epithelium immunostained for CAIX (brown). A. COPD persistent sputum producer (grade 3); B. COPD non-producer (grade 2 with areas of grade 1); C. Healthy smoker (grade 2 with areas of grade 1); D. Healthy non-smoker (grade 1) and E. Isotype control. Scalebar = 50μm.
Correlation of HIF-1α to CAIX expression

CAIX expression was strongly correlated to HIF expression in the 19 patients (3 COPD persistent sputum producers, 3 COPD non-producers, 6 S and 7 HNS) who did not receive oxygen ($r = 0.85$, $p < 0.0001$) (Figure 6.4). Furthermore, areas of increased HIF-1α expression also showed increased CAIX expression in these patients (Figure 6.5).

**Figure 6.4:** Correlation between HIF-1α and CAIX expression in the bronchial epithelium. Correlation of percentage positive areas of immunostaining for HIF-1α and CAIX in the bronchial epithelium in 19 subjects (3 COPD persistent sputum producers, 3 COPD non-producers, 6 healthy smokers and 7 healthy non-smokers) who had not received oxygen during bronchoscopy.

**Comparison of CAIX and HIF-1α immunostaining**

**Figure 6.5:** Representative photomicrographs of a section of bronchial epithelium immunostained for HIF-1α (A) and CAIX (B). The same area is immunostained in both the sections. Scalebar = 500μm.
CAIX expression in the bronchial surface epithelium

CAIX expression scores were significantly higher in COPD persistent sputum producers [9.90 ± 2.64] compared to COPD non-producers [4.3± 3.47](p = 0.002), S [5.00 ± 11.79] (p = 0.01) and HNS [2 ± 1.51](p = 0.0002). There was a significant difference in CAIX expression between COPD non-producers and S compared to HNS (p = 0.04 and p = 0.03 respectively). There was no difference in CAIX expression between COPD non-producers and S (p = 0.69) (Figure 6.6).

Figure 6.6: CAIX expression in the bronchial surface epithelium in COPD and control groups. CAIX IHC score calculated by multiplying the staining intensity grade with the grade for the percentage of bronchial surface epithelium immunostained. Columns represent geometric means, error bars represent ±1SD.
Correlation of CAIX immunoreactivity with mucin expression and clinical parameters

CAIX expression had a weak positive correlation to MUC5B expression (r = 0.33, p = 0.03) and MUC5AC expression (r = 0.27, p = 0.08). An inverse correlation was found between CAIX expression and FEV₁% predicted (r = -0.46, p = 0.001). A weak positive correlation was found between CAIX expression and pack year history (r = 0.34, p = 0.03). CAIX expression was higher in current smokers compared to ex-smokers (p = 0.05). Figure 6.7 illustrates these results.
Figure 6.7: Correlations of CAIX expression in the bronchial epithelium. Figures showing (a) correlation between CAIX and mucin expression; (b) correlation between CAIX expression and FEV$_1$% predicted & pack years; (c): Effect of smoking status on CAIX expression.
6.3 To investigate if HIF-1α can upregulate MUC5B expression in vitro

Preliminary experiments

Initial experiments were run to assess if DMOG upregulated HIF and MUC5B expression in vitro. DMOG was added at 1 mM concentration to the left sided wells of an eight well chamber slide and after 24 hours the chamber slide was fixed in ice cold acetone: methanol (1:1, vol:vol) and HIF-1α and MUC5B expression studied using the immunohistochemistry protocols previously optimised for these. The only modification was that PBS was used as buffer for washing and antibody dilution instead of TBS and heat induced antigen retrieval step was omitted. Also, 100 mM glycine in PBS for five minutes was used to reduce the disulfide bonds in the MUC5B wells (Harrop et al. 2012).
Result

DMOG caused expression of both HIF-1α and MUC5B at 24 hours (Figure 6.8).

Figure 6.8(a): Representative photomicrographs of HIF-1α staining in DMOG positive (A) and negative sections (B) at 24 hours. Scalebar = 50μm.

Figure 6.8(b): Representative photomicrographs of MUC5B staining in DMOG positive (A) and negative (B) sections at 24 hours. Scalebar = 50μm.
Time course experiment to assess HIF-1α and MUC5B expression

The experiment was designed using four different time points (1, 4, 16 and 24 hours), chosen based on previous studies that have shown that DMOG causes stabilisation of HIF-1 from four hour onwards (Ockaili et al. 2005). Preliminary experiments had shown that DMOG caused both HIF and MUC5B expression at 24 hours.

1mM DMOG was added to HIF and MUC5B positive and negative wells of the chamber slides at 1, 4, 16 and 24 hour time points. The slides were then fixed in ice-cold acetone: methanol (1:1) and immunocytochemistry for HIF-1α and MUC5B carried out according to previously optimised protocols. The design of the chamber slide used for the experiment is shown in Figure 6.9 below.

<table>
<thead>
<tr>
<th>(A) HIF +</th>
<th>(B) HIF +</th>
</tr>
</thead>
<tbody>
<tr>
<td>(C) HIF -</td>
<td>(D) HIF -</td>
</tr>
<tr>
<td>(E) MUC5B +</td>
<td>(F) MUC5B +</td>
</tr>
<tr>
<td>(G) MUC5B -</td>
<td>(H) MUC5B -</td>
</tr>
</tbody>
</table>

Figure 6.9: Design of the chamber slide used for the time course experiment. The shaded left sided wells (A, C, E and G) are DMOG positive and the right sided wells (B, D, F and H) are DMOG negative. Wells B and F are designed to see if HIF and MUC5B primary antibodies cause any staining in the absence of DMOG. Wells C and G were designed to see if DMOG itself causes any non-specific staining compared to control DMOG negative wells (D and H).

Digital micrographs were obtained using a Nikon Eclipse 80i microscope (Nikon UK Ltd., Surrey, UK) equipped with a QImaging digital camera (Media Cybernetics, Marlow, UK).
6.3.2 Results

For each time-point, no staining was observed in the DMOG only positive wells (C and G) compared to the control DMOG negative wells (D and H). Thus DMOG itself did not cause any non-specific staining.

Also for each point, no significant staining was observed in the HIF and MUC5B positive, DMOG negative wells (B and F). Thus, in the absence of DMOG, HIF-1α and MUC5B were not expressed.

At 1 hour, no staining was observed in the HIF positive, DMOG positive (A) or the MUC5B positive, DMOG positive (E) well.

At 4 hours, strong intracellular staining was seen in the HIF positive, DMOG positive well (A) but no staining was observed in the MUC5B positive, DMOG positive well (E).

