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A clinico-pathological study of COPD

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES
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A CLINICO-PATHOLOGICAL STUDY OF COPD

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Smokers with and without COPD and non-smoking controls underwent detailed clinical evaluation followed by quantitative high-resolution computed tomography (HRCT), sputum induction and endobronchial biopsy analysis.
The relationship between clinical and HRCT indicators of COPD severity was examined. Expiratory HRCT measurements and the expiratory/inspiratory mean lung density ratio (indicators of peripheral airway dysfunction) correlated more closely with airflow obstruction than did inspiratory HRCT measurements (which reflect emphysema severity).
Proximal airway inflammation was related, for the first time, to small airways dysfunction and emphysema (as determined by HRCT). Sputum neutrophils correlated with peripheral airways dysfunction but not with emphysema severity, while submucosal CD8+ T cell counts correlated weakly with airflow obstruction and HRCT measurements of emphysema.
Neutrophils and the Epidermal Growth Factor Receptor (EGFR) are implicated in the generation of epithelial goblet cell hyperplasia. Epithelial ErbB receptor and mucin expression were characterised, for the first time, in smokers. EGFR, ErbB3 and MUC5AC expression were elevated in smokers. ErbB3 expression correlated with MUC5AC expression and was expressed by mucin producing cells. No correlation was observed between mucin or ErbB receptor expression and either COPD severity or neutrophil counts.
The neutrophil chemokines Interleukin 8 (IL-8) and growth related oncogene - α (GRO-α) are possible contributors to neutrophilic inflammation in COPD. Both were measured in sputum and epithelium. Expression of both was increased in sputum, but not epithelium, of smokers with COPD. However, sputum IL-8 was negatively associated with current smoking habit while neither chemokine correlated with either disease severity or neutrophilic inflammation.
The results suggest that peripheral airway dysfunction is a key determinant of COPD severity and that sputum neutrophilia is an indicator of peripheral airway dysfunction severity. Smoking *per se* is associated with enhanced ErbB receptor and mucin expression by proximal airway epithelium with evidence of a novel assocation between ErbB3 and MUC5AC. Increased levels of IL-8 and GRO-α are observed in airway secretions in COPD, but, in the case of IL-8, current smoking habit may have an important negative influence on measurements.

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Abbreviations

ARDS Adult respiratory distress syndrome

ATS American thoracic society

AEC Amino ethyl carbazole

AR Amphiregulin

α-1 AT α-1 antitrypsin

Va Alveolar volume

BSA Bovine serum albumin

BTS British thoracic society

BHR Bronchial hyper-responsiveness

BL Bronchial lavage

BLF Bronchial lavage fluid

BAL Bronchoalveolar lavage

BALF Bronchoalveoalar lavage fluid

CSE Cigarette smoke extract

CT Computed tomography

COPD Chronic obstructive pulmonary disease

DAB Diaminobenzoate

DEP Diesel exhaust particles

DMEM Dulbecco's modified Eagles medium

E/I ratio Ratio of the MLD on expiration to inspiration

ELISA Enzyme Linked Immuno-sorbent Assay

ECP Eosinophilic cationic protein

EGF Epidermal growth factor

EGFR Epidermal growth factor receptor

ERS European respiratory society

ECM Extracellular matrix

ERV Expiratory reserve volume

FBS Fetal bovine serum

FEV_1 Forced expiratory volume in 1 second

FVC Forced vital capacity

FRC Functional residual capacity

GOLD Global strategy for the diagnosis, management and prevention of chronic obstructive pulmonary disease

GMA Glycolmethacrylate

GM-CSF Granulocyte macrophage colony stimulating factor

GRO-α Growth related oncogene α

He Helium

HGF Heparin-binding growth factor

HRCT High resolution computed tomography

HU Hounsfield units

HIV Human immunodeficiency virus

IGF Insulin-like growth factor 1

IDL Interactive Data Language

ICAM-1 Intercellular adhesion molecule 1

IL-8 Interleukin 8

IL-1 Interleukin 1

IC Inspiratory vital capacity

LTB4 Leukotriene B4

LTC4 Leukotriene C4

MEFV Maximal expiratory flow volume curve

MMP Matrix metalloprotease

MLD Mean lung density

mRNA Messenger RNA

mAb Monoclonal antibody

MIP Monocyte inflammatory protein

MCP Monocyte chemotactic factor

NK Natural killer

NF-κB Nuclear factor κB

PEFR Peak expiratory flow rate

%LAA Percentage of lung tissue < -950HU

PAS Periodic acid-schiff

PI 3-kinase Phosphatidyl inositol 3-kinase

PDGF Platelet derived growth factor

PCNA Proliferating cell nuclear antigen

PGE2 Prostaglandin E2

RV Residual volume

TLCO Carbon monoxide gas transfer

TLC Total lung capacity

TGF Transforming growth factor

TBS TRIS-buffered saline

TNF Tumour necrosis factor

V/Q Ventilation-perfusion

Chapter 1

General introduction to COPD

Chronic obstructive pulmonary disease (COPD), a condition that arises almost exclusively as a result of long-term cigarette smoking, is a leading cause of morbidity and mortality in modern society. It may be defined, according to the recommendations of the American thoracic society (ATS), as *a disease state characterised by the presence of airflow obstruction due to either or both of the conditions chronic bronchitis or emphysema. The airflow obstruction is generally progressive, may be accompanied by airway hyperreactivity, and may be partially reversible* (1). A second, more recent, definition has emerged from the consensus workshop report, *Global strategy for the diagnosis, management and prevention of chronic obstructive pulmonary disease* (GOLD). COPD is defined in this report as *a disease state characterised by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases* (2). Both definitions are useful. The ATS statement highlights the dual pathology of COPD, comprising both chronic bronchitis and emphysema. The GOLD definition, on the other hand, recognises the singular aetiology of COPD (ie. cigarette smoke) and the importance of airway inflammation. Chronic bronchitis is defined clinically, as the presence of *cough productive of sputum for most days for a minimum of three months per year for at least two successive years which cannot be attributed to other pulmonary or cardiac causes* (3). The hypersecretion can occur in the absence of airflow limitation. Emphysema, on the other hand, is defined anatomically, as *permanent enlargement of the airspaces distal to the terminal bronchioles accompanied by destruction of their walls and without obvious fibrosis.* Destruction in this scenario is defined as a lack of uniformity in the pattern of respiratory airspace enlargement such that the orderly appearance of the acinus and its components is disturbed or lost.

As highlighted by the 1995 ATS statement, both chronic bronchitis and emphysema may be present without causing clinically detectable airflow obstruction and in such cases are not strictly classified as COPD (1).

1:1 Epidemiology and risk factors

Since the latter half of the 20th century COPD has assumed enormous importance in terms of economic, social and health care services impact. The prevalence of the disease is uncertain. It varies widely between countries and, as many patients have only mild symptoms or are symptom free, diagnosis may only occur late in the

disease or not at all. However, figures from the United States estimated that the prevalence of COPD ranged from 4-6% in male smokers (1). The disease is ranked as the fourth leading cause of death in the western world (4). Statistics from individual countries bear this out, with COPD associated death ranked fourth in both the U.S (1) and Canada (5). COPD morbidity varies, such that many patients are only mildly affected while, in advanced cases, COPD associated debility may be severe enough to necessitate wheelchair transport and continuous oxygen therapy. Recurrent chest infections, physician visits, medications, visit to the emergency department and hospitalisations form a major burden to health services. Added to these are the indirect costs of, for example, work absenteeism and early retirement.

1:1:1 Smoking

COPD occurs as a direct result of chronic cigarette smoking (6, 7) and this, it is estimated, accounts for 85-90% of the risk of disease development (1). Smoking is directly associated with airflow obstruction, the physiological hallmark of COPD, and the most accurate indicator of prognosis (8). Under normal circumstances an age related decay in lung function occurs of approximately 30mls FEV_1/year. Cigarette smoking accelarates this to an average of 45ml/year but, due to the wide variation in individual susceptibility, some smokers remain unaffected while others experience an annual decline of up to 70-120 mls (9, 10). Susceptible smokers will, therefore, if they continue to smoke, eventually develop clinically significant airflow obstruction (11). For reasons as yet undetermined, only 15-20% of cigarette smokers fall into this category (11). There is evidence, however, that stopping smoking reduces the rate of functional decay to approximate that of a non-smoker (9, 12, 13). In addition to the links between smoking and airflow obstruction, observations from both pathological and thoracic imaging studies suggest that the prevalence of mild, clinically silent, COPD among smokers is much underestimated (14, 15). Thus chronic smokers who do not develop clinically important COPD are not immune to the effects of smoking but develop more subtle changes in lung function that are too mild to have clinical impact (16). Why constitutive susceptibility to cigarette smoke varies among smokers is unknown. Inherited factors are likely to be responsible in most cases, while, in a small minority, the environmental effects of occupational or industrial dust inhalation may have an added effect.

1:1:2 α –1 antitrypsin deficiency

α –1 antitrypsin deficiency, an inherited condition whereby individuals are deficient in the enzyme α-1 antitrypsin (α-1 AT), is the next most important risk factor after smoking, though as a relatively rare condition its impact on COPD epidemiology is small. Individuals homozygous for the defective gene are at increased risk of developing COPD, due to the unopposed actions of neutrophil proteases normally inhibited by α-1 AT. Deficiency of this enzyme, however, has a population prevalence of no more than one in several thousand and accounts for less than 1% of cases of COPD (1).

1:1:3 Dust and chemical exposure

Exposure to occupational dusts and gases (17), industrial fumes (18), and environmental air pollution (19) increase the risk of developing COPD, although the overall impact is much less than that of smoking. Particular occupations associated with increased risk, even in non-smokers, include both coal and gold mining (20).

1:1:4 Socioeconomic factors

Statistics suggest a greater risk for COPD development in subjects coming from a poorer socioeconomic background. Whether this reflects differences in smoking habits, pollutant exposure, poor nutrition or other factors is unknown. Poor diet itself has been proposed as a risk factor with some animal studies indicating an increased likelihood of developing emphysema where nutrition is sub-optimal (21).

1:1:5 Airway hyperresponsiveness

Bronchial hyper-responsiveness (BHR) was raised as a possible risk factor as part of the "Dutch hypothesis" that smoking, when superimposed on a predisposition to airway hyper-responsiveness, was more likely to lead to the development of COPD (22). BHR in COPD is common, with studies estimating prevalences ranging from 45% (23) to 70% (24). However BHR severity in COPD tends not to be as significant as that found in asthmatics with a similar degree of airflow obstruction (23). Although there is evidence that bronchial hyperreactivity is inversely related to FEV_1 and may be predictive of an accelerated decline in lung function (9, 23, 24), there is no firm evidence of its role as a separate risk factor for COPD.

1:2 Clinical and physiological features of COPD

1:2:1 Symptoms and signs

The symptoms of COPD are productive cough, wheeze, dyspnoea and recurrent chest infections. Symptom severity, however, is variable. Patients with mild disease may be asymptomatic, or limited on performance of demanding exercise or physical labour only. Severe disease, on the other hand, may be associated with movement restricting debility and an inability to carry out normal activities of daily living. Physical signs may be absent in mild COPD. Auscultatory wheeze, reduced breath sounds and chest hyperinflation are the most common findings in more severe disease. With progressing severity, weight loss and cyanosis may be observed. Additional physical signs, arising as a result of disease complications, include stigmata of carbon dioxide retention such as drowsiness, asterixus, chemosis, papilloedema, peripheral vasodilatation and a bounding pulse. Signs of cor-pulmonale and pulmonary hypertension may be present including peripheral oedema and a raised jugular venous pressure (26). Few clinical features are exclusive to COPD therefore confident diagnosis always requires pulmonary function testing.

1:2:2 Pulmonary function tests

Pulmonary function tests are primarily used, in the diagnosis of COPD, to demonstrate the presence and severity of airflow obstruction. Large airway spirometry, with measurement of the forced expiratory volume in one second (FEV_1), forced vital capacity (FVC) and FEV_1/FVC ratio is the mainstay of disease diagnosis. The FEV_1 in particular is a useful prognostic indicator for mortality, while the FEV_1/FVC ratio, although a more sensitive marker of airways obstruction, is inferior as a predictor of survival because the FVC itself tends to decrease also with progression of disease (8). Carbon monoxide gas transfer (TLCO) measurements provide a useful reflection of impairment of gas exchange in emphysema (27) and, as COPD is associated with lung hyperinflation, lung volume measurement may be helpful to suppport the diagnosis (28).

Other lung function indicators of COPD are not used as routinely in clinical practice. These include compliance measurements and tests more suitable for the detection of small airways obstruction such as expiratory flow rates at low lung volumes, closing volume and single breath nitrogen washout techniques (29). Of these, the most

commonly used is the maximal expiratory flow volume curve (MEFV) which indicates peripheral airways obstruction by demonstrating reduced flow at the end of the forced expiratory manoeuvre. There is, however, an extremely wide range of normal for flow rates at low lung volumes so use of flow values close to the end of the FVC manoeuvre are less satisfactory at distinguishing "normal" from "abnormal" subjects than values obtained at higher lung volumes (30).

1:2:3 Bronchodilator reversibility testing

Bronchodilator reversibility testing, performed by administration of a nebulised β2 agonist, is useful for the establishment of both the post-bronchodilator FEV_1, a useful prognostic indicator, and to exclude the presence of asthma, which is usually characterised by a substantial post bronchodilator increase in FEV_1 (26). More recent evidence suggests that measurement of lung volumes pre and post bronchodilator may add sensitivity to the assessment of bronchodilator responsiveness as subjects who do not demonstrate an FEV1 improvement may experience a reduction in volumes with possible improvement in symptoms (28).