At 16 and 24 hours, strong staining was seen in both the HIF positive, DMOG positive well (A) and the MUC5B positive, DMOG positive well (E). These results are shown in Figure 6.10.
Figure 6.10 (a): Representative photomicrographs of HIF-1α and MUC5B expression at 1, 4, 16 and 24 hour time points in the DMOG+, HIF+ (A) and DMOG+, MUC5B+ (E) wells. Scalebar = 50μm.
Figure 6.10 (b): Representative photomicrographs of HIF-1α and MUC5B expression at 1, 4, 16 and 24 hour time points in the DMOG-, HIF+ (B) and DMOG-, MUC5B+ (F) wells. Scalebar = 50μm.
Figure 6.10 (c): Representative photomicrographs of HIF-1α and MUC5B expression at 24 hours in the DMOG+, HIF- (C) and DMOG+, MUC5B- (G) wells. Scalebar = 50μm.

The experiment was run three times to ensure reproducibility and the same result was obtained each time.
6.4 Discussion

This is the first study to report increased expression of carbonic anhydrase IX (CAIX), which is a stable downstream marker of hypoxia inducible factor-1α (HIF-1α), in the bronchial epithelium of COPD persistent sputum producers compared to COPD sputum non-producers and healthy controls.

Comparison of HIF-1α immunostaining in COPD patients compared to controls was not possible due to the expression of HIF-1α being affected by the use of oxygen during bronchoscopy; HIF-1α has a short half-life (< 10 minutes under normoxic conditions) and gets degraded immediately on exposure to oxygen (Salceda and Caro 1997; Zagorska and Dulak 2004).

Carbonic anhydrase IX (CAIX) is a transmembrane protein that acts as a pH regulator in tissue that has become hypoxic due to increased expression of HIF-1α (Beasley et al. 2001). CAIX has a much longer half-life (38 hours) (Rafajova et al. 2004a) than HIF-1α so it was anticipated that even those patients who had received oxygen during bronchoscopy would express CAIX. CAIX was therefore used as an alternate marker of hypoxia.

CAIX expression highly correlated to HIF-1α expression both in terms of quantitative immunohistochemical scores and qualitative staining distribution. The data suggest that CAIX can be used to elucidate areas of HIF-1α expression. CAIX expression was found to be significantly higher in COPD persistent sputum producers compared to all other subject groups. Based on the good correlation between CAIX and HIF-1α immunostaining, it may be deduced also that HIF-1α expression is increased in COPD persistent sputum producers compared to all other subject groups.

A recent study has shown that HIF-1α can drive goblet cell hyperplasia and increased MUC5AC expression in COPD (Polosukhin et al. 2011). In accordance with this, in the previous chapter, significantly higher grades of PAS staining were found in COPD persistent sputum producers compared to COPD non-producers. MUC5AC expression was also found to be higher in the COPD persistent sputum producers compared to COPD non-producers with a trend towards statistical significance. Smoking is another cause of HIF-1α activation (Yu et al. 2012a) and significantly higher expression of CAIX was found in smokers with normal lung function compared to non-smokers.
The time course experiment examining the expression of HIF-1α and MUC5B \textit{in vitro} showed that HIF-1α can potentially increase MUC5B expression in bronchial epithelial cells. The relatively short time in which HIF-1α could cause MUC5B expression implies that HIF-1α may directly upregulate the \textit{MUC5B} gene. Another reason of increased MUC5B expression in persistent sputum producers may be HIF-1α driving goblet cell hyperplasia (Polosukhin et al. 2011). The main source of epithelial MUC5B in the sputum producers is likely to be goblet cells although there may be some contribution from the basal cells (Casalino-Matsuda et al. 2009). Increased number of goblet cells could lead to increased MUC5B expression resulting in mucus hypersecretion in this phenotype of COPD.

In addition to hypoxia, HIF-1α can also be stabilised and activated in response to growth factors and pro-inflammatory cytokines, such as IL-1β, EGF and TNF-α (Jiang et al. 2010; Dery et al. 2005b), vascular hormones (Richard et al. 2000) and viral proteins (Wakisaka et al. 2004); these are relevant pathophysiological triggers in the lungs of COPD patients. Whereas tissue hypoxia is associated with protein stabilisation and increase in half-life, normoxic activation occurs via increased HIF-1α protein synthesis or its modification. As CAIX is only activated by HIF-1α under hypoxic conditions, consequently, these findings present a strong case that hypoxia is a driving force for increased HIF-1α and subsequent CAIX expression.

In the first study, significantly higher levels of TNF-α were found in COPD persistent sputum producers compared to sputum non-producers. TNF-α has been shown to induce expression and transcriptional activity of HIF-1α under normoxic conditions and amplifies HIF-1α activation in hypoxia (Jiang et al. 2010). Elevated levels of this pro-inflammatory cytokine could be a mechanism of HIF-1α upregulation in COPD persistent sputum producers. Furthermore, TNF-α induces \textit{MUC} gene expression via ERK or the p38 MAPK pathway \textit{in vitro} (Song et al. 2003) and analysis of signal transduction pathways in cells with HIF-1α activation shows increased ERK1/2 phosphorylation (Polosukhin et al. 2011). Therefore, it is plausible that TNF-α activates HIF-1α which in turn induces mucin gene expression via the ERK pathway.

Levels of IL-6 and IL-13 were also elevated in COPD persistent sputum producers. IL-6 can cause increased MUC5B expression (Chen et al. 2003) and has also been shown to
increase HIF-1α expression by increasing HIF-1α translocation to the nucleus (Kockar et al. 2012). It is therefore likely that IL-6 increases MUC5B expression through pathways involving HIF-1α activation.

Furthermore, HIF-1α contains a conserved STAT6 motif in its promoter region (Evans et al. 2009) and IL-13 may act via STAT6 to upregulate MUC5AC and MUC5B (Yoshisue and Hasegawa 2004; Kuperman et al. 2002).

The data suggest that upregulation and increased expression of HIF-1α is one possible mechanism that causes increased expression of MUC5AC and MUC5B and thus mucus hypersecretion in COPD persistent sputum producers and pro-inflammatory cytokines such as IL-6, IL-13 and TNF-α found elevated in this phenotype may activate mucin genes by pathways involving HIF-1α. The fact that COPD persistent sputum producers have increased CAIX and HIF-1α expression also implies that the lungs of this phenotype are more hypoxic and these patients thus have increased severity of disease.