1:2:4 Radiology

Although plain chest x-ray may display characteristic features of emphysema such as narrowing of the cardiac silhouette, bullae or an overall decrease in lung markings it is not sufficiently sensitive to detect mild disease. Radiological confirmation of COPD is achieved most accurately using computed tomography (CT), obtained either at normal or high resolution (HRCT). Although not routinely used for this purpose in clinical practice, this test has been shown to be both sensitive and specific for the diagnosis of emphysema (31) and is the most sensitive radiographic method with which to image the disease (32).

1:2:5 Staging of COPD in clinical practice

In clinical practice COPD is staged according to the severity of airflow obstruction. The ATS, British thoracic society (BTS) and European respiratory society (ERS) have all published staging guidelines based upon the degree of FEV_1 impairment. For example, according to the BTS (26) an FEV_1 of >80% predicted is normal, 60-79% represents mild impairment, 40-59% represents moderate impairment, and <40%

represents severe impairment. The ERS, on the other hand, has selected cut-offs of 70% and 50% to define moderate and severe disease (33) while the ATS uses 50% and 35% respectively (1). More recently, following a Global workshop, the GOLD guidelines were introduced (2). In this document COPD was initially categorised into four stages of severity, taking into account not only FEV_1 impairment, but also recognising the significance of a reduced FEV_1/FVC ratio as an early indicator of airflow obstruction. This has now been expanded (July 2003) to 5 stages. The GOLD stages, as defined in 2001 (2), are listed below. In the updated guidelines stage II has been divided into stages II and III demarcated by an FEV_1 cut-off of 50% predicted, while the old stage III is now classed as stage IV. For the purposes of this thesis, the 2001 guidelines have been used, as these were current when the work was carried out.

Stage 0: At risk – Subjects have cough and sputum production but normal spirometry.

Stage I: mild COPD – Subjects have only mild airflow limitation where the FEV_1 remains > 80% predicted but the FEV_1/FVC ratio is reduced < 70%. These subjects may or may not have symptoms.

Stage II: Moderate COPD – The FEV_1 is < 80% and > 30% predicted and FEV1/FVC ratio is < 70%.

Stage III: Severe COPD – The FEV_1/FVC ratio is < 70%. The FEV_1 is < 30% or < 50% plus there is evidence of respiratory failure, or clinical signs of right heart failure.

1:3 Evaluation of COPD for research purposes

1:3:1 Overview

Accurate assessment of COPD pathology *in vivo* is hampered by the heterogenous, often "silent", nature of the disease, and the limitations of currently available investigations. There is no substitute for the gold standard of pathological examination of resected or post mortem lung specimens. In clinical practice, lung function tests are employed in combination to detect physiological hallmarks of COPD such as airflow limitation and impaired alveolar transfer of oxygen. Although useful indicators of disease severity and prognosis, these investigations provide only limited information concerning the underlying pathology. HRCT, although not routinely used for this purpose in clinical practice, allows non-invasive detection and quantification of emphysema, and evaluation of peripheral airways dysfunction.

However, the strength of the relationship between HRCT findings and both lung morphology and lung function impairment in COPD varies depending on HRCT methods and mode of interpretation.

1:3:2 Limitations of the clinical approach in the research setting

The complex clinical nature of COPD is problematic for the researcher. Disease severity varies considerably from one individual to another, and asymptomatic "silent" disease is common (14, 15). Few clinical features are exclusive to COPD, with other lung conditions capable of presenting in a similar fashion. Moreover, among clinicians, considerable inter-observer variability exists in eliciting physical signs in COPD, while mild cases may lack symptoms altogether and have no clinical evidence of airflow limitation (34, 35). None of the clinical investigations in common use can give a completely accurate picture of the disease. Spirometry confirms the presence of airflow obstruction and hence is important for clinically defining the disease. However, although airflow obstruction severity is associated with increased mortality, it is a poor predictor of symptom severity (36) and inadequate for the detection of mild or early forms of COPD (37, 38). TLCO impairment, although an indicator of alveolar damage, does not specifically indicate emphysema and may occur in association with other pathologies including lung fibrosis and pulmonary vascular disease. In addition, TLCO impairment occurs by separate, additional, mechanisms in current smokers (16, 39-41) and therefore is of limited specificity even in the absence of other lung conditions.

Pathological interpretation of the clinical indicators of COPD is difficult. As both remodelling of the peripheral airways, and destruction of the lung parenchyma may impact upon lung function, linkage of the commonly used lung function tests to the underlying pathology is not straightforward *in vivo.* Although COPD is defined by airflow obstruction, controversy still exists as to whether peripheral airways disease (29, 42, 43) or emphysema severity itself (44-46) is the principal contributor. Moreover emphysema is commonly found in smokers who have not developed airflow obstruction (37, 38). Thus, although in clinical practice COPD diagnosis often depends simply on history taking, physical examination and demonstration of airflow obstruction and reduced gas transfer, characterisation of research subjects based on

these criteria alone invites error and misclassification and fails to provide an adequate insight into the underlying pathology.

1:3:3 High resolution computed tomography (HRCT) in COPD

With CT or HRCT the lungs are displayed as a series of radiographic cross sections that can be depicted as a set of images on film, for interpretation by a radiologist, or stored for computerized analysis. HRCT data are based on the variable absorption of X-rays by tissues, measured in Hounsfield units (HU). The attenuation value of a pixel depicted on a scan, expressed in HU, has a linear relationship with the density of the tissue represented by that pixel. In emphysema the destruction of lung parenchyma results in a greater air-tissue ratio, therefore emphysematous tissue manifests on CT as areas of reduced density (32). Although quantification of disease severity can reliably be performed by visual scoring of CT films (31, 47, 48), computerised analysis of lung density may be used also, and has the advantage of negating inter and intra-observer variability (14). Based on the premise that, as emphysema severity increases, parenchymal tissue density is reduced, investigators have shown that both the mean lung density (MLD) (obtained by averaging the CT values of all the pixels) (49-51) and the percentage of lung tissue below -950HU (%LAA)(a HU value chosen on the basis of radiological/pathological correlation studies of emphysema) (52) are significantly altered in smokers with emphysema. The value of these measurements as indicators of parenchymal destruction and gas trapping is discussed in the methods chapter (section 2:3:2:2).

1:3:4 The effects of inspiration vs expiration on HRCT interpretation in COPD

Interpretation of HRCT densitometry in COPD must be carried out with caution. In subjects with airflow obstruction, air trapping can lead to an apparent reduction in lung density. As a result the relationship with disease severity can vary depending on CT methodology, specifically whether or not scans are performed on inspiration or expiration (50, 53-55). Which modality is superior has been a matter of some debate. Investigators have shown that the %LAA at full inspiration provides an objective measure of the morphological extent of emphysema (52, 56) and that the correlation is superior to that achieved with expiratory CT (56). In contrast there is evidence that expiratory HRCT densitometry readings correlate more closely than inspiratory measurements with indicators of functional impairment including airflow obstruction

(55, 57) and TLCO reduction (49, 54, 55). Peripheral airway dysfunction and instability, which, as suggested by a number of studies, may have a significant impact upon airflow limitation in COPD (42, 43), is believed to be the underlying cause of this discrepancy. Inspiratory CT densitometry does not detect peripheral airways dysfunction. Expiratory CT readings, on the other hand, are affected because collapse of peripheral airways on expiration results in trapping of air distal to the obstruction (53, 56, 58, 59). Investigators have gone on to show that the average ratio of HRCT mean lung density at full expiration to that in full inspiration (E/I ratio) may be a useful method of indirectly evaluating gas trapping and, therefore, small airways dysfunction in smokers with COPD (53, 60). Thus, while HRCT performed on inspiration, can provide an insight into the pathological severity of emphysema, expiratory HRCT also sheds light on mechanisms of functional impairment. Future studies may employ this mode of investigation more commonly as an aid to the clinical phenotyping of COPD patients

1:4 The Pathology of COPD

In COPD pathological changes are found both in the airways and the lung parenchyma itself. Remodelling, a process of sustained disruption of structural morphology, occurs in the airway wall, while emphysema affects the lung parenchyma and refers to the destruction of alveolar tissue.

1:4:1 Epithelial changes

In the epithelium, exposure to cigarette smoke induces changes that include loss of ciliary length (61), disintegration of intercellular tight junctions (62, 63), and atrophy and shedding of columnar cells (64). In addition, focal squamous cell metaplasia (29, 65), where cells adopt a flattened, squamous phenotype, is a typical finding in the epithelium of smokers. The extent of this metaplastic change has been shown to correlate with tobacco smoke exposure (66) and it is reversible on smoking cessation (67). Goblet cell metaplasia, the process by which airway epithelial cells differentiate to become non-ciliated mucus producing goblet cells, occurs also and is considered in more detail below.

1:4:2 Goblet cell metaplasia and submucosal gland hypertrophy

Mucus, a viscoelastic gel normally produced in the airways by surface epithelial goblet cells and submucosal gland mucous cells, is believed to play an important role in mucosal hydration and protection from both irritants and bacteria (68). Hypersecretion of mucus, a classical hallmark of chronic bronchitis (69), may overwhelm the mucociliary apparatus, obstruct the airways and provide a favourable milieu for bacterial colonisation (70). The mucus producing apparatus of the airways is enhanced in two ways. Epithelial goblet cell hyperplasia occurs such that goblet cells become more numerous in the proximal airways, and there is a more even distribution of these cells across the spectrum of airway size. Therefore the smaller (< 400 microns in diameter) airways, which normally hold only a sparse population of mucous cells, become important contributors to excess mucus in this disease (71-73). In addition, enlargement of tracheobronchial glands occurs due to an increase in both the number and size of mucus secreting cells (74). In the glands, also, a variable degree of replacement of serous with mucous acini occurs (75). The elevated mucous/serous acini ratio results in a more gel like, thicker mucus, lacking in lysozymes and antiproteases, which normally provide protection against both infection and proteolytic injury (76). Partial or complete occlusion of the small (< 2mm in diameter) airways with mucous plugs may occur (77), and replacement of surfactant lining the small airways with mucous results in increased surface tension at the air-liquid interface further predisposing to airway collapse (46). The result is chronic productive cough, airflow limitation and a tendency towards recurrent respiratory tract infection (70).

1:4:3 Extracellular matrix changes

Studies of the large airways in COPD have not, to date, revealed an equivalent to the subepithelial fibrosis observed in asthma but appear to show, rather, a basement membrane thickness within the normal range (64, 78). Exceptions may exist, however, in a subset of patients with COPD who display some overlap with the asthmatic phenotype. These individuals, who have a bronchoalveoalar lavage fluid (BALF) eosinophilia and significant corticosteroid reversibility, also have demonstrable basement membrane thickening, though the composition remains unknown (79). Further studies are needed to properly define the clinical and pathological characteristics of this sub-group. Excess matrix deposition in COPD has,

however, been identified in the peripheral, non-cartilaginous airways (<2 mm in diameter), resulting in peribronchiolar fibrosis (29, 71).

1:4:4 Smooth muscle hypertrophy

Airway smooth muscle changes occur in COPD, particularly in the peripheral airways, resulting in a large increase in peri-bronchiolar smooth muscle mass (44, 45, 73, 80). Studies generally describe these changes under the heading of hypertrophy but the extent to which hyperplasia plays a role is unclear. Estimates of smooth muscle enlargement in the larger airways in COPD have varied (81). Some investigators have observed no abnormality in smooth muscle area (82) while others have demonstrated up to a two-fold increase in bronchial smooth muscle thickness, a finding attributable to both hyperplasia and hypertrophy (83).

1:4:5 Parenchymal destruction

Emphysema is characterised by destruction of the most peripheral bronchioles, the adjacent alveolar walls and their capillary networks resulting in replacement of normal tissue by dilated, non-functional, air spaces. Although a number of patterns exist two are predominant, as identified and described many years ago in post mortem studies of human lungs (84). *Centrilobular* emphysema, by far the most common type of emphysema, is characterised by destruction of the central portion of the acinus centred around the terminal bronchiole (ie. involving the respiratory bronchioles and the adjacent alveoli). The areas of destruction are surrounded by relatively normal lung parenchyma. The damage is distributed more frequently in the upper lung regions in mild cases, but may progress to appear throughout the lung (85). A second form, *panacinar* emphysema, is characterised by homogenous destruction of all the alveolar walls beyond the terminal bronchioles resulting in dilatation of all the airspaces throughout the secondary lung lobules. This type is associated with α-1 AT deficiency and tends to be more severe at the lung bases (85).

1:5 Pathophysiology of COPD

1:5:1 Expiratory airflow limitation

The hallmark physiological effect of COPD is expiratory airflow limitation and this is believed to occur via a number of mechanisms. Dysfunction of the peripheral airways,

with a tendency toward closure and collapse, arises as a result of remodelling of the peripheral airway wall and/or reduced support from the emphysematous surrounding parenchyma (86-88). Loss of elastic recoil, due to emphysema, is also believed to be responsible, as, because the elastic tissue that is stretched on inspiration is diminished, the driving force on expiration is lessened (89). The relative importance, however, of the two main pathological changes, peripheral airways remodelling and emphysema, as determinants of the severity of airflow obstruction in COPD remains unknown. Large airway changes, on the other hand, have not been shown to contribute significantly to expiratory airflow limitation (90).

Much of our knowledge concerning the mechanisms underlying airflow obstruction in COPD has been obtained by comparing the morphology of lung tissue, obtained at resection or post-mortem, with lung function *in vivo*. Studies have demonstrated that, although small, peripheral bronchi and bronchioles of less than 2mm in diameter, defined as either membranous (non alveolated airways) or respiratory bronchioles (partially alveolated airways), account for less than one quarter of total airways resistance in normal lungs (77), they are an important site of airflow obstruction in COPD due to either remodelling or obliteration (91, 92). Fibrosis, airway wall oedema, smooth muscle thickening and mucus overproduction all contribute, via thickening of the airway wall, narrowing of the lumen and mucus plugging (77, 92-94). In addition, replacement of the normal airway surfactant lining by mucus may result in increased surface tension and, therefore, increased small airway instability (46). Evidence from older post mortem studies (95) and more recent CT studies (42, 43) suggests that, for some patients at least, small airways narrowing is more important than emphysema as a contributor to airflow obstruction in COPD. For example, in a study of post mortem lungs taken from subjects with severe airways obstruction, some of the patients were observed to have little or no emphysema (95). Similarly, some HRCT studies have shown that the majority of smokers with chronic bronchitis and airflow obstruction had either only trivial emphysema or none at all, while some subjects with marked emphysema had relatively good lung function (42, 43). However, although some structure-function studies of the small airways have shown strong correlations between small airways morphologic disruption and chronic airflow limitation (29), other investigators have found associations that were either

only moderate (44, 94) or poor (43). Thus, the contribution of the remodelling *per se* to airflow obstruction remains uncertain.

Emphysema may give rise to airflow obstruction by a number of mechanisms. Destruction of parenchymal tissue is believed to result in loss of elastic recoil and obliteration of small conducting bronchioles (77, 96) while the stability of the remaining small airways is decreased due to loss of supporting radial traction forces normally applied by surrounding parenchyma (86). Thus, emphysema may itself lead to peripheral airways dysfunction independantly of the effects of remodelling or mucus plugging. In contrast to the studies cited in the above paragraph, some investigators have used HRCT to show that emphysema is highly prevalent in smokers with COPD and that its extent and severity are significantly associated with the severity of airflow obstruction (97). However, although a number of morphological studies have shown strong correlations between emphysema severity and airflow obstruction that heavily outweighed the predictive value of small airways abnormalites in estimating lung function (44-46), others have demonstrated a poor relationship between airflow obstruction and either CT or morphologically scored emphysema (31, 95, 98).

Although their relative importance may remain a source of conjecture, it is likely that both peripheral airways remodelling and emphysema contribute towards airflow obstruction in COPD, via a combination of mechanisms. Given the heterogenous nature of the disease it is also possible that the predominant mechanism varies from one individual to another, depending on the principal pathology that is present.

1:5:2 Reduced gas exchange and circulatory abnormalities

The capacity of the lungs to exchange oxygen and carbon dioxide across the alveolar-capillary basement membrane is compromised in COPD (99). In more advanced cases the damage is so profound that respiratory failure develops with a reduction in arterial oxygen concentration (hypoxaemia) and, in later stages, an elevated level of arterial carbon dioxide (hypercapnia) (100). The principal contributing factors are destruction of alveolar units, resulting in a reduced total functional alveolar-capillary basement membrane area (27) and ventilation-perfusion (V/Q) mismatching (101). Three main patterns of V/Q abnormality have been identified in COPD; High V/Q with no low V/Q and no shunting of blood, Low V/Q with no high V/Q and no shunting, and both

low and high V/Q (101). Uneven distribution of inspired gas, due to peripheral airways disease, can result in areas with the disproportionately low V/Q ratio and vascular shunting (102). Alternatively, a high V/Q ratio occurs in the presence of increased physiological dead-space, which may be the result not only of alveolar wall destruction, but also pulmonary vasoconstriction, perhaps occurring as a direct response to nicotine or to inflammatory mediators (16). More chronic pulmonary vascular abnormalities that occur in COPD also have the capacity to interfere with the gas exchange properties of pulmonary vessels. These include pulmonary arteriolar smooth muscle hypertrophy, intimal thickening and endothelial dysfunction (45). Pulmonary hypertension may arise in severe cases, and is associated with the development of right ventricular hypertrophy (46) and, eventually, right ventricular failure (103).

1:5:3 Increased lung volumes and gas trapping

Parenchymal destruction with the resultant loss of elastic recoil results in overdistension of the lungs. Thus, the lung pressure-volume curve is displaced to larger lung volumes. '*k* 'is a constant quantifying the rate of change in the slope of the Pressure/Volume curve and is an index of pulmonary elasticity. Thus an increased *k* value indicates increased static lung compliance. Both age (104) and emphysema (105) are associated with higher *k* values. This alteration in the pressure-volume curve leads to an increase in total lung capacity (TLC) and also contributes toward increases in residual volume (RV) (volume of air remaining in the lungs after a maximal expiratory effort) and functional residual capacity (FRC) (volume remaining after normal tidal expiration) (105). Added to this, peripheral airways dysfunction and gas-trapping results in closure of peripheral airways earlier on expiration. Thus, closure of small airways occurs at larger lung volumes than would otherwise be the case. This also contributes toward an elevated RV and lowers the inspiratory vital capacity (IC). The tendency of patients with COPD to contract their inspiratory muscles, in an attempt to minimise dynamic compression of the airways during expiration, may also contribute towards a raised FRC and a reduced IC.

1:6 Airway inflammation in COPD

1:6:1 Overview

COPD is characterised by chronic airway inflammation, upon which both major pathological processes of the disease, airway remodelling and parenchymal destruction depend (65). Exposure of the airways to cigarette smoke results in an inflammatory phenotype in which, current evidence suggests, neutrophils, macrophages and CD8+ve T lymphocytes play a key role. These cells are thought to employ a variety of mechanisms, including cytokine production, proteolytic enzyme release and the generation of reactive oxygen species to induce parenchymal tissue destruction, airway remodelling and a sustained inflammatory response. Numerous investigations, employing animal models, *in vivo* analyses and *in vitro* experiments, have led to this understanding.

1:6:2 Previous studies of COPD inflammation

Post-mortem studies, performed as long ago as 1957, have shown that emphysema is found in association with bronchial inflammation. Centrilobular emphysema in particular is always accompanied by chronic bronchiolitis ie. inflammation of the respiratory bronchioles leading to the emphysematous lesions (84). Numerous pathological examinations of bronchi and bronchioles in smokers have confirmed that active inflammation is present in the small peripheral airways in association with both the remodelling and destructive changes in COPD (29, 44, 46, 73, 93). In addition, correlations have been demonstrated between the severity of bronchiolar inflammation and indicators of functional impairment (27, 73, 93). Moreover, evidence has been put forward that a cause-effect relationship exists between small airways inflammation and centrilobular emphysema. This includes work demonstrating that the severity of inflammation in the small airways increases in parallel with emphysema severity (73, 106, 107). Also, studies have shown that areas of smokers' lungs where bronchiolar inflammation is most marked share the same distribution as emphysematous regions, with the upper lobes more severely affected (106, 108), and there is evidence that small airways disease temporally precedes centrilobular emphysema (29). Thus, it has been hypothesised that parenchymal destruction in centrilobular emphysema is the result of an inflammatory reaction that spreads centrifugally from the bronchioles to involve the parenchyma thus explaining

why alveolar ducts are often destroyed despite relative preservation of the more distal alveoli (106).

Animal models, *in vivo* analyses and *in vitro* experiments have suggested roles for specific inflammatory cells in the airway response to smoking and in the generation of parenchymal tissue destruction and airway remodelling. The results of some of these studies are considered in more detail below. The techniques of bronchoscopy and sputum induction, in addition to analysis of resected lung, have been used to describe airways inflammation *in vivo* in COPD and have helped confirm dominant positions in the inflammatory hierarchy for neutrophils (109-111), macrophages (78, 112, 113) and CD8+ T lymphocytes (114-116). Other inflammatory cells such as mast cells (112), eosinophils (117) and natural killer cells (112) have been credited with less importance.

Although numerous studies have been carried out, a clear profile of COPD airways inflammation has yet to emerge. There are a number of possible reasons. Firstly, there are the natural limitations of the techniques available. The predominant pathology in COPD is found in the peripheral airways and lung parenchyma, areas not easily accessible without the use of either resected or post mortem lung specimens. Therefore, to what extent proximal airways inflammation, detected by analysis of bronchial biopsy samples, reflects events in the distal lung is uncertain. Although BALF and induced sputum may provide a more distal representation of airway inflammation the correlation between luminal and tissue inflammation is, similarly, unknown. Moreover the results are dependant on the protocol in use, as inflammatory cell numbers in the lung vary depending with the region sampled, with neutrophils more numerous in the proximal airways while macrophages predominate in distal airways and alveoli. Digital subtraction angiography has shown that bronchial lavage fluid (BLF) (fluid aspirated back from the initially instilled aliquots) samples proximal airways, whereas successive aliquots (bronchoalveolar lavage (BAL)) may reflect distal airway and alveolar events (118). Thus, in healthy smokers neutrophil percentages decline in successive BAL aliquots (119). Sputum induction, similarly, is prone to sampling effects. In subjects with COPD induced sputum samples contains higher percentages of neutrophils and eosinophils and lower percentages of macrophages and lymphocytes than BALF, most likely because it samples more proximal airways (110, 120). However, increasing the duration of sputum induction

results in a progressive lowering of sputum neutrophils and eosinophils and an elevation in macrophages, possibly due to increasingly peripheral airways sampling (121). Thus standardisation of induction duration is important if reliable comparisons are to be made between different centres.

Another obstacle to consensus regarding the inflammatory phenotpye in COPD is that many prominent studies have had conflicting results. Some investigations showing CD8+ T lymphocyte infiltration of the airways (114, 115) have not been confirmed by others (110, 112). Macrophage infiltration has similarly been both confirmed (78, 122) and refuted (115, 123) while studies measuring neutrophils, eosinophils and mast cells in airway wall and sputum have also produced inconsistent results. Tables 1:1, 1:2 and 1:3 illustrate the way the results of inflammatory cell measurements in bronchial biopsy, induced sputum and lavage samples have diverged from one another in some recent studies.

A third impediment is the heterogeneiety of the disease itself. Subject characterisation in many studies has depended on symptoms and spirometry only to differentiate subjects with COPD from healthy smokers (110, 115, 123). In some cases TLCO (111, 114) and visually interpreted CT imaging were used also (124, 125) but these studies were exceptional. In no study has quantitative HRCT been combined with measurements of proximal airways inflammation. Thus, although airways inflammation has been linked with functional impairment, no data exists directly comparing proximal airways inflammation with distal airways and parenchymal pathology.

Another factor, not taken into account by many studies, but of potential importance, is the effect of current smoking. Although not recorded as frequently, this may be as important as pack year history in determining airway inflammation. Rennard and colleagues, for example, have demonstrated that short-term smoking reduction is associated with a reduction in airway inflammation in heavy smokers (126). Turato and colleagues, on the other hand, demonstrated that ex-smokers with symptoms of chronic bronchitis had a similar degree of airway inflammation to current smokers (127). Thus it is possible, but by no means certain, that differences in current smoking history may have contributed to the variability in results between different studies.

Sample processing and cell quantification methods have varied between studies. Biopsies, for example, have been variously embedded in paraffin (128) or OCT (114) and Tissue Tek (78). Inflammatory cells have been quantified in some studies in the

submucosa as a whole (110) where as others have limited their interest to a subepithelial zone 100μm deep to the reticular masement membrane (114). Thus, to date, despite great interest in the area, diverse methods and, often, diverse results, have contributed to uncertainty with regard to the most important inflammatory cells in COPD. However, evidence supporting the most likely candidates is strong, and considered in more detail in the next section.

1:6:3 Inflammatory cell types

Current evidence suggests that neutrophils, macrophages and CD8+ve T lymphocytes play a key role in the pathogenesis of COPD. These cells are thought to employ a variety of mechanisms, including cytokine and chemokine production, proteolytic enzyme release and the generation of reactive oxygen species to induce parenchymal tissue destruction, airway remodelling and a sustained inflammatory response.

1:6:3:1 Neutrophils

Neutrophils are front line defensive cells of the immune system and a source of reactive oxygen metabolites, inflammatory cytokines (eg. Tumour necrosis factor α (TNF-α)), chemokines (eg. Interleukin 8 (IL-8)), lipid mediators (eg. Leukotriene B4 (LTB4)), anti-bacterial peptides and tissue damaging enzymes, including the serine protease neutrophil elastase, metalloproteases and lysozyme (129-131). A central role has been ascribed to neutrophils and their products in both the generation of mucous metaplasia in chronic bronchitis and the destruction of lung tissue in emphysema. Animal models, *in vitro* work and clinical studies have suggested that neutrophil products induce mucus hypersecretion by both an acute secretagogue effect and by augmentation of the bronchial mucus producing apparatus. In hamsters, neutrophil elastase induces both secretory granule discharge by airway goblet cells and epithelial goblet cell metaplasia (132). *In vitro*, neutrophil elastase is a potent secretagogue for cultured bovine tracheal submucosal gland cells (133). The mucin proteins are the major macromolecular components of mucus and mucin gene expression has been proposed as the principal factor governing the differentiation of epithelial cells into goblet cells (68). Neutrophil elastase and reactive oxygen species have been shown to independantly increase epithelial mucin mRNA and protein expression *in vitro* (134-136), possibly via ligand independent transactivation of the epidermal growth factor

receptor (EGFR) (134). Moreover, in asthma a recent study has shown that the epithelial mucin, MUC 5AC, and EGFR are co-localised in airway epithelial goblet cells (137). As severe forms of asthma are associated with both massively increased mucus production and neutrophilic airway inflammation (138-141), it has been hypothesised that neutrophil-driven goblet cell metaplasia may be a component of asthma airways remodelling. COPD, like severe asthma and other diseases including bronchiectasis and cystic fibrosis, all provide circumstantial evidence implicating the neutrophil in excessive mucus production, as all are associated both with this and with neutrophilic airways inflammation (109, 138, 139, 142, 143).