This study however has certain limitations. One disadvantage of CAIX being used as a prognostic biomarker for HIF-1α lies in the fact that two separate mechanisms have been shown to control CAIX expression, both dependent on HIF-1α activity and decreased O2 levels but differing with respect to the amount of HIF-1α required. True hypoxia is defined as the mechanism that is driven by HIF-1 and requires HIF-1α stabilisation to increase the total amount of HIF-1. Mild hypoxia, although still requiring some HIF-1 activity, induces additional pathways, including the involvement of activated PI3-K (Kaluz et al. 2002), but is not associated with an additional increase in HIF-1α levels (Kaluz et al. 2003). Varying amounts of HIF-1α have been detected even under normoxic conditions in normal tissue (Stroka et al. 2001) or cell lines (Zhong et al. 1998), and this presumably can provide the small level of HIF-1 activity that is necessary for activation of CA9 under conditions of mild hypoxia. This could explain why HIF-1α and CAIX was also expressed in non-smokers in this study. The existence of additional pathways resulting in CAIX expression is also confirmed by study of CAIX expression in tumor sections, where CAIX expression was found to extend beyond the hypoxic regions (Wykoff et al. 2000; Olive et al. 2001). Therefore, although DMOG was used in this study to simulate hypoxic conditions in vitro, it is likely that the expression of CAIX may have been overestimated to some extent.
Another factor is that in many tissues, HIF-1α may not be sufficient to induce CAIX and only several genetic changes leading to chromatin remodelling will ‘uncover’ the CA 9 gene and make it inducible by HIF-1α (Ilie et al. 2010). It is therefore possible that some HIF positive areas could not be determined by CAIX expression in these biopsy specimens.

In conclusion, although immunostaining for HIF-1α was optimised successfully, the quantification of HIF-1α in the biopsies was not deemed reliable as the expression of HIF-1α was affected by oxygen therapy during bronchoscopy in some of the patients. CAIX was therefore used to elucidate areas of HIF-1α expression and this study showed that the 1) expression of HIF-1α assessed via its downstream marker CAIX is significantly increased in the COPD persistent sputum producers compared to control groups and 2) HIF-1α can potentially cause increased MUC5B expression. These results provide an avenue for further investigating the role of HIF and CAIX in mucus hypersecretion in COPD.
CHAPTER 7: DISCUSSION AND CONCLUSION

Over the last decade there has been increasing interest in improved definition of COPD phenotypes that will allow specific subgroups of patients with different prognosis or treatment response to be identified. The discovery of such phenotypes will allow novel therapies to be specifically targeted at the most appropriate patients.

In order to understand chronic bronchitis in COPD with greater precision, this thesis has investigated the clinical characteristics and airway inflammatory and mucosal profile of a subphenotype of chronic bronchitis which has mucus hypersecretion throughout the year as its main presenting symptom i.e. the COPD ‘persistent sputum producer’. The key findings of the work done in this thesis are now discussed.

7.1.1 COPD persistent sputum producers: more severe disease characteristics

This work has demonstrated that COPD persistent sputum producers have worse lung function and decreased exercise capacity compared to COPD sputum non-producers; i.e.. those COPD patients who produce sputum only during an exacerbation. They also have increased symptoms and greater impact from the disease on their day to day lives, higher rate of bacterial colonization and increased number of exacerbations. These findings in COPD persistent sputum producers and the underlying mechanisms for them are probably inter-related.

The worse lung function in this group may be due to the spontaneous mucus hypersecretion which causes airflow obstruction by several mechanisms: mucus hypersecretion causes luminal occlusion (Hogg et al. 2004); epithelial layer thickening encroaches on the airway lumen (James and Wenzel 2007); and increased mucus alters surface tension, thereby predisposing to airway collapse (Macklem et al. 1970). In keeping with this, recent studies have shown that COPD patients with chronic bronchitis have worse lung function compared to those without chronic bronchitis (de Oca et al. 2012) and chronic bronchitis becomes more frequent as the severity of COPD increases (Agusti et al. 2010; de Oca et al. 2012; Lu et al. 2010).

Airflow obstruction occurs largely because of increased resistance in the small airways (Jeffery 2001) and mucus hypersecretion in the small airways has been shown to correlate
with the degree of airflow obstruction (Hogg et al. 2004; Kim et al. 2008a). This knowledge has led to doubt about the relevance of large airway pathology in causing airflow obstruction, such as changes in bronchial epithelium and submucosal glands. Sputum symptoms are probably a reflection, at least in part, of large airway pathology. However, central airway pathology in terms of mucus hypersecretion is probably linked to peripheral airway pathology because both occur as a consequence of the same factors such as cigarette smoke exposure.

Poor mucus clearance in the central airways in COPD persistent sputum producers could affect the health and sterility of the small airways because excessive mucus retention centrally will adversely affect mucus clearance from the peripheral airways. This phenomenon is probably restricted to patients with severe chronic bronchitis and a possible reason why some previous studies have failed to show an association between symptoms of chronic bronchitis and mucus hypersecretion in the small airways is that these studies have enrolled patients with varying symptoms of chronic bronchitis including those with milder symptoms (Hogg et al. 2004; Caramori et al. 2004).

Airway obstruction is a risk factor for bacterial colonization (Monso et al. 1999; Kim et al. 2008b). Bacterial pathogens may enter the lower respiratory tract because of impaired mucociliary clearance, enhanced mucus secretion, disruption of normal ciliary activity and airway epithelial injury. These bacteria may persist by further impairing mucociliary clearance. These bacteria are also in a constant state of turnover, releasing extracellular products, undergoing lysis with release of a variety of proteins, peptidoglycan and lipooligosaccharide (LOS), which is a potent inflammatory stimulus (Gu et al. 1995). Bacterial products in the airways can also cause neutrophil migration into the airways, and elastase released from these neutrophils can act synergistically with bacterial products and cause further inhibition of ciliary function (Sethi and Murphy 2001a). As bacterial products cause neutrophil influx and degranulation in the airways and lung parenchyma, they contribute to chronic inflammation, parenchymal lung damage, and progressive small airway obstruction (Amitani et al. 1991; Hiemstra et al. 1998).

The incidence of bacterial colonization increases with increasing severity of COPD (Zalacain et al. 1999) and airway bacterial load correlates with markers of airway inflammation in stable chronic bronchitis (Hill et al. 2000). Therefore, the importance of
bacterial colonization-induced inflammation could increase as airflow obstruction becomes more severe. Furthermore, bacterial colonization of the lower respiratory tract has been shown to occur not only in patients with established COPD, but also in smokers who have chronic bronchitis but no significant airway obstruction (Soler et al. 1999). Therefore, bacterial colonization of the lower airways appears to be an early phenomenon in the course of the disease and may be ‘driving’ this phenotype of COPD persistent sputum producers.

Bacterial colonization in the stable state has also been shown to increase the frequency of COPD exacerbations (Sethi et al. 2002). Potential pathways by which bacteria could contribute to the pathogenesis of acute exacerbations include (1) primary infection of the lower airways, (2) secondary infection of the airways after an antecedent viral infection, and (3) bacterial antigens inducing bronchial hyper-reactivity and eosinophilic inflammation (Sethi and Murphy 2001b). Increased number of exacerbations cause further decline in lung function (Donaldson et al. 2002) and health status (Seemungal et al. 1998).