A role for the neutrophil in emphysema pathogenesis has seemed likely since the discovery that individuals with inherited α1-AT deficiency were at increased risk of developing emphysema. This led to the theory that excessive proteolytic tissue digestion causes emphysema with the neutrophil, acting via proteolytic enzyme release, as the perpetrator of this protease/antiprotease imbalance (131). In support of this hypothesis purified preparations of neutrophil elastase have been shown to produce emphysema in animal models (21). Supportive observations in human studies include the demonstrations of neutrophil elastase in emphysematous tissue (144), increased products of elastase activity in urine and plasma from patients with COPD (145) and a correlation between the extent of emphysema and the level of elastase in peripheral blood neutrophils in cigarette smokers (146). Moreover, *in vitro*, peripheral blood neutrophils from subjects with emphysema have been shown, by analysis of digestion of iodine-125-radiolabelled fibronectin, to digest more extracellular connective tissue protein than those taken from control subjects (147).
The *in vivo* evidence that neutrophils play a role in COPD is abundant. Studies employing induced sputum (110, 111, 148) and BAL (117, 149, 150) have consistently demonstrated increased numbers of neutrophils, expressed either as relative (%) or absolute counts, in the lungs of smokers with COPD compared to controls. Likewise, elevated levels of neutrophil derived enzymes have been repeatedly demonstrated in sputum, BLF and BALF taken from subjects with COPD (124, 148, 151-153). Studies of bronchial biopsies and lung resection specimens, however, have often failed to demonstrate a corresponding increase in neutrophilic infiltration of the bronchial wall (78, 114, 117), although there are some notable

exceptions (112, 113). One proposed theory is that neutrophil migration from the vasculature to the airway lumen is swift, such that no accumulation occurs in the submucosa.

1:6:3:2 Macrophages

Macrophages, the most prevalent phagocytic cell in the lung, account for the majority of inflammatory cells recovered bronchoscopically by both BL and BAL regardless of whether the subjects are non-smokers, healthy smokers or smokers with airways disease (122, 150). Compounds released by macrophages include reactive oxygen species, chemotactic factors (eg. IL-8, LTB4, complement factors), proinflammatory cytokines (eg. Interleukin 1β (IL-1β), TNF-α), smooth muscle constrictors (eg. Leukotriene C4 (LTC4)) mucus gland activators (LTB4) and extracellular matrix proteins (eg. fibronectin). Included also is an array of matrix metalloprotease enzymes (MMPs), which, when combined, can degrade a similar spectrum of extracellular matrix (ECM) proteins to neutrophils (131). In addition, although macrophages don't transcribe the neutrophil elastase gene, their ability to internalise the enzyme has led to the proposal that macrophage released neutrophil elastase can add further to the cell's protease potential (154). Although macrophages have the theoretical potential to induce mucus hypersecretion via products with secretagogue activity, such as LTB4 and interleukin 1 (IL-1), most studies have focussed on their possible role, particularly with regard to MMP production, in causing emphysema. In dog models aerosolised homogenates of alveolar macrophages produce emphysema (21). In mice, over-expression of MMP-1 causes emphysema-like airspace enlargement, while deficiency of MMP-12 appears to be protective against cigarette smoke induced lung destruction (155). *In vivo,* MMP-12 has been demonstrated by immunohistochemistry and *in situ* hybridisation to be present in the lungs of subjects with emphysema but not healthy controls, while, *in vitro,* cultured alveolar macrophages taken from COPD subjects express elevated amounts of both MMP-1 and MMP-9 when compared to normal subjects (154).

Studies of emphysematous lung tissue from human subjects have demonstrated a direct relationship between alveolar macrophage density in the parenchyma and the severity of lung destruction (156). Moreover, numerous investigations have found macrophage numbers to be elevated in the bronchial submucosa (78, 110, 112, 114,

157) and bronchial glands (113) of subjects with COPD, although there have been exceptions, that have found counts in both the large (113) and small airway walls (115) to be similar to those of healthy smokers. Macrophage counts in induced sputum or BALF in COPD vary depending on whether relative or absolute cell counts are used. Relative macrophage counts are often reduced in COPD (110, 111, 125), a reflection, most likely, of elevated neutrophil percentages. Absolute counts, on the other hand, have been shown by a number of investigators to be elevated both in sputum (148) and BALF (122, 150, 158).

1:6:3:3 T lymphocytes

In asthma much interest has focussed on the CD4+ T cell as the proposed orchestrator of an immune response to allergens that is skewed toward a TH2 phenotype (159). In COPD, however, it is the CD8+ve cell that has attracted most interest following the observations that they are overabundant in both the airways and alveolar walls of smokers with COPD. An increase in total T cells (CD3+ve cells), which includes both CD4+ve and CD8+ve cells, has been shown to occur in the alveolar walls in emphysema (107, 116) with CD8+ve cells predominating over CD4+ cells (116). These findings compliment studies demonstrating elevated CD8+ve cell numbers in both the large (114) and small (115) airways of smokers with COPD.

What role these inflammatory cells play in emphysema development is the source of some speculation. An important function of CD8+ve cells is believed to be elimination of viral infection either by cytolysis of infected cells or by the induction of apoptosis (160, 161). There is evidence, however, that tissue damage can occur as a by-product of this activity. In respiratory syncitial virus (RSV) infected mice, for example, excessive activation of CD8+ T cells results not only in viral clearance but also potentially lethal pulmonary damage (162). Moreover in humans infected with the human immunodeficiency virus (HIV), high numbers of CD8+ve cells in alveolar lavage have been shown to be associated with an accelerated onset of emphysema induced by smoking (163). Whether CD8+ve cell induced lung damage occurs directly or indirectly is uncertain. Lytic substances released by CD8+ve cells such as perforin and granzyme have the potential to damage the lung interstitium (164). Alternatively with studies showing that increased numbers of structural cells undergo apoptosis in emphysematous lungs (165) in addition to findings by Majo and colleagues of a correlation between the number of CD8+ve T cells in the alveolar

walls of smokers and the number of cells undergoing apoptosis, it has been hypothesised that CD8+ve cells induce emphysema by induction of structural cell apoptosis (116).

Prior activation of T cells, by the presentation of antigen, is generally required before they can infiltrate non-lymphoid tissue such as the lung. Although the nature of the proposed antigen in COPD is unknown, two hypotheses have been put forward. Enelow and colleagues demonstrated that recognition of a lung "autoantigen" by T cells can produce lung injury in the absence of a viral stimulus, and this damage was mediated or amplified by non-antigen specific inflammatory cells eg. macrophages (166). It has been hypothesised, therefore, that repetitive damage to the lung, as a result of chronic smoking and inflammation, results in structural alteration in self-antigens allowing them to be recognised by T cells (116). Alternatively, it has been postulated that a persistant intracellular pathogen may provide a foreign antigenic stimulus. One such candidate is adenovirus as demonstrated by Retamales and colleagues who found a 41 fold increase in alveolar epithelial cells expressing the adenoviral transactivating protein E1A in severe emphysema compared with healthy smokers with an associated increase in intercellular adhesion molecule 1 (ICAM-1) expression in the alveolar epithelium (167).

The function of CD4+ve cells in COPD is unknown. It has been hypothesised that their actions as T helper cells, priming CD8+ cytotoxic responses, maintaining their memory and ensuring their survival, may be as important to the development of emphysema as the CD8+ve cells themselves (116).

1:6:3:4 Natural killer (NK) T lymphocytes

NK cells are a distinct population of specialised cytotoxic lymphocytes that target transformed or virus infected cells (168, 169). Although the function of NK cells in COPD has not been widely studied, one investigation has suggested that they are found in increased numbers in the large airway submucosa of smokers with COPD, and the authors hypothesised that excessive NK cell recruitment in COPD occurs due to repeated viral or bacterial infections (112). However, a recent study measuring lymphocyte sub-populations in the alveolar walls of non smokers, healthy smokers and smokers with emphysema demonstrated no elevation in NK cell numbers in

subjects with emphysema (116). Further studies are required to determine whether or not these cells play an important role in COPD.

1:6:3:5 Eosinophils and mast cells

Although eosinophils and mast cells are both believed to be important effector cells in asthma, neither of these two inflammatory cell types has been ascribed a prominent role in COPD pathogenesis. Some studies have demonstrated elevated numbers of eosinophils in the sputum (125), BALF (150) and airway wall (117, 123) of COPD subjects. Moreover, in exacerbations of chronic bronchitis, increased eosinophilic infltration of the submucosa has been observed, at a level similar to that found in asthmatics (170). Eosinophilic cationic protein (ECP) a potent, eosinophil derived, tissue damaging protein has also been found in increased amounts in both BAL (171) and induced sputum (148, 172, 173) in COPD. Other investigators, however, have found no increase in either eosinophil numbers or activity in smokers with COPD (78, 111, 113, 114, 117, 174) and some reports have suggested that infiltrating eosinophils in COPD do not display evidence of activation. For example, Lacoste and colleagues found that eosinophils appeared to be less degranulated, and localised deeper in the submucosa, in COPD subjects than in subjects with asthma (117). Rutgers and colleaues showed that although eosinophil numbers in BALF were increased in COPD, the concentration of ECP when expressed per eosinophil was not different from that of healthy subjects (110). It has been hypothesised, therefore, that the influx of these cells, if it occurs, is merely a by-product of the ongoing smoking-induced inflammation. One possible candidate mediator for this is the chemokine IL-8, which is known to be present in increased amounts in COPD airways. Although normally associated with neutrophil chemotaxis, this agent has been shown to exert a chemotactic effect on primed eosinophils (175, 176). In addition a positive correlation has been demonstrated between IL-8 levels and ECP in BALF of chronic bronchitis (152).

Although, as outlined above, the importance of the eosinophil to COPD pathogenesis remains uncertain, a number of recent reports have suggested that elevated numbers of eosinophils in sputum (125, 177) and BALF (79) taken from COPD subjects are predictive of a response to steroid treatment. Whether these subjects had an eosinophilic variant of COPD or concomitant asthma is a matter for speculation.

These results do suggest, however, that eosinophils may play a role in a clinical subset of COPD that has phenotypical features in common with that of asthma.

The ability of mast cells to release an array of mediators has invited speculation regarding their possible role in COPD inflammation. Possible mast cell contributions could include neutrophil recruitment via the release of chemotactic factors, tissue injury by the actions of the secreted enzymes tryptase, chymase and elastase and mucus hypersecretion via the potent secretagogue action of mast cell chymase (178). Some studies of COPD airways have revealed increased mast cell numbers in the airway wall (179, 180). In addition one study, demonstrating that mast cell degranulation in the bronchial gland layer appeared more marked in subjects with chronic bronchitis compared to healthy controls, suggested that increased mast cell activity may be present also (180). These findings are countered, however, by reports displaying no evidence of mast cell abundance in either the airways or parenchyma in COPD subjects (78, 112, 113, 170, 174). Further studies are needed, therefore, to establish whether or not mast cells play a significant role in COPD pathogenesis.

Cell type	Elevated	Not elevated
CD3+ve cells	Fournier 1989 (epithelium) γ Saetta 1993 (submucosa) γ O'Shaughnessy 1997 (submucosa) γ Distefano 1996 (submucosa) θ	Saetta 1996 Distefano 1998 Lams 1998 Rutgers 2000
CD4+ve cells	Fournier 1989 (epithelium) χγ	Saetta 1993 Saetta 1997 O'Shaughnessy 1997 Distefano 1998 Saetta 1998 Lams 1998 Rutgers 2000 Distefano 1996 θ
CD8+ve cells	Fournier 1989 (epithelium) χγ Saetta 1998 (small airways) χ O'Shaughnessy 1997 (submucosa) γ Majo 2001 (alveolar wall) γχ	Saetta 1993 Saetta 1997 Dostefano 1998 Lams 1998 Rutgers 2000 De Boer 2000 Distefano 1996 θ
CD8+/CD4+ ratio	Lams 1998 (small airways) π Saetta 1997 (bronchial glands) χ O'Shaughnessy 1997(submucosa) γ	Saetta 1993 Rutgers 2000 Saetta 1997

Table 1:1 Studies of CD3+, CD4+ and CD8+ cells in the airway wall in COPD. Each article is denoted by the first author name and the year of publication.