Thus mucus hypersecretion in persistent sputum producers results in greater airflow obstruction ultimately resulting in a higher number of exacerbations compared to COPD sputum non-producers; this is in keeping with the findings of recent studies (Burgel et al. 2009; Miravitlles et al. 2000). Exacerbations further increase airway inflammation as well as result in mucus hypersecretion, both of which cause airflow obstruction, thus setting up a vicious cycle resulting in lung damage. Chronic cough and mucus over-production over time therefore leads to excessive decline in FEV\textsubscript{1}, as shown in the study by Vestbo et al. (Vestbo et al. 1996).

Identification of the COPD persistent sputum producer phenotype is likely to be important in assessing the risk of future exacerbations. The development of novel methods to reduce or eradicate bacterial colonization represent opportunities of future intervention in this group of patients.

Mucus hypersecretion has significant physiologic effects such as impaired pulmonary function (disease severity), respiratory symptoms (dyspnoea and wheezing), and exacerbations, resulting in worse health related quality of life (Fuhrman et al. 2009). However, it is also possible that patients who have complaints of chronic cough and
sputum production are more likely to describe worse respiratory symptoms to health care providers. The work in this thesis re-inforces the importance of mucus hypersecretion being involved in worse symptoms and clinical outcomes in COPD.

**7.1.2 COPD persistent sputum producers have more airway inflammation**

The persistent sputum producers had higher total sputum neutrophil and eosinophil counts as well as a number of pro-inflammatory cytokines such as eotaxin, GM-CSF, IL-6, IL-13, MCP-1, MCP-4 and TNF-α compared to COPD non-producers indicating that this group of patients has more airway luminal inflammation. Increased airway inflammation is associated with an increased tendency to produce sputum. Neutrophils and eosinophils can directly cause mucus hypersecretion (Kohri et al. 2002; Burgel et al. 2001). Previously, it has been shown that bronchial lumen neutrophilia in chronic bronchitis is associated with worse lung function and more sputum production (Thompson et al. 1989a).

The inflammatory cytokines found elevated in these patients have biological roles that may of importance in COPD and mucus hypersecretion. TNF-α is a potent neutrophil stimulant (Ferrante 1992) and GM-CSF prolongs neutrophil survival (Laan et al. 2003). IL-6, IL-13, MCP-1 and TNF-α all induce mucin gene upregulation (Chen et al. 2003; Zhu et al. 1999; Monzon et al. 2011; Song et al. 2003), and so may be responsible for the mucus hypersecretion seen in this phenotype. Eotaxin and MCP-4 cause eosinophil recruitment in the airway lumen (Garcia-Zepeda et al. 1996).

Chronic inflammation also causes parenchymal destruction leading to small airway disease (Cosio Piqueras and Cosio 2001). The higher number of neutrophils are likely to cause greater alveolar destruction and emphysema in COPD persistent sputum producers (Shapiro et al. 2003) resulting in reduced diffusion capacity and a trend towards increased residual volume as seen in this thesis, and suggestive of increased hyperinflation in this group compared to COPD sputum non-producers. Again, this finding seems to pertain to those with severe chronic bronchitis i.e. the persistent sputum producers and a recent study using a mixed population of patients with varying symptoms of chronic bronchitis failed to identify this difference (Kim et al. 2011).

Previous studies on cytokines in chronic bronchitis have shown significantly higher levels of IL-4, IL-8, IL-13, MCP-1, MIP-1β and TNF-α in the central airways of subjects with
chronic bronchitis (Capelli et al. 1999; Miotto et al. 2003a; Chanez et al. 1996; Ma et al. 1999). This appears to be the first study to show higher levels of eotaxin, GM-CSF, IL-6 and MCP-4 in COPD sputum producers compared to non-producers, and it is likely that some of these cytokines through their actions described above are key factors in the disease mechanisms underlying this phenotype of COPD patients with persistent sputum production. Bacteria in the airways release lipooligosaccharide (LOS) which can potentially increase the secretion of IL-6 and TNF-α from bronchial epithelial cells (Khair et al. 1996) and thus increased bacterial colonization in this phenotype may contribute to elevated levels of IL-6 and TNF-α. These cytokines may play a role in activation and migration of neutrophils to sites of inflammation and also in mucin upregulation.

Of particular importance are eotaxin and MCP-4 both of which are eosinophil chemoattractants. Eosinophils cause mucus hypersecretion which would favour bacterial colonization. The inefficient removal of bacteria from the lower respiratory tract results in prolonged contact between the bacterial antigens and airway lymphoid tissue, resulting in the emergence of IgE antibodies to bacterial antigens, which could induce further eosinophil infiltration and mast cell degranulation on repeated exposures to the bacterial antigens (Clementsen et al. 1995; Clementsen et al. 1990). Mast cells release histamine which increases bronchial epithelium permeability, stimulates mucus secretion, and induces bronchoconstriction. This finding is supported by other studies showing that COPD patients with chronic bronchitis have higher sputum eosinophils than patients without these symptoms (Snoeck-Stroband et al. 2008) and eosinophil numbers increase further during exacerbations (Saetta et al. 1994). Eotaxin and MCP-4 may therefore be the two most important cytokines defining this phenotype of COPD and this possibly distinct biomarker profile of COPD persistent sputum producers could be utilized to investigate therapies to suppress inflammation and mucus hypersecretion in COPD.

It is important to note that it is difficult to ascertain which cytokines found elevated in persistent sputum producers are ‘cause or effect’ of mucus hypersecretion i.e. if a particular cytokine is the reason for increased mucus secretion or is the level of the cytokine elevated because of mucus hypersecretion resulting in increased bacterial colonization and airway inflammation. However, these mechanisms are inter-related and it
is likely that some of these cytokines may be both the cause and effect of mucus hypersecretion in persistent sputum producers.

7.1.3 Induced sputum measurements are repeatable over short term period in the COPD persistent sputum producers

The repeatability of sputum in COPD is an important issue, as measurements that are more repeatable over time have greater applicability as biomarkers compared to those with naturally high variability. This thesis has demonstrated that unlike spontaneous sputum, there is moderate to good repeatability of induced sputum neutrophil, macrophage as well as eosinophil cell count and percentages over an eight week interval in COPD persistent sputum producers, though ICC values for sputum percentages were lower compared to sputum cell counts. This is in keeping with a previous study that shows that repeatability of sputum differential cell counts becomes lower over time from analysis performed at one week compared to three months (Boorsma et al. 2007). These induced sputum parameters can therefore be used as reliable scientific endpoints in the assessment of airway inflammation in COPD persistent sputum producers and as a means to monitor therapeutic control that could increase patient’s quality of life based on downstream monitoring.