γ: COPD compared to non-smokers

χ: COPD compared to healthy smokers

π: All smokers compared to non-smokers

θ: Chronic bronchitis with airflow obstruction compared to without airflow obstruction

Cell type	Elevated	Not elevated
Neutrophils	Saetta 1997 (glands and epithelium) γ Distefano 1998 (submucosa) χ Retamales 2001 (alveolar walls) γ	Lacoste 1993 Saetta 1993 O'Shaughnessy 1997 Saetta 1998 Lams 1998 Distefano 1996 Rutgers 2000 Rutgers 2000 Turato 2002
Macrophages	Saetta 1993 (submucosa) γ Saetta 1997 (bronchial glands) γ Rutgers 2000 (submucosa) γ Distefano 1998 (submucosa) χ Rutgers 2000 (submucosa) χ O'Shaughnessy 1997 (submucosa) γ Distefano 1996 (submucosa) θ	Saetta 1997 Lams 1998 Saetta 1998
Eosinophils	Lams 1998 (small airways) π Rutgers 2000 (submucosa) χ Lacoste 1993 (submucosa) γ	Saetta 1993 Saette 1996 Saetta 1997 Distefano 1996 O'Shaughnessy 1997 Distefano 1998
Mast cells	Pesci 1994 (epithelium and glands) γ Lamb 1982 (small airways) π Grashoff 1997 (small airways) χ	Saetta 1997 Distefano 1998 Saette 1996 O'Shaughnessy 1997 Distefano 1996

Table 1:2: Studies of neutrophils, macrophages, eosinophils and mast cells in the airway wall in COPD. Each article is denoted by the first author name and the year of publication

γ: COPD compared to non-smokers

χ: COPD compared to healthy smokers

π: All smokers compared to non-smokers

θ: Chronic bronchitis with airflow obstruction compared to without airflow obstruction.

Cell type	Elevated	Not elevated	Reduced
Neutrophils	Peleman 1999 (% cells) γ Rutgers 2000 (% cells) γχ Fujimoto 1999 (% cells) γ Keatings 1997 (% cells) γ Keatings 1997 (x 10^6/ml) γ Stanescu 1996 (% cells) χ Linden 1993 (x 10^4/ml) γ Martin 1985 (% cells) χ Kuschner 1996 (x10^3 cells/ml) π Lacoste 1993 (% cells) γ Capelli 1999 (% cells) γ χ Capelli 1999 (absolute) γ π	Rutgers 2000 (% cells) χ Pesci 1994 (% cells) γ Capelli 1999 (absolute) χ	
Eosinophils	Keatings 1997 (x 10^6/ml) γ Fujimoto 1999 (% cells) γ Rutgers 2000 (% cells)χ Linden 1993 (x 10^4/ml) γ Rutgers 2000 (% cells) χ	Peleman1999 (% cells) γ Lacoste 1993 (% cells) γ Pesci 1994 (% cells) γ Kuschner 1996 (x10^3/ml) π Capelli 1999 (% cells) γ χ	
Macrophages	Keatings 1997 (x 10^6/ml) γ Linden 1993 (x 10^4/ml) γ Kuschner 1996 (x10^3 cells/ml) π Capelli 1999 (absolute) π	Pesci 1994 (% cells) γ Keatings 1997 (% cells) γ Capelli 1999 (% cells) γ χ Capelli 1999 (absolute) χ	Martin 1985 (% cells) χ Lacoste 1993 (% cells) γ Peleman 1999 (% cells)γ Fujimoto 1999(% cells)γ Rutgers 2000 (% cells) χ

Table 1:3 Studies of neutrophils, macrophages and eosinophils in sputum, bronchial lavage fluid or bronchoalveolar lavage fluid in COPD. Each article is denoted by the first author name and the year of publication.

γ = COPD compared to non smokers

χ = COPD compared to healthy smokers

π = All smokers compared to non smokers

θ = Chronic bronchitis with airflow obstruction compared to without airflow obstruction.

1:7 The bronchial epithelium and neutrophilic inflammation in COPD

1:7:1 Pro-inflammatory and structural epithelial changes in COPD

Bronchial epithelial cells serve not only a barrier function but have the potential to play a role in airway inflammation and remodelling processes, via alterations in both their structure and function. Upon activation, the range of agents expressed by the epithelium includes autacoid mediators (nitric oxide and the arachidonic acid metabolite, prostaglandin E2 (PGE2)), chemokines (IL-8, growth related oncogene α (GRO-α), monocyte inflammatory protein α (MIP-1α)) and growth factors (transforming growth factor α (TGF-α), insulin-like growth factor 1 (IGF-1) and platelet derived growth factor (PDGF) (181). Investigations into asthma pathogenesis have pointed to the bronchial epithelium as an important source of such mediators in response to environmental stimuli including allergens and viruses (182), while the increased epithelial expression of adhesion molecules (183) and growth factor receptors (184, 185) have been taken as evidence that epithelial injury and repair is ongoing in this disease. It has been proposed that interactions between epithelial repair responses and those of underlying fibroblasts and myofibroblasts play a role in the development of asthmatic large airway subepithelial fibrosis (185).

In chronic smokers, the airway epithelium is the front line recipient of constant injury, both directly from cigarette smoke exposure, and from products, for example neutrophil elastase (186), released by recruited inflammatory cells. As a result, significant changes occur in the morphology and phenotype of airway epithelial cells. As outlined in section 1:4:1, structural changes include loss of columnar cell ciliary length (61), disintegration of intercellular tight junctions (62, 63), atrophy and shedding of columnar cells (64), focal squamous cell metaplasia (66, 187) and goblet cell metaplasia (44, 45, 94, 188). However, epithelial cell function, also, may be affected by smoking with possible contributing effects to the inflammatory state of the airways in COPD. *In vitro*, cigarette smoke, or its constituents, induces enhanced epithelial expression of the pro-inflammatory cytokines IL-8, granulocyte macrophage colony stimulating factor (GM-CSF) and TNF-α (189-192). *In vivo*, expression of both IL-8 (128) and the adhesion molecule ICAM-1 (193, 194) is augmented in the epithelium of COPD subjects. Further *in vitro* work has suggested that bronchial epithelium in COPD patients shows greater susceptibility to the effects of cigarette smoke than in healthy smokers. Specifically, cultured epithelial cells

taken from COPD subjects, when exposed to cigarette smoke, have been shown to be more prone to increases in transepithelial permeability and reduction in intracellular glutathione levels (and thus reduced anti-oxidant activity), and to release higher levels of the inflammatory cytokine IL-1β and ICAM-1, than cells from their healthy counterparts (195). These results fuel speculation that differential responses of bronchial epithelial cells to cigarette smoke exposure is a determinant of COPD development.

The importance of epithelial mediator production, compared to production by infiltrating inflammatory cells, towards COPD inflammation remains unknown. Mediators that have been demonstrated in increased quantities in either sputum, BALF, lung tissue or blood of COPD subjects include chemokines and pro-inflammatory cytokines such as IL-8 (148, 152, 171), MIP-1α (112), LTB4 (196), TNF-α (109), GMCSF (197) and monocyte chemotactic factor 1 (MCP-1) (158), growth factors such as transforming growth factor β (TGF-β) (197) and endothelin-1 (198) and extracellular matrix glycoproteins such as fibronectin (199). Any of these agents may be produced by a variety of cell types and studies have not, so far, identified their major sources in COPD.

1:7:2 Interleukin 8 and Growth related oncogene α

Neutrophil infiltration of the lung in COPD is believed to be a complex process mediated by an array of molecules including adhesion molecules, inflammatory cytokines and chemotactic factors. The latter group includes lipid mediators (200), complement components (201), and members of the chemokine family of proteins ie. chemokines (202). Chemokines are low molecular weight signalling proteins that direct the migration of inflammatory cells from blood vessels to the inflammatory focus (202). They are produced by a variety of structural cells, including epithelial cells, endothelial cells, smooth muscle and fibroblasts as well as inflammatory cells. A total of 39 human chemokines have been identified, and these are divided into the following 4 subclasses based on the number and spacing of conserved cysteines in their sequence; CC (28 identified), CXC (15 identified), C (2 identified) and CXXXC (1 identified), in which X denotes the number of non-cysteine residues between the first two conserved cysteines.

The biological effects of chemokines are mediated by their interaction with specific transmembrane cellular receptors. A variety of chemokine receptors have been identified, distributed to varying degrees on different cell types. These are classed according to their preferred ligand. Thus receptors CCR1 - CCR11 bind, variously, the CC chemokines while CXCR1 – CXCR5 bind CXC chemokines. There is a degree of redundancy in the chemokine superfamily as, in some cases, one ligand can bind several receptor subtypes, and one receptor can bind several chemokines (203, 204).

IL-8 and GRO-α are both neutrophil chemokines, belonging to the CXC family. Structurally, IL-8 and GRO-α are very similar, with receptor binding and activation domains located at the N terminus of the molecules, where, in both, the ELR sequence (Glu4-Leu5-Arg6), which is essential for neutrophil chemotactic activity, is located (205, 206). The two chemokines differ from one another in both the NH2-terminal region containing the ELR motif and the NH2 terminal loop region. The difference in structure of the NH2 terminal region between the two is important in determining their differing receptor selectivity. CXCR1 and CXCR2 are the G-protein coupled receptors for both of these chemokines. CXCR1 receptors are located on neutrophils, macrophages, mast cells and dendritic cells. CXCR2 receptors are found on neutrophils, macrophages, epithelial cells and endothelial cells (207). IL-8 binds both receptors with high affinity although the CXCR1 receptor is most highly selective for IL-8. GRO-α binds both the CXCR1 and CXCR2 receptors but has much greater affinity for the CXCR2 (204). Although these chemokines possess a number of biological effects on various cells, they predominantly act upon neutrophils.
IL-8 was the first leukocyte subtype-selective chemoattractant to be discovered (208) and is one of the most potent activators and chemoattractant mediators known for neutrophils, but also has some lesser chemoattractant activity for T lymphocytes, eosinophils and basophils. It is an extremely stable protein and maintains its biological activity, even in the presence of significant changes in pH and proteolytic enzymes, suggesting that once produced it may exert a prolonged biological activity (209). GRO-α was first reported as a growth factor for human melanoma cells (210) and, likewise, displays chemotactic activity for not only neutrophils but also T cells and basophils (210, 211). Studies *in vitro* have shown that both IL-8 and GRO-α

stimulate transendothelial migration of neutrophils, the release of proteolytic enzymes (212) and activation of the respiratory burst with reactive oxygen species formation (209, 213, 214). However, although GRO-α is a strong inducer of neutrophil chemotaxis and activation (215), the respiratory burst response by neutrophils is much weaker than that to IL-8 (212). This observation is in keeping with work showing that, although both the CXCR1 and CXCR2 receptors trigger intracellular calcium changes, chemotaxis and granule exocytosis, activation of the respiratory burst is only observed after stimulation of CXCR1 (212, 216).

In vivo evidence that CXC chemokines potentiate neutrophilic inflammation has come mainly from animal models. Intra-dermal injection of human IL-8 induces a rapid and concentration dependant infiltration of neutrophils in mice, rats, rabbits, dogs and sheep (213). Rabbit models have employed a human monoclonal antibody, which is able to neutralise rabbit IL-8 with high potency. Anti - IL-8 antibodies have been shown, in rabbits, to reduce alveolar damage occurring due to smoke exposure (217), neutrophil influx and lung endothelial injury following acid aspiration (218), endotoxin mediated influx of neutrophils into the pleural space (219) and neutrophilic infiltration and lung injury in lung reperfusion injury (220). In a rabbit model of the adult respiratory distress syndrome (ARDS), massive neutrophil infiltration was accompanied by raised IL-8 levels in BALF and increased immunohistochemical expression of IL-8 by the lungs. Treatment with monoclonal antibody to IL-8, however, almost entirely prevented both neutrophil influx and pathological lung changes in these animals (221). In addition, antagonists to CXCR2, which is activated by both IL-8 and GRO-α, have been shown to inhibit neutrophilic inflammation in a subcutaneous air pouch and in the peritoneal cavity of mice in response to pro-inflammatory agonists (222).

1:7:3 Regulation of IL-8 and GRO-α expression

Although the IL-8 gene is controlled at both the transcriptional and post-transcriptional level there is evidence that regulation of these cytokines is mainly transcriptional (223). Activation of the nuclear factor κB (NF-κB) transcription factor, followed by binding to the NF-κB gene element, is the most crucial step for transcription of both IL-8 and GRO-α. The NF-κB gene element is present in a

multitude of genes. In resting cells NF-κB is present in the cytoplasm bound to its inhibitor IκBα. After cellular activation, and through proteolytic phosphorylation and degradation of IκBα by IκB kinases, NF-κB is released and translocated to the nucleus where it can induce expression of those genes that contain NF-κB elements (223). In the case of both IL-8 and GRO-α transcriptional regulation is dependant on multiple factors recognising not only NF-κB but also adjacent, but not identical, DNA binding elements (224). IL-8 induction requires the CAAT/enhancer binding protein (C/EBP) site adjacent to the NF-κB site for complete cytokine induction (225). Thus in many cases, depending on the stimulus, a co-operative effect is required between NF-κB and other transcription factors to induce IL-8 gene transcription (226). Activation of GRO-α expression, similarly, requires additional gene elements, located adjacent to NF-κB in the immediate upstream region (224). The additional transcription factors required for induction of this chemokine are not well described however.

1:7:4 Previous studies of IL-8 and GRO-α in COPD

Clinical studies have provided evidence of a role for IL-8 in a number of lung disorders that are associated with neutrophilic inflammation. Elevated IL-8 levels have been demonstrated in the sputum of patients with cystic fibrosis (139), in tracheal aspirates and induced sputum from patients with poorly controlled asthma (227, 228) and in BALF from subjects with idiopathic lung fibrosis, sarcoidosis and ARDS (229, 230). In these studies levels of IL-8 correlated with neutrophil counts and/or neutrophil chemotactic activity in the same samples. In addition elevated levels of this cytokine have been found in the induced sputum (110, 148), BLF (152, 171) and BALF (231) of healthy smokers (148), smokers with COPD (110) and smokers with subclinical emphysema without airflow obstruction (232). GRO-α has not been widely investigated *in vivo* in smokers. One study failed to show significant differences in GRO-α levels in the BALF of healthy smokers as compared to non-smokers (233). A more recent investigation suggested that BALF GRO-α levels might actually be reduced in healthy smokers compared to non-smokers, while neither group differed significantly from COPD smokers (234). In this same study, however GRO-α levels were measured in induced sputum also, and were significantly elevated in COPD compared to the other two groups and correlated with sputum neutrophil

absolute counts. BALF recovery was significantly lower in the COPD subjects in this study than in the control groups. The authors suggested that the discrepancy between sputum and BALF may be the result of problems inherent in BAL such as dilution of chemokines and failure of standardisation of technique due to clinical constraints. Although there is *in vitro* evidence that polymorphonuclear cells (PMNs) from subjects with COPD show enhanced chemotaxis compared to PMNs from control subjects (147) the importance of the contribution made by either IL-8 or GRO-α toward neutrophil chemotaxis in the lungs of smokers remains uncertain. Analysis of *purulent* sputum taken from subjects with cystic fibrosis, bronchiectasis and chronic bronchitis has shown, however, that a significant proportion of neutrophil chemoattractant activity in sputum is attributable to IL-8 and, to a lesser extent, LTB4 (196, 235). GRO-α was not investigated in these studies.

1:7:5 The bronchial epithelium as a producer of IL-8 and GRO-α in COPD.

In COPD, bronchial epithelial chemokine production, enhanced due to prolonged exposure to cigarette smoke, has a possible role in the generation of neutrophilic airway inflammation. Both IL-8 and GRO-α are produced by bronchial epithelium, and their expression is augmented by exposure to toxic stimuli. Evidence for this effect includes *in vitro* observations that cigarette smoke (190), diesel exhaust particles (DEP) (236), *Haemophilus influenza* endotoxin (237), *pseudomonas aeruginosa* products (238) and neutrophil elastase (237) all induce IL-8 production or release in cultured bronchial epithelial cells. In primary epithelial explant cultures, exposure of cells from COPD subjects to cigarette smoke and DEP induced significant increases in IL-8 release, a consequence not observed in cells taken from smokers without lung function impairment when exposed to the same stimuli (239). *In vivo,* exposure to diesel exhaust particles augments bronchial epithelial expression of both IL-8 and GROα (240). Immunohistochemical and *in situ hybridisation* analysis of resected lung tissue has shown that IL-8 messenger RNA (mRNA) and protein levels are significantly raised in bronchiolar epithelium from COPD subjects compared to healthy smokers, and that the levels of both correlated inversely with measurements of airflow obstruction (128). These investigations have fueled speculation that inter-subject variability in the IL-8 response to cigarette smoke by bronchial and/or alveolar epithelial cells is a determinant for the early development of

emphysema (232). Epithelial expression of GRO-α in COPD has not been explored to date.

Smoking probably augments epithelial chemokine production via a number of mechanisms. Evidence from a number of *in vitro* studies has linked epithelial IL-8 release with epidermal growth factor receptor (EGFR) activation. The epidermal growth factor ligand (EGF) initiates release of IL-8 from bronchial epithelial cells, consistant with its ability to activate NF-κB (241). In addition a more recent study has shown that cigarette smoke extract (CSE) induces expression and release of several EGFR ligands, in a dose dependant manner, including TGF-α and amphiregulin (242). Critically these investigators also showed that anti-EGFR antibodies partially block CSE induced IL-8 release, thus suggesting a causal relationship between autocrine EGFR ligand release and IL-8 production in response to CSE (242). Alternatively TNF-α, a pro-inflammatory cytokine found in increased amounts in COPD lung secretions (109), has also been shown to augment epithelial IL-8 gene expression *in vitro* (237) and may thus play a similar role in smokers *in vivo*. Neutrophil elastase itself has been shown to induce IL-8 expression in cultured epithelial cell lines (223, 243) thus perpetuating the cycle of inflammation with recruited neutrophils promoting further neutrophil recruitment via stimulation of IL-8 release.

Despite the evidence above, studies showing elevated luminal IL-8 and GRO-α levels in smokers' lungs have not, thus far, identified the principal source of this chemokine. Both IL-8 and GRO-α are produced, not only by epithelium, but also by two of the major inflammatory cell types that infiltrate the airways in COPD, monocytes/macrophages (244) and neutrophils (245). Other possible sources exist also, including T lymphocytes, mast cells and smooth muscle cells for IL-8 and airway fibroblasts for GRO-α (213).

1:8 Neutrophils, ErbB receptors and goblet cell metaplasia in smokers

1:8:1 Overview

Activation of the epidermal growth factor receptor (EGFR), a member of the ErbB family of growth factor receptors, which is expressed on normal bronchial epithelium

(246), may play a role not only in heightened epithelial chemokine expression, as outlined above (242), but also in the development of epithelial goblet cell metaplasia (247). It has been speculated that an important cause-effect relationship exists between both such epithelial changes and airways inflammation, particularly neutrophilic inflammation. In the sections below I have explored the evidence for this and the possible relevance of these processes to changes in the airways of cigarette smokers

1:8:2 Epithelial Goblet cell metaplasia

As mentioned in section 1:4:2: The pathology of COPD, epithelial goblet cell metaplasia occurs in the airways of cigarette smokers both with and without COPD (44, 45, 94, 188). Enhanced expression and transcription of mucin genes, that encode the mucin glycoproteins, is believed to be the rate-limiting step for this process (68). Mucin proteins, the major macromolecular components of mucus, are heavily glycosylated structures, consisting primarily of a polypeptide backbone and oligosaccharide chains attached to serine and/or threonine residues. They can be classified into two broad groups; the secreted mucins, which are found in secreted mucus and the membrane-bound mucins, which are believed to participate in cell-cell signalling and modulation of adhesion. Of the four secreted mucins identified (MUC2, MUC5AC, MUC5B, and MUC6), three (MUC2, MUC5AC and MUC5B) are found in the lung. MUC5AC, is believed to be the major secreted mucin of the bronchial epithelium, and has been shown by *in situ* hybridisation to be strongly expressed by bronchial epithelial cells (248). MUC2, also, is found in airway epithelium but its expression levels are lower than that of MUC5AC (248). MUC5B, unlike MUC5AC and MUC2, is produced mainly by submucosal glands, while its expression in airway epithelium is very limited (248, 249). Both environmental and host factors, acting on the epithelium, have been shown to stimulate mucin secretion and mucin gene upregulation. These include infectious agents (250), environmental pollutants including the cigarette smoke constituent, acrolein (251), inflammatory mediators (252) and cell degranulation products (253, 254). To date, no quantitative comparisons between non-smokers and smokers, with or without COPD, in terms of airway mucin protein expression have been performed.

1:8:3 ErbB receptors in bronchial epithelium

The EGFR (ErbB1), ErbB2 and ErbB3 are members of a structurally homologous transmembrane tyrosine kinase growth factor receptor family that, unlike their other family member, ErbB4 are normally expressed by bronchial epithelium (246). ErbB receptors are believed to play a fundamental role in the processes of epithelial repair and differentiation in response to injury (255). The EGFR, the prototype and most studied member, is the target of six functionally distinct ligands, the EGF family of growth factors, that include epidermal growth factor (EGF), transforming growth factor α (TGF-α), amphiregulin (AR), heparin-binding growth factor (HGF), betacellulin and epiregulin. ErbB2 has no known ligand while ErbB3 and ErbB4 are low and high affinity receptors, respectively, for the neuregulins. Binding of the EGFR by one of its ligands stabilises it in dimeric form comprising either EGFR homodimers or EGFR/ErbB heterodimers involving one of the other members of the ErbB family. This phenomenon of heterodimerisation allows recruitment of the intracellular signalling apparatus utilised by both receptor types involved, facilitating induction of a broader range of signals, thus increasing the repertoire of ErbB receptor activation (256). There is much *in vitro* and *in vivo* evidence that EGFR activation plays a key role in stimulation of cell motility, migration, proliferation and differentiation (255), all processes important in the process of epithelial repair. Studies in rodents have shown that expression of EGFR is increased in bronchiolar epithelial cells after bleomycin and naphthalene induced lung injury (257, 258). In dermal wounds (259) and carcinoma (260) increased EGFR expression results in epithelial proliferation.

In asthma, studies showing that EGFR expression is elevated on damaged epithelium have provided convincing evidence that epithelial injury and repair is an on-going process in the disease (185). However, in asthma EGFR expression is elevated not only on damaged but also on both morphologically intact epithelium (184, 185) suggesting the presence of a "stressed" epithelium exhibiting an abnormal, heightened repair response (185). The co-expression of the EGFR with its fellow receptors ErbB2 and ErbB3 in both normal and asthmatic airway epithelium, supports the view that these receptors can interact to form heterodimers that can regulate intracellular signalling and response to injury (261).

It is likely, also, that ErbB receptor signalling plays a role in the restitution of the epithelial barrier in response to cigarette smoke induced damage. It has been reported that cigarette smoking results in enhanced EGFR expression in airways epithelium *in vivo* (262) and that, in smokers, EGFR expression is increased in areas of squamous metaplasia compared with normal epithelium (263). However, expression levels of the other ErbB receptors in cigarette smokers remains unknown and no study has yet profiled the expression of any ErbB receptors in bronchial epithelium in COPD.

1:8:4 ErbB receptor involvement in induction of goblet cell metaplasia

Several lines of evidence suggest that epithelial mucin gene upregulation, and subsequent differentiation of epithelial cells into mucus producing goblet cells, is mediated via EGFR activation. Studies in animal models have shown that foreign body-induced upregulation of epithelial MUC5AC expression is EGFR-dependent (264). In humans, EGFR immunoreactivity has been demonstrated in several mucin producing cell types including mucous neck cells, and mucous secreting pyloric gland cells in human gastric mucosa (265) while in asthmatic subjects both the EGFR and its EGF ligand are expressed in increased amounts in bronchial *glandular* tissue compared with controls (184). The EGFR ligands, EGF and TGF-α, induce synthesis of mucous glycoconjugates in the stomach (266). Moreover, in the airways, goblet cells have been shown to express EGFR, and this is co-localised with MUC5AC protein in the bronchial epithelium of both asthmatics and normal subjects (137). Despite the information above the role, if any, of the other ErbB receptors, either singly, or as part of a heterodimer combination, in the induction of goblet cell hyperplasia, remains unexplored.

1:8:5 Neutrophil mediated ErbB activation and goblet cell hyperplasia

As outlined in section 1:6:3:1: Airway inflammation in COPD, evidence from a variety of sources, including clinical studies and animal models, has linked neutrophilic inflammation with pathological mucus hypersecretion by the airways. Neutrophil driven EGFR activation, resulting in epithelial mucin gene expression and goblet cell metaplasia may be a key component. *In vitro,* augmentation of MUC5AC gene and protein expression by cultured H292 epithelium occurs when exposed to supernatants of neutrophils activated by IL-8, TNF-α and FMLP (134). In addition,

also in cultured cell lines, both neutrophil elastase and reactive oxygen species, both produced by neutrophils, independantly increase epithelial mucin mRNA and protein expression (134-136). Although blocking the effects of the EGFR ligand EGF failed to inhibit MUC5AC synthesis occurring due to exposure to activated neutrophils, upregulated epithelial MUC5AC protein expression can be blocked by inhibitors selective for the EGFR (134). It has been hypothesised, therefore, that ligand-independent EGFR transactivation, which is known to occur in response to oxidative stress, is the mechanism whereby neutrophilic inflammation augments epithelial mucin gene expression (134).

1:8:6 Alternative mechanisms of ErbB activation

Alternative or additional mechanisms are also likely to play a role in smoking induced ErbB receptor activation and goblet cell metaplasia. Oxidant stress occurs not only as a result of neutrophilic inflammation but also as a direct consequence of cigarette smoke exposure. In cultured cell lines exogenous hydrogen peroxide augments both EGFR tyrosine phosphorylation and MUC5AC expression (134). Anti-oxidants, however, only partially inhibit mucin synthesis by cultured epithelial cells exposed to cigarette smoke (267). Cigarette smoke may stimulate the EGFR directly, as cigarette smoke extract (CSE) has been shown to cause ligand independent phosphorylation of EGFR (267). In addition, cigarette smoke may induce autocrine epithelial expression and release of several EGFR ligands, including TGF-α and AR, supporting the possibility that autocrine ligand release and binding are also potential stimulants of MUC5AC expression (242). Another possibility is that other cell types, for example macrophages, which are known to produce TGF-α (268), are sources of EGFR ligands in smokers' lungs. Thus, cigarette smoking, independent of neutrophilic inflammation, has the potential to both directly and indirectly enhance EGFR activation (242). Further studies are needed to establish the importance of each of these mechanisms *in vivo* in smokers.

Chapter 2

Methods

2:1 Clinical assessment

2:1:1 Recruitment and screening

Poster and newspaper advertisements were used inviting the participation of both non-smokers and currently smoking individuals in the Southampton area. Particular areas that were targeted included hospital outpatients departments, GP sugeries, pubs and clubs. In addition, a number of radio advertisements were used. All those recruited were between the ages of 40 and 65 and smokers had smoked at least 20 pack years of cigarettes. No volunteer had any history of allergy, asthma, cardiovascular disease or chest disease other than COPD. The study was approved by the Southampton University and General Hospital ethics committee, and volunteers gave their informed consent in writing.