7.1.4 MUC5B is increased in the bronchial epithelium of COPD persistent sputum producers

This thesis has demonstrated for the first time that increased MUC5B expression in the bronchial epithelium is a feature which is unique to the phenotype of COPD patients with persistent sputum production and this could account for the mucus hypersecretion in this group of patients. So far increased MUC5B expression in the bronchial epithelium has been reported in other inflammatory lung diseases (Kamio et al. 2005; Chen et al. 2001) and studies on COPD groups have not identified this (Caramori et al. 2009; O'Donnell et al. 2004b). The reason is that previous studies conducted in mixed populations of COPD patients have overlooked the possibility that these mucins may have unique expression profiles in the subset of COPD patients with severe chronic bronchitis. MUC5B expression had an inverse correlation to FEV1% predicted, supporting the fact that mucin over-expression and hypersecretion in the large airways results in increased airflow obstruction in this group.
Expression of MUC5AC in the bronchial epithelium was also increased in COPD persistent sputum producers compared to sputum non-producers with a trend towards statistical significance ($p = 0.07$). Unlike MUC5B expression, the expression of MUC5AC correlated with pack year history and was higher in current smokers compared to ex-smokers. This finding supports previous studies that have shown that cigarette smoke upregulates MUC5AC expression (Rose and Voynow 2006). Therefore whilst MUC5AC expression was related to both smoking and persistent sputum production, the expression of MUC5B was only related to persistent sputum production.

The over-expression of these mucins in COPD persistent sputum producers may be related to the increase in pro-inflammatory cytokines; IL-6, IL-13, MCP-1 and TNF-α which have been shown to cause mucin gene upregulation (Song et al. 2003; Chen et al. 2003; Zhu et al. 1999; Monzon et al. 2011). It is likely that these cytokines also play an important role in mucin over-expression in the submucosal glands which predominantly secrete MUC5B. Other reasons for the altered mucin profile in COPD persistent sputum producers as previously discussed in Chapter 5 could be MUC5B gene polymorphisms, altered post-translational modification of mucin genes and changes in mucin glycosylation in this group of patients.

7.1.5 HIF-1α and CAIX expression is increased in the bronchial epithelium of COPD persistent sputum producers

This is the first study to report increased HIF-1α and CAIX expression in COPD sputum producers compared to sputum non-producers. Few authors have been able to achieve immunostaining for HIF-1α, and this was successfully done as part of the work in this thesis. However, quantification of HIF-1α was not possible as expression levels were affected by oxygen therapy during bronchoscopy in some patients. CAIX which is a downstream marker of HIF-1α was therefore used to elucidate areas of HIF-1α expression and good correlation was found between CAIX and HIF-1α expression in the biopsies. This work demonstrates that CAIX can be reliably used as a stable marker of HIF-1α expression in COPD patients.

HIF-1α is a marker of hypoxia and its over-expression in COPD persistent sputum producers implies that the lungs of this phenotype are more hypoxic and these patients thus
have increased severity of disease. CAIX expression positively correlated to MUC5AC and MUC5B expression. Previous studies have shown that HIF-1α can drive goblet cell hyperplasia and increase MUC5AC expression in COPD (Polosukhin et al. 2011) and this is in keeping with the higher grades of PAS staining (indicative of goblet cell hyperplasia) found in this group compared to sputum non-producers, as well as the positive correlation of CAIX to MUC5AC expression. The correlation of CAIX expression to MUC5B can be explained by the finding below.

7.1.6 HIF-1 upregulates MUC5B expression in bronchial epithelial cells in vitro

Another novel finding of this thesis is the establishment of a potential relation between HIF-1α and MUC5B expression. The time course experiment to assess the expression of HIF-1α and MUC5B in vitro shows that HIF-1α may be a regulator of MUC5B gene expression and could thus have an important role in the increased expression of MUC5B in COPD persistent sputum producers. Other then hypoxia, HIF-1α is regulated by inflammatory cytokines such as TNF-α (Jiang et al. 2010) and as previously discussed in Chapter 6, it is likely that in addition to TNF-α, pro-inflammatory cytokines such as IL-6 and IL-13 found elevated in COPD persistent sputum producers also increase MUC5B expression through pathways involving HIF-1α activation. Targeting HIF-1α activation could thus be a potential avenue for limiting mucus hypersecretion in COPD.

In conclusion, increased airway inflammation and epithelial remodelling in COPD persistent sputum producers create hypoxic conditions that leads to HIF-1α activation. It is likely that this is then followed by a co-operative regulation of MUC5B and MUC5AC expression by HIF-1α and inflammatory cytokines such as IL-6, IL-13, MCP-1 and TNF-α as well as the regulation of HIF-1α by hypoxia and cytokines such as TNF-α found elevated in COPD persistent sputum producers, resulting in mucus hypersecretion in this group of patients. Eotaxin and MCP-4 as eosinophil chemoattractants may also have a significant role to play in the mucus hypersecretion. Increased mucus secretion results in mucus plugging of the small airways predisposing them to bacterial colonization which further increases airway inflammation and exacerbation rates, resulting in a vicious cycle amplifying mucus production over time. This ultimately results in worse clinical characteristics such as reduced lung function, exercise capacity and worse quality of life (Figure 7.1).
It is possible that increased bacterial colonization in the stable state resulting in the increased airway inflammation and increased number of exacerbations in this group accounts for some of these effects. Another contributing factor could be defective immunity predisposing these patients to recurrent infections. However, it seems likely that persistent mucus hypersecretion *per se* affects health status and lung function in these patients.

It is important to note that the positive findings in this thesis relate specifically to COPD patients with persistent sputum production, and cannot be generalized to all patients with chronic bronchitis. It has shown that “splitting” patients into discrete phenotypes depending upon the frequency of sputum production can uncover potential mechanisms restricted to subgroups of patients. The COPD ‘persistent sputum producer’ is a severe subphenotype of chronic bronchitis with distinct clinical characteristics and airway biomarker profile and worse prognosis. This phenotype needs more directed therapy targeting chronic mucus production not only to improve symptoms but also to reduce risk, improve quality of life and outcomes and reveals potential targets for the development of these novel therapies.
Figure 7.1: Disease mechanisms in COPD persistent sputum producers (the red arrows are indicative of the mechanisms proposed in this thesis): Amplification of mucus production results from a vicious cycle of airway inflammation, mucus hypersecretion, airways obstruction and colonization. This is regulated by inflammatory cells such as neutrophils and eosinophils, the transcription factor HIF-1α and inflammatory cytokines such as eotaxin, GM-CSF, IL-6, IL-13, MCP-1, MCP-4 and TNF-α.
7.2 Limitation and future directions

Within this thesis, there are a few areas of weakness that in the future could be addressed to strengthen this project. The study population mainly comprised of moderate COPD patients with the majority being GOLD II, and the inclusion of more severe patients would allow further consolidation and expansion of the results already obtained. It would also have been useful to have had HRCT results on all COPD persistent sputum producers to rule out any significant bronchiectasis prior to their enrollment on the study. However, those patients who had HRCT were confirmed to have none or minimal amount of bronchiectasis secondary to COPD. Also, the presence of bacterial colonization via sputum culture was not assessed in all the subjects.