2:1:2 Clinical evaluation and questionnaire administration

E ach subject gave a detailed clinical, occupational and smoking history and underwent a physical examination. As part of their smoking history both current smoking habit and pack year history was quantified. Symptom scores and quality of life were assessed using the St. Georges' respiratory disease questionnaire (269, 270).

2:1:3 Allergen skin prick testing

Skin prick test sensitivities to a panel of common aeroallergens were determined for each subject. The following allergen extracts were used; Dermatophagoides pteronyssimus, mixed grass pollens, mixed tree pollens, feathers, cat dander, dog fur, aspergillus spores and candida spores. Saline (0.9% sodium chloride) and histamine (1mg/ml) were used to exclude non-specific responses and to provide a positive control.

2:1:4 Histamine bronchial provocation challenge

Non-specific bronchial hyper-reactivity was measured using the histamine bronchial provocation challenge. The test was carried out in all subjects whose FEV_1 was greater than 60% predicted. In those whose FEV_1 was less than 50% bronchodilator responsiveness only was measured. Nebulised histamine acid phosphate (Sigma Co, Poole, Dorset, UK) dissolved in saline was administered by a method modified from that of Chai and colleagues (271). The degree of airways responsiveness to histamine

was expressed as the cumulative Pc20 – the concentration producing a 20% fall in FEV_1.

2:1:5 Bronchodilator responsiveness

Bronchodilator responsiveness was measured in all subjects. A positive test was defined as an increase in FEV_1 of at least 15% or 200ml of the baseline value following administration of the bronchodilator. The FEV_1 was recorded in each subject, followed by the administration of 6 puffs of salbutamol via a spacer device. Twenty minutes later spirometry was repeated in an identical fashion. The result of the test was expressed as the percentage change in FEV_1 from the original value.

2:2 Pulmonary function testing

Pulmonary function tests were all carried out during one visit. They included spirometry, carbon monoxide gas transfer measurement and lung volume assessment.

2:2:1 Spirometry

Spirometric measurements are the foremost tests for both diagnosis of COPD in clinical practice, and for classification of disease severity. Spirometry was performed according to standardised guidelines, outlined by the ATS (272). Subjects took the fullest possible inspiration through the mouth and then exhaled forcibly into the spirometer mouthpiece, blowing as hard as possible for at least the first second and continuing until there was no breath left when the residual volume had been reached. When three technically satisfactory blows had been obtained, the highest values were recorded. The following parameters were measured.

- FEV_1 (forced expiratory volume in one second, measured in litres), the maximal volume of air that can be exhaled in one second from the point of maximum inhalation. A value of < 80% predicted is taken to indicate airflow obstruction (273). The severity of FEV_1 impairment is a useful prognostic indicator of survival in COPD (8).
- FVC (forced vital capacity) is the total volume that can be forcibly exhaled from maximal inhalation until the point where no further air can be expired (ie. residual volume has been reached).

- FEV_1/FVC ratio. Expression of the forced expiratory volume in one second as a percentage of the forced vital capacity is more sensitive for the detection of airways obstruction than FEV_1 alone. A value of < 70 suggests airflow obstruction (2). The FEV_1/FVC ratio is not a valuable predictor of mortality in COPD because the FVC itself tends to decrease also with progression of disease (8). Flow rate measurements at 50% expiration (MEF 50) were also noted. Reduced flow rates at lower lung volumes are suggestive of peripheral airways obstruction but, as the normal range is wide, are less satisfactory at distinguishing "normal" from "abnormal" subjects (30).

2:2:2 Carbon monoxide gas transfer

Measurement of the transfer factor for carbon monoxide (TLCO) was performed to assess the efficiency of alveolar gas exchange. In emphysema, alveolar and/or microvascular destruction results in reduced functional surface area available for gas exchange, and can be assessed by measuring the degree to which diffusion of radiolabelled carbon monoxide (CO), measured as a surrogate for oxygen, is impaired. TLCO impairment is a recognised feature of emphysema (95) and has been repetitively shown to be useful for both detection of emphysema and quantification of disease severity (27, 37, 94). However the test is non-specific and results can be altered in a variety of pulmonary disorders, including lung fibrosis, vasculitis and pulmonary haemorrhage. Moreover, current cigarette smoking itself can lower TLCO values in the absence of emphysema (41). Interpretation of TLCO impairment, therefore, must be carried out with caution, and in the context of other clinical investigations.

Measurement of TLCO in our study was carried out via the *single-breath carbon monoxide test*, performed as follows; each subject was seated and, following application of a noseclip, exhaled completely to residual volume via a mouthpiece. He/she was then switched to a reservoir bag containing gases, including CO and helium (He), at known concentrations (0.3% CO, 14% He, 18% O_2) from which they rapidly inhaled to total lung capacity. The subject held his/her breath for approximately 10 seconds at maximal inspiration before making a rapid and complete expiration. The first 750mls, assumed to be contaminated with anatomic dead space

gas, was discarded and the next 500mls was collected for analysis of CO and He. The TLCO value was determined via the following steps.

1. The first step was to work out the rate of disappearance of the inhaled CO from alveolar gas (kCO (units:min^{-1}). This was done by dividing the difference between the alveolar concentrations of CO at the beginning and end of breath holding time by the duration of breath holding time.
2. The next step was to calculate the lung volume through which the CO diffused during breath holding, the alveolar volume (Va (ml STPD). Va is calculated by the helium dilution method. As helium does not cross the blood gas barrier, and the inspired concentration is known, the alveolar volume can be calculated from the new dilution of helium in the expired air.
3. From these results the TLCO is calculated and expressed as follows; TLCO = kCO x Va per unit pressure = {kCO x Va}/ Pb {$mmol.min^{-1}$ k Pa^{-1} in SI units} where Pb is barometric pressure minus water vapour pressure at 37 degrees.

All TLCO measurements were carried out by trained pulmonary function technicians.

2:2:3 Measurement of lung volumes

Measurement of lung volumes is performed in clinical practice as an adjunctive test for COPD assessment. Taken on their own these measurements are of limited diagnostic value but, in the context of spirometry and TLCO, can provide valuable additional information as to the severity of emphysema and gas trapping. The Gold standard for lung volume assessment is Body plethysmography. In clinical practice, however, the less accurate but more practical helium dilution method is employed. This was the method of choice for our study. The subject re-breathes from a closed circuit spirometer containing He as an indicator gas at a known concentration. As both the spirometer volume and the initial concentration of He are known then measurement of the new concentration of helium allows calculation of the volume of the total system (ie lungs and spirometer) and hence the total volume of the lungs. The following volumes were calculated; IC, FRC, TLC, obtained by adding the FRC and the IC and RV, obtained by subtracting the expiratory reserve volume (ERV) from the FRC. The RV may be expressed as a percentage of the TLC (RV/TLC) to compensate for differences in the size of patients. The RV, in particular, is an indicator of

peripheral airways dysfunction as its value may be raised in the presence of expiratory gas trapping.

2:3 High-resolution computed tomography (HRCT)

2:3:1 Principles of HRCT

High resolution computed tomography (HRCT) is a radiographic test that allows visualisation of lung parenchyma in great detail. With a HRCT scan the lungs are divided into a series of radiographic cross sections. These can be displayed as a set of images on film, for interpretation by a radiologist, or stored for computerized analysis. HRCT data are based on the variable absorption of X-rays by tissues, measured in Hounsfield units (HU). The attenuation value of a pixel depicted on a scan, expressed in HU, has a linear relationship with the density of the tissue represented by that pixel. The HU scale has been set such that water has a value of 0 HU, and the range of density ranges from -1000HU, the value for air and +1024HU the value for dense bone. CT images are displayed across various HU ranges depending on the tissue of interest. A 16 level grey scale is set to span this HU range such that images of varying density can be distinguished from one another by the observer by their different level of greyness. Any pixels with values above the upper limit of the window width will appear white while those with values below the lower limit will appear black. The window used for viewing the lung parenchyma and airways is usually –600 to –700HU with a window width of between 1000–1500HU (32).

HRCT scans were obtained on a General Electric Hi-speed CTi scanner, at 10mm intervals with a collimation (slice thickness) of 1mm. All scans were carried out in the supine, feet first position, on full inspiration and expiration, from the lung apices to the costophrenic angles. Scanning voltage was 140kV and tube current was 250mA. Hard copy images were photographed at a window level of –650HU and a window width of 1500HU.

2:3:2 Analysis of HRCT

The scans were evaluated for the presence of emphysema both qualitatively by a blinded consultant radiologist and quantitatively by computerised density mask analysis.

2:3:2:1 Radiologists' appraisal

In emphysema the destruction of lung parenchyma results in a greater air-tissue ratio, therefore emphysematous tissue will manifest on a CT scan as areas of reduced density. On a CT scan such regions will be depicted as "blacker" than their surroundings. Two consultant cardiothoracic radiologists, DD and CP, based at Southampton General Hospital, performed, in a blinded fashion, a visual assessment of each subjects' HRCT scan for the presence or absence of emphysema. In addition, the films were screened to ensure the absence of any other disease processes. Formal quantification of emphysema by visual scoring was not performed.

2:3:2:2 Computerised density analysis

A computer programme was used to quantify HRCT lung density parameters. Three parameters were measured, % area of low attenuation (%LAA), mean lung density (MLD) and the ratio of the MLD on expiration to inspiration (E/I ratio). The %LAA was measured by a density mask programme that highlights and measures all pixels within a given range, and was defined as the percentage of lung area with attenuation values of less than -950 Hounsfield units in density. It has been previously reported that the percentage of lung parenchyma below this level, on inspiratory HRCT scan, correlates well with morphological emphysema severity (52, 56). The MLD is the average attenuation value of all pixels in the scan. As lung tissue density is decreased in areas of emphysema, a reduction in MLD provides an indication of emphysema severity (57, 97). However, MLD values are also influenced by the effects of gas trapping and hyperinflation, such that smokers with peripheral airways dysfunction may have reduced readings also, in the absence of emphysema (50, 51). Finally, the E/I ratio provides an indication of the discrepancy between MLD measurements taken at full inspiration and those at full expiration. In subjects with expiratory air-trapping the MLD on expiration will be reduced relative to that on inspiration. Studies comparing the ratio of one to another (E/I ratio) with lung function indicators of airflow obstruction and gas trapping have found that this value provides an indirect measurement of peripheral airways dysfunction (53, 60).

2:3:2:3 Image processing methods for computerised density analysis

A program was written in IDL (Interactive Data Language, Research Systems Inc, Boulder, CO, USA) to enable reasonably automated image processing of the CT images to be carried out. The images were converted into a suitable file format (interfile) and read by the program. The operator was then prompted to set an appropriate grey scale window width and level to enable structures in the lungs to be clearly visualised. Next, the operator drew around the trachea or bronchi in each cross-section. These were set to a high pixel value of 5000 HU. This was to effectively "block off" the trachea and bronchi so that their volume would not be included in the automatically calculated lung volume.

Once the lungs had been identified several parameters could be calculated from their corresponding histogram of pixel values. The program automatically calculated the total volume of the lungs, by multiplying the number of voxels identified as being lung tissue by the individual voxel volume. It also calculated the number of lung voxels below –950 HU, the percentage that this was of the total lung volume, and the mean CT number of the voxels within the lung, which equals the MLD.

2:4 Sputum induction and processing

Sputum induction was carried out, as previously reported (274), via the inhalation of aerosolized hypertonic saline (4.5%). Subjects were seated in an induction chamber, nebulised salbutamol was administered via a spacer, and the peak expiratory flow rate (PEFR) was recorded. Saline was administered via a nebuliser. After every five minutes the nebuliser was stopped and PEFR noted. Sputum was expectorated into a Petri dish. The induction period was kept constant, 20 minutes, for all subjects regardless of the quality of the sample obtained.

Unselected sputum was processed according to the following protocol:

1. Place expectorated sputum in petri dish on ice and process within 1 hour, removing portions for microbiological analysis.
2. Pre weigh a 50ml Falcon tube
3. Using a cell scraper, scrape the sputum from the petri dish, leaving any obvious saliva behind, into falcon tube
4. Weigh the tube plus sample and calculate actual weight of expectorate
5. Add an equal weight of 0.01M D.T.E solution

6. Place on bench rocker for 30 minutes, aspirate every 5 minutes with pasteur pipette for complete homogenisation
7. Filter contents of tube through a 100μg filter to remove mucus, use a second filter if first blocks
8. Centrifuge the filtrate @1500rpm for 10 minutes @ 4°C to pellet cells
9. Aliquot supernatant (250μl) into labelled eppendorfs (10 if possible) and any extra into a single tube, and store for future analysis @ -80°C
10. Resuspend the cell pellet in 1ml of PBS
11. Remove 10μl of cell suspension and mix with 90μl of Trypan blue in eppendorf
12. Use a manual haemocytometer to record respiratory cells and squamous cells, viable and dead
13. Calculate total count per ml and dilute with PBS to obtain approx. 1 million cells per ml for slides with optimum density
14. Assemble cytospins and add 70μl of diluted cell suspension to 4 slides
15. Spin @ 400rpm for 5 minutes, check quality of slides prior to staining, repeat if necessary
16. Stain slides with "Diff-Quick" stain, following guidelines
    I. 5 seconds – dry for 30 seconds
    II. 5 seconds – drain
    III. 30 seconds – rinse – air dry, check quality of slides, restain if necessary
    IV. Mount in DPX, and coverslip

Differential cell counting was performed on the obtained cytospins by counting 600 cells in each cytospin in a blinded fashion. The mean of the two scores was used for analysis. Absolute inflammatory cell numbers per 20 minutes induction were calculated by multiplying the cell percentage with the total (non-squamous) cell number in the sputum.