This work showed that induced sputum is repeatable in stable COPD persistent sputum producers over short term. However, it would be interesting to look at the repeatability of these inflammatory biomarkers over an extended period of time. Especially as it can be argued that due to occurrences such as patient exacerbations, COPD can be a variable disease and one might expect the repeatability to be changeable over longer periods in comparison to the observation at an eight week interval.

The bronchial biopsies used for immunohistochemistry studies contained bronchial rings with surface bronchial epithelium but not bronchial submucosal glands. It was therefore not possible to assess the expression of mucins in the submucosal glands, which are the main site of MUC5B production and in COPD subjects have also been shown to express MUC5AC (Caramori et al. 2009). These glands may show differential expression of MUC5B and MUC5AC in the persistent sputum producers compared to sputum non-producers.

The final experimental chapter showed that HIF-1α can cause increase MUC5B expression and the short time over which this happens implies that HIF-1α may directly act on the MUC5B gene. It would be useful to confirm this using chromatin immunoprecipitation studies in the future.
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Lung physiology and airway inflammation in COPD patients with persistent sputum production


COPD NICE clinical guideline 2010.


Lung physiology and airway inflammation in COPD patients with persistent sputum production


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APPENDIX 1

Preparation of Antigen Retrieval Solutions used in the immunohistochemistry studies

Tris Buffered Saline (TBS)

- 10 x TBS (0.5M Tris Base, 9% NaCl, pH 7.6)
- Trizma base – 61g
- NaCl – 90g
- Distilled water – 1000ml
- Mix to dissolve and adjust pH to 7.6 using concentrated hydrochloric acid.
- Dilute 1:10 with distilled water prior to use.

TBS-tween (TBSt)

- 10 x TBS Tween 20 (0.5M Tris Base, 9% NaCl, 0.1% Tween 20, pH 7.6)
- Trizma base – 61g
- NaCl – 90g
- Distilled water – 1000ml
- Mix to dissolve and adjust pH to 7.6 using concentrated hydrochloric acid and then add 1 ml Tween-20. Dilute 1:10 with distilled water prior to use.

pH 9 -Tris-EDTA (1 mM) Retrieval solution

- Dissolve 0.37 g Diaminoethanetetra-acetic acid disodium salt and 1.21 g of Trizma base in 1L of ultra-pure water.
- Adjust to pH 9 with a few drops of 1 M NaOH or 1 M HCl.

pH 6 - Citrate Buffer (10 mM) Retrieval solution

- Dissolve 2.1 g of Citrate acid monohydrate in 1 L of ultra-pure water.
- Adjust to pH 6 with a few drops of 1 M NaOH or 1 M HCl.

TNB blocking buffer

- 0.5 gm blocking reagent (supplied in TSA kit, Perkin Elmer)
- 100 mls TBS
- Dissolve 0.5% blocking reagent (supplied in TSA kit) in small increments to blocking buffer (TBS) whilst stirring. Heat upto 60˚C with continuous stirring to completely dissolve the blocking reagent.
4% Paraformaldehyde

- Paraformaldehyde – 4g (Sigma Aldrich)
- 1 x PBS – 100mls
- Dissolve 4g paraformaldehyde in small increments to 100 ml PBS whilst stirring. Heat up to 60°C with continuous stirring. At 60°C add few drops of 1M NaOH until solution goes clear. Allow to cool. Adjust to pH 7.4-7.6 with a few drops of 1M NaOH or 1M HCl.

Gill’s Modified Haematoxylin

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<td>De-ionised Water</td>
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<td>Sodium Iodate</td>
<td>0.4 g</td>
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<td>1,2 Ethanediol (Ethylene Glycol)</td>
<td>0.5 L</td>
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<td>Bronopol BNPD (2-Bromo 2-Nitropropane 1,3 Diol)</td>
<td>1.48 g</td>
<td>Sigma Aldrich</td>
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<td>80 % Acetic Acid</td>
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<td>Magnesium Sulphate (Heptahydrate)</td>
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<td>Haematoxylin</td>
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<td>Aluminium Sulphate</td>
<td>70 g</td>
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Method

- Heat 400 mL of water to 30 °C in a beaker.
- Add the following reagents sequentially: Magnesium Sulphate, Sodium Hydrogen Carbonate and Bronopol BNPD.
- Ensure that each chemical has dissolved before addition of the next.
- In a second beaker, heat 400 mL of water to 30 °C and dissolve the Aluminium Sulphate whilst heating to 40-50 °C whilst stirring.
- Mix the solutions from the two beakers and add the 1,2 Ethanediol whilst mixing vigorously.
- Add the Haematoxylin to 45 mL of water. Add this to the beaker containing the other reagents and add the Sodium Iodate and Acetic Acid.
- Add 630 mL of water and mix well.
MATERIALS AND EQUIPMENT

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PRE-PREPARED CHEMICALS AND REAGENTS

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<tr>
<th>Chemical / Reagent</th>
<th>Source</th>
<th>Code</th>
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<tr>
<td>1,2 Ethanediol (Ethylene Glycol)</td>
<td>Sigma Aldrich</td>
<td>146750025</td>
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<td>80 % Acetic Acid</td>
<td>Sigma Aldrich</td>
<td>320099</td>
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<td>ABC</td>
<td>Vector</td>
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<tr>
<td>Acetone</td>
<td>Sigma Aldrich</td>
<td>179124-500ML</td>
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<tr>
<td>Aluminium Sulphate</td>
<td>Acros</td>
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<tr>
<td>Borax</td>
<td>Fisher Scientific</td>
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<td>Bronopol BNPD</td>
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<td>DTT</td>
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<td>D-5545</td>
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<td>EDTA</td>
<td>Sigma Aldrich</td>
<td>E5134-100G</td>
</tr>
<tr>
<td>Eosin</td>
<td>Leica Microsystems</td>
<td>30610</td>
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<tr>
<td>Eosin Y</td>
<td>Leica Microsystems</td>
<td>01601E</td>
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<tr>
<td>FBS</td>
<td>PAA-Labs</td>
<td>A15-144</td>
</tr>
<tr>
<td>Goat Anti Mouse</td>
<td>Vector</td>
<td>BA-2020</td>
</tr>
<tr>
<td>Goat Anti Rabbit</td>
<td>Vector</td>
<td>BA-1000</td>
</tr>
<tr>
<td>Glycine</td>
<td>Sigma Aldrich</td>
<td>50046</td>
</tr>
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<td>H2O2</td>
<td>Sigma Aldrich</td>
<td>H1009</td>
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<td>Sigma Aldrich</td>
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<td>Horse Anti Mouse</td>
<td>Vector</td>
<td>BA-2000</td>
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<td>IMS</td>
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<td>Iodoacetamide</td>
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<td>L-Glut</td>
<td>Gibco</td>
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<td>M1775-1GA</td>
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<td>Mouse IgG1 (Control)</td>
<td>Dako</td>
<td>X0931</td>
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<td>NaCL</td>
<td>Sigma Aldrich</td>
<td>S5886</td>
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<td>NaOH</td>
<td>Sigma Aldrich</td>
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<td>Normal Goat Serum</td>
<td>Vector</td>
<td>S-1000</td>
</tr>
<tr>
<td>Normal Horse Serum</td>
<td>Vector</td>
<td>S-2000</td>
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<td>PBS</td>
<td>Sigma Aldrich</td>
<td>P1408</td>
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<td>P6148</td>
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<td>Pen / Strep</td>
<td>Gibco</td>
<td>15140-122</td>
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<td>Periodic Acid</td>
<td>Surgipath</td>
<td>3803812</td>
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<td>Vector</td>
<td>BA-5000</td>
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<td>Rapi-diff</td>
<td>GCC Diagnostics</td>
<td>SP300B</td>
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<td>Sigma Aldrich</td>
<td>R0883</td>
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<td>Schiff's Reagent</td>
<td>Leica Microsystems</td>
<td>03800E</td>
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APPENDIX 2(a)

Table summarizing the different pre-treatments attempted for optimization of the HIF-1α antibody (ab1, Abcam, Cambridge)

<table>
<thead>
<tr>
<th>Primary Antibody Conc. (µg/ml)/Dilution Factor</th>
<th>Blocking Step</th>
<th>pH</th>
<th>Hydrogen Peroxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 (1:50)</td>
<td>1.5 % &amp; 5 % Serum</td>
<td>pH 6</td>
<td>3 % in Methanol &amp; 3 % in TBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 9</td>
<td>3 % in Methanol &amp; 3 % in TBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 8</td>
<td>3 % in Methanol &amp; 3 % in TBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 6</td>
<td>0.3 % in Methanol &amp; 0.3 % in TBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 9</td>
<td>0.3 % in Methanol &amp; 0.3 % in TBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 8</td>
<td>0.3 % in Methanol &amp; 0.3 % in TBS</td>
</tr>
<tr>
<td>2.5 (1:100)</td>
<td>1.5 % &amp; 5 % Serum</td>
<td>pH 6</td>
<td>3 % in Methanol &amp; 3 % in TBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 9</td>
<td>3 % in Methanol &amp; 3 % in TBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 8</td>
<td>3 % in Methanol &amp; 3 % in TBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 6</td>
<td>0.3 % in Methanol &amp; 0.3 % in TBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 9</td>
<td>0.3 % in Methanol &amp; 0.3 % in TBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 8</td>
<td>0.3 % in Methanol &amp; 0.3 % in TBS</td>
</tr>
<tr>
<td>1.25 (1:200)</td>
<td>1.5 % &amp; 5 % Serum</td>
<td>pH 6</td>
<td>3 % in Methanol &amp; 3 % in TBS</td>
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<td></td>
<td></td>
<td>pH 9</td>
<td>3 % in Methanol &amp; 3 % in TBS</td>
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<tr>
<td></td>
<td></td>
<td>pH 8</td>
<td>3 % in Methanol &amp; 3 % in TBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 6</td>
<td>0.3 % in Methanol &amp; 0.3 % in TBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 9</td>
<td>0.3 % in Methanol &amp; 0.3 % in TBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 8</td>
<td>0.3 % in Methanol &amp; 0.3 % in TBS</td>
</tr>
</tbody>
</table>
APPENDIX 2(b)

Table summarizing the different pre-treatments attempted for optimization of the HIF-1α antibody (BD Biosciences, Oxford, 610958)

<table>
<thead>
<tr>
<th>Primary Antibody Conc.(µg/ml)/Dilution Factor</th>
<th>Blocking Step</th>
<th>pH</th>
<th>Hydrogen Peroxide</th>
<th>Use of TSA kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 (1:100) 1.5 %, 5 % &amp; 10 % Serum</td>
<td>pH 6</td>
<td>3 % in Methanol &amp; 3 % in TBS</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 9</td>
<td>3 % in Methanol &amp; 3 % in TBS</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 8</td>
<td>3 % in Methanol &amp; 3 % in TBS</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 6</td>
<td>0.3 % in Methanol &amp; 0.3 % in TBS</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 9</td>
<td>0.3 % in Methanol &amp; 0.3 % in TBS</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 8</td>
<td>0.3 % in Methanol &amp; 0.3 % in TBS</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2.5 (1:100) TNB</td>
<td>pH 6 (5 &amp; 15 mins)</td>
<td>0.3 % and 1 % in PBS</td>
<td>TSA Kit</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 9 (5 &amp; 15 mins)</td>
<td></td>
<td>TSA Kit</td>
<td></td>
</tr>
<tr>
<td>2.5 (1:100) TNB &amp; 10 % Serum</td>
<td>pH 6 (5 &amp; 15 mins)</td>
<td></td>
<td>TSA Kit</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 9 (5 &amp; 15 mins)</td>
<td></td>
<td>TSA Kit</td>
<td></td>
</tr>
<tr>
<td>1.25 (1:200) TNB</td>
<td>pH 6 (5 &amp; 15 mins)</td>
<td>0.3 % and 1 % in PBS</td>
<td>TSA Kit</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 9 (5 &amp; 15 mins)</td>
<td></td>
<td>TSA Kit</td>
<td></td>
</tr>
<tr>
<td>1.25 (1:200) TNB &amp; 10 % Serum</td>
<td>pH 6 (5 &amp; 15 mins)</td>
<td></td>
<td>TSA Kit</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 9 (5 &amp; 15 mins)</td>
<td></td>
<td>TSA Kit</td>
<td></td>
</tr>
<tr>
<td>0.63 (1:400) TNB</td>
<td>pH 6 (5 &amp; 15 mins)</td>
<td>0.3 % and 1 % in PBS</td>
<td>TSA Kit</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 9 (5 &amp; 15 mins)</td>
<td></td>
<td>TSA Kit</td>
<td></td>
</tr>
<tr>
<td>0.63 (1:400) TNB &amp; 10 % Serum</td>
<td>pH 6 (5 &amp; 15 mins)</td>
<td></td>
<td>TSA Kit</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 9 (5 &amp; 15 mins)</td>
<td></td>
<td>TSA Kit</td>
<td></td>
</tr>
<tr>
<td>0.31 (1:800) TNB</td>
<td>pH 6 (5 &amp; 15 mins)</td>
<td>0.3 % and 1 % in PBS</td>
<td>TSA Kit</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 9 (5 &amp; 15 mins)</td>
<td></td>
<td>TSA Kit</td>
<td></td>
</tr>
<tr>
<td>0.31 (1:800) TNB &amp; 10 % Serum</td>
<td>pH 6 (5 &amp; 15 mins)</td>
<td></td>
<td>TSA Kit</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 9 (5 &amp; 15 mins)</td>
<td></td>
<td>TSA Kit</td>
<td></td>
</tr>
<tr>
<td>0.25 (1:1000) TNB</td>
<td>pH 6 (5 &amp; 15 mins)</td>
<td>0.3 % and 1 % in PBS</td>
<td>TSA Kit</td>
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</tr>
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<td></td>
<td>pH 9 (5 &amp; 15 mins)</td>
<td></td>
<td>TSA Kit</td>
<td></td>
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<tr>
<td>0.25 (1:1000) TNB &amp; 10 % Serum</td>
<td>pH 6 (5 &amp; 15 mins)</td>
<td></td>
<td>TSA Kit</td>
<td></td>
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<tr>
<td></td>
<td>pH 9 (5 &amp; 15 mins)</td>
<td></td>
<td>TSA Kit</td>
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</table>
APPENDIX 3(a)

Grading criterion for Haematoxylin & Eosin (H&E) staining of bronchial biopsy specimens

Representative photomicrographs of H&E staining showing A. Grade 1 (normal epithelium with no extra layers of cells); B. Grade 2 (hypertrophic; enlarged/elongated columnar cells and enlarged/swollen basal cells); C. Grade 3 (hyperplastic; indicating increased cell numbers throughout, with normal appearing basal cells but extra rows of cells above basal cells); D. Grade 4 (metaplastic; indicating increased cell numbers throughout, enlarged basal cells with extra rows of cells above basal cells); E. Grade 5 (squamous cell metaplasia) and F. Grade 6 showing an epithelial cyst. Scalebar = 50μm
APPENDIX 3(b)

Grading criterion for Periodic acid Schiff (PAS) staining of bronchial biopsy specimens

Representative photomicrographs of PAS staining showing A. Grade 1 (normal epithelium with occasional PAS+ cells); B. Grade 2 (PAS+ cell hypertrophy); C. Grade 3 (PAS + cell hyperplasia); D. Grade 4 (epithelial metaplasia with PAS+ cells throughout mucosa); E. Grade 5 (epithelial metaplasia/squamous plates with loss of PAS+ cells); F. showing PAS positive submucosal glands. Scalebar = 50μm.
APPENDIX 3(c)

Grading criterion for staining intensity of HIF-1α and CAIX in bronchial biopsies

A. CAIX: Grade 3 (Severe), on right  A. HIF: Grade 3 (Severe)

B. CAIX: Grade 2 (Moderate)  B. HIF: Grade 2 (Moderate)

C. CAIX: Grade 1 (Mild)  C. HIF: Grade 1 (Mild)

Representative photomicrographs of staining intensity gradation used for calculation of HIF-1α and CAIX IHC scores.
APPENDIX 4(a)

Sputum cytokines between persistent sputum producers and non-producers
(not statistically different between the two groups)

Columns represent mean. Error bars represent standard deviation
Unit of measurement – pg/ml.
APPENDIX 4(b)

Table summarizing the difference between sputum cytokines from COPD persistent sputum producers and non-producers

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Persistent sputum producer</th>
<th>Non-producer</th>
<th>p value</th>
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</thead>
<tbody>
<tr>
<td>GM-CSF*</td>
<td>0.99 (3.4)</td>
<td>0.83 (2.5)</td>
<td>0.01</td>
</tr>
<tr>
<td>TNF-α</td>
<td>77.6 (76.1)</td>
<td>36.8 (18.7)</td>
<td>0.04</td>
</tr>
<tr>
<td>MCP-1</td>
<td>512.6 (434.1)</td>
<td>334.1 (261.5)</td>
<td>0.03</td>
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<tr>
<td>IL-6</td>
<td>362.6 (238.3)</td>
<td>237.2 (154.1)</td>
<td>0.05</td>
</tr>
<tr>
<td>MCP-4</td>
<td>337.2 (368.6)</td>
<td>206.6 (185.5)</td>
<td>0.03</td>
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<tr>
<td>Eotaxin</td>
<td>263.1 (194.4)</td>
<td>160.3 (50.1)</td>
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<td>IL-13</td>
<td>27.5 (20.3)</td>
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<td>IL-8</td>
<td>9219 (5560)</td>
<td>4953 (3381)</td>
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<tr>
<td>IL-1β</td>
<td>168.1 (572.3)</td>
<td>63.3 (142.7)</td>
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<td>MDC</td>
<td>188.6 (150.7)</td>
<td>209 (177.5)</td>
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<tr>
<td>MIP-1β</td>
<td>632.0 (972.0)</td>
<td>267.4 (253.1)</td>
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<td>TARC</td>
<td>278.7 (352.3)</td>
<td>354.5 (337.1)</td>
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<td>IL-17</td>
<td>2.31 (7.8)</td>
<td>1.0 (1.4)</td>
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<tr>
<td>IFN-γ</td>
<td>28.0 (40.7)</td>
<td>19.7 (10.0)</td>
<td>0.32</td>
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<tr>
<td>IL-12p70</td>
<td>6.1 (12.6)</td>
<td>3.4 (1.5)</td>
<td>0.30</td>
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<tr>
<td>IL-2</td>
<td>14.2 (8.1)</td>
<td>11.8 (6.8)</td>
<td>0.95</td>
</tr>
<tr>
<td>IL-4</td>
<td>6.0 (16.3)</td>
<td>4.2 (5.4)</td>
<td>0.83</td>
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<tr>
<td>IL-5</td>
<td>13.4 (10.6)</td>
<td>21.4 (18.9)</td>
<td>0.11</td>
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<tr>
<td>IL-10</td>
<td>28.3 (35.3)</td>
<td>34.0 (41.2)</td>
<td>0.61</td>
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<tr>
<td>Eotaxin -3</td>
<td>885.2 (1211)</td>
<td>417.1 (336.8)</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Data represented as mean (± SD) in pg/ml. * 25 patients had GM-CSF levels below the lower limit of detection.
Lung physiology and airway inflammation in COPD patients with persistent sputum production