2:5 Bronchoscopy

Bronchoscopy was conducted according to approved international guidelines under local anaesthesia (275). Subjects abstained from smoking for four hours prior to the procedure. Pre-medication consisted of nebulised salbutamol 2.5mg and ipratropium

bromide 500μg, and 50μg fentanyl and 600μg atropine given intravenously. Local anaesthesia was achieved using topical 10% lignocaine. A flexible fibreoptic bronchoscope (Olympus BF Type XT20) was used and introduced via the mouth. Endobronchial biopsies were taken from the middle lobe and right lower lobe sub-carinae.

2:6 Immunohistochemistry

2:6:1 Principle

Immunohistochemistry is the use of labelled antibodies as specific reagents for the localisation of tissue antigens *in situ.* The streptavidin-biotin complex (SABC) technique is a sensitive method for the detection of antigens. This method exploits the high affinity of streptavidin for biotin (Dissociation constant 10^{-19} M). Avidin is a large glycoprotein from egg white that has a very high affinity (four binding sites per molecule) for biotin, a vitamin of low molecular weight found in egg yolk. The streptavidin-biotin method requires a biotinylated antibody as a link antibody, which is produced by covalently binding biotin to the antibody. Open sites on streptavidin from the avidin-biotin complex binds to the protein on the link antibody. The sequence of reagent application is primary antibody, biotinylated secondary antibody and preformed streptavidin-biotin complex. Streptavidin or biotin is then coupled to a peroxidase such as horseradish peroxidase and a colour substrate is developed with amino ethyl carbazole (AEC) (for inflammatory cell staining), which colours the reaction product red or diaminobenzoate (DAB) (for epithelial staining) which colours it brown. Successful immunostaining requires tissue antigens to be made insoluble and yet their antigen sites must be available to the applied antibody without any great alteration in their tertiary structure. In addition the tissue architecture must be preserved so that immmunoreactive cells, for example, may be identified in context. For my thesis I used the validated and widely employed technique of embedding mucosal biopsies into glycolmethacrylate (GMA) resin (276) which offers a number of advantages over conventional frozen section or paraffin embedding techniques.

2:6:2 Advantages of the GMA resin embedding technique

- The morphology of GMA embedded sections are superior to that of frozen or paraffin embedded sections.

- GMA embedding allows tissue sections to be cut much thinner (1-2μm) than either frozen (usually 8μm) or paraffin (usually 4μm) sections while allowing the effective demonstration of antigens for light microscopy. These ultrafine thin sections allow taking sequential sections 1-2μm apart which can be used to co-localise two different antigens of interest on the same cells
- Addition of protease inhibitors to acetone during fixation, pre-treatment with, and addition of methyl benzoate to the GMA infiltrating solution, and the addition of ammonia to the water on which the sections are floated out, offers improved demonstration of antigens in the GMA embedded tissue sections.
- GMA resin, being hydrophobic, does not need to be removed prior to immunostaining.
- Trypsinization is not necessary for the demonstration of antigens.

Mucosal biopsies have an abundance of proteolytic enzymes which may cause digestion of the tissue antigens very soon after the biopsy. Fixation with cold acetone (-20°C) along with the addition of protease inhibitors are therefore necessary while fixing mucosal biopsies.

2:6:3 Material for GMA embedding and tissue staining

- JB4 embedding kit (Park scientific), containing solutions A and B and benzoyl peroxide (GMA resin).
- Acetone (Merck, Cat No. 10003 4Q), used for tissue fixation and processing.
- Phenylmethylsulfonyl fluoride (Sigma).
- Iodoacetamide (sigma, Cat No. I-6125), protease inhibition.
- Locking media – Dulbecco's modified Eagles medium (sigma) and Foetal calf serum and bovine serum albumin (Sigma).
- Methyl benzoate (Merck), for processing.
- 0.1% Sodium azide, peroxidase inhibition.
- 0.3% Hydrogen peroxide, peroxidase inhibition.
- Tris buffered saline, made by mixing sodium chloride (80gm) + Tris (6.05gm) + 1M HCL (38ml) in 1 litre of distilled water, adjusting the pH to 7.65 and adding to 9 litres of distilled water to give a final pH of 7.6.

- Tris/HCL buffer, made by mixing 0.2M Tris (12ml) + 0.1M HCL (19ml) + distilled water (19ml) and adjusting pH to 7.6.
- Biotinylated labelled second stage antibodies.
- Streptavidin-biotin-peroxidase complex.
- Mayer's haematoxylin.
- Toluidine Blue.
- Aminonethyl carbazole (AEC), prepared by mixing AEC (0.4%)(1ml) with 30% hydrogen peroxide and acetate buffer.
- Diaminobenzoic acid (DAB)

2:6:4 Immunohistochemistry method

2:6:4:1 GMA embedding and processing and cutting

The endobronchial biopsies obtained at bronchoscopy were processed into GMA resin for immunohistochemical staining according to Britten and co-workers (276). Each biopsy was placed in ice-cooled acetone containing protease inhibitors – phenylmethyl-sulphonyl fluoride (2nM) and iodoacetamide (2nM) – cooled rapidly to -20°C and left overnight. The following day each sample was put in acetone at room temperature for 15 minutes and then in methyl benzoyl for further 15 minutes. The tissue was then immersed in GMA JB4 solution A (Polysciences, Northampton, UK) at 4°C for 7 hours, during which time the GMA solution was changed three times. The tissue was finally embedded in GMA resin (prepared by mixing GMA monomer, N.N-dimethylaniline in PEG 400 and benzoyl peroxide), which was left for polymerisation overnight at 4°C. The blocks were then stored in air-tight containers at -20°C until used for immunostaining.

2:6:4:2 Toluidine blue staining

Three biopsies were taken per subject. Sections were treated with toluidine blue and tissue morphology was assessed under a light microscope. This was to ascertain the quality of submucosal and epithelial tissue present in each biopsy so that the best sample could be selected for immunostaining.

2:6:4:3 Immunostaining

The full staining protocol is outlined below. The GMA sections were cut at 2μm thickness and floated onto ammonia water (1:500), placed onto 0.01% poly-l-lysine glass slides, and allowed to dry at room temperature for 1 hour. The sections were treated with 0.1% sodium azide and 0.3% hydrogen peroxide in distilled water to block endogenous peroxidase. Nonspecific antibody binding was blocked by applying blocking medium consisting of Dulbecco's modified Eagles medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and 1% (w/v) bovine serum albumin (BSA) for 30 min followed by the primary monoclonal antibody (mAb). The first antibodies were titrated before use and conditions established which gave specific staining. After rinsing in TRIS-buffered saline (TBS), biotinylated rabbit anti-mouse or swine anti-rabbit or rabbit anti-goat (to detect the sheep antibody) IgG Fab immunoglobulins (all from Dako Ltd., High Wycombe, UK) were applied for 2h, followed by the streptavidin-biotin horseradish peroxide complex (Dako Ltd.) for another 2h. After rinsing in TBS, peroxidase was detected with 3,3-diaminobenzidine (DAB). All sections were counterstained with Mayer's haematoxylin. Isotype-matched antibody controls were negative in all cases.

In addition to the above, for chapter 5, GMA sections were cut and stained with Periodic acid-schiff (PAS) to quantify goblet cell numbers. Also, double staining of the bronchial epithelium for ErbB3 and PAS was performed to determine whether ErbB3 expression was present on goblet cells. This involved following the standard immunohistochemistry protocol for ErbB3, but, instead of the final counterstaining step, the PAS technique was applied.

2:6:4:4 Antibodies

For chapter 4 sections were stained with antibodies to the following inflammatory cell populations; CD4+ cells (clone leu-3a + leu-3b, Dako, Denmark)(277), CD3+ cells (Clone UCHT1 Dako, Denmark)(278), CD8+ cells (Clone DK25, Dako, Demark)(279), neutrophils (neutrophil elastase, clone NP57, Dako, Cambridge UK (280), macrophages (CD 68+ cells, clone PG-M1 Dako, Denmark)(281), eosinophils (EG2 pharmacia, Sweden)(282), mast cells (AA1, Dako, Denmark)(283) and natural killer cells (CD56 clone moc-1 Dako, Denmark)(112).

For chapter 5 sections were stained with the following antibodies; anti-EGFR, 1:800 (an Ig fraction from a sheep polyclonal antiserum, obtained from immune serum raised against EGFR purified from A431 squamous carcinoma cell membranes), anti-ErbB2, 1:20 (mouse monoclonal, clone 42, Catalog E19420) and anti-ErbB3, 1:20 (rabbit polyclonal, Catalog E38530) (Transduction laboratories – supplied by Affiniti Research products Ltd., Exeter, UK) (246, 261), anti-MUC5AC, 1:400 (clone 21M1, mouse monoclonal to the carboxy-terminal region of MUC5AC, a generous gift from Dr J.M Bara)(284) anti-MUC2 (clone Ccp58, Catalog NCL-MUC2 mouse monoclonal antibody to the tandem repeat region of MUC 2, Novocastra laboratories, Newcastle-upon-Tyne, UK) (285), anti-MUC5B (EU-MUC5B, a mouse monoclonal antibody raised against a peptide located in the centrally located repeated cysteine domains of MUC5B , produced by the EU consortium BMH4-CT98-3222 (286) anti-neutrophil elastase (clone NP57, Dako, Cambridge UK) (280).
For chapter 6 sections were stained with antibodies to the two chemokines IL-8 (Q-Biogene, Harefield, UK) and GRO-α (R&D Systems, Abingdon, UK).

2:6:4:5 Immunostaining protocol

Day – 1

1. Remove blocking media from fridge.
2. Remove slides kept in foil in freezer and place on tray.
3. Prepare sodium azide solution (0.1% 5ml Sodium Azide + 50µl 30% H2O).
4. Pour 150µl sodium azide on each slide and keep for 30 minutes.
5. Prepare primary antibody dilutions
6. Wash slides with TBS – 3 times for 5 mins each.
7. Drain slides and apply 150µl blocking media per slide and keep for 30 mins.
8. Drain slides and apply 150µl primary antibodies at appropriate dilutions and cover with cover slips avoiding air bubbles.
9. Incubate at room temperature overnight.

Day – 2

1. Prepare Stage II antibodies (Biotin A mouse) in TBS at 1:300 dilution.
2. Prepare Stage III antibodies (Streptavidin + Biotin in horse radish peroxidase) in Tris HCL at 1:200 dilution.
3. Wash slides with TBS – 3 times for 5 mins each.
4. Drain slides. Apply 150µl Stage II antibody and incubate for 2 hours.
5. Wash slides with TBS – 3 times for 5 mins each.
6. Drain slides. Apply 150µl Stage III antibody and incubate for 2 hours.
7. Wash slides with TBS – 3 times for 5 mins each.
8. Drain slides. Apply 150µl AEC/DAB
9. Incubate for 20 mins for AEC or 10 mins for DAB.
10. Wash with TBS and rinse in running water – 5 mins.
11. Counterstain with Meyer's Haematoxylin for 90 secs.
12. Wash in running water, drain and apply crystal mount.
13. Bake in oven at 80°C for 10 mins. Cool slides and mount in DPX.

Name	Marker	Cells	Dilution	Source
NE	Elastase	Neutrophils	1:1000	Dako, High Wycombe, UK
CD68	CD68	Macrophages	1:100	Dako, High Wycombe, UK
EG2	ECP	Eosinophils	1:500	Pharmacia, Upsalla, Sweden
AA1	Tryptase	Mast cells	1:100	Dako, High Wycombe, UK
CD3	CD3	T-cells	1:100	Dako, High Wycombe, UK
CD4	CD4	T-cells	1:10	Dako, High Wycombe, UK
CD8	CD8	T-cells	1:100	Dako, High Wycombe, UK
EGFR	EGFR	Epithelium	1:800	University of Southampton
ErbB2	ErbB2	Epithelium	1:20	Transduction laboratories, UK
ErbB3	ErbB3	Epithelium	1:20	Transduction laboratories, UK
MUC5AC 21M1s	MUC5AC	Epithelium	1:400	Dr. J.M. Bara
EuMUC5B	MUC5B	Epithelium	1:20	EU consortium BMH4-CT98-3222
MUC2	MUC2	Epithelium	1:20	Novocastra, Newcastle, UK
IL-8	IL-8	Epithelium	1:200	Q-Biogene, Harefield, UK
GRO-α	GRO-α	Epithelium	1:25	R&D Systems, Abingdon, UK
Biotinylated anti-mouse	Secondary Antibody		1:300	Dako, High Wycombe, UK
Biotinylated anti-rabbit	Secondary Antibody		1:600	Dako, High Wycombe, UK
Biotinylated anti-goat	Secondary Antibody		1:10,000	Dako, High Wycombe, UK
Streptavidin biotin horse radish peroxidase	Third stage antibody		1:200	Dako, High Wycombe, UK

Table 2.1: Antibodies used for immunohistochemistry studies

2:6:5 Inflammatory cell measurement in biopsy samples

Quantification of inflammatory cells was carried out in both the epithelium and submucosa using a light microscope, and expressed as the number of cells per mm^2 submucosal area and the number of cells per mm epithelial length. Both submucosal area and epithelial length were assessed by computerised image analysis (Image associates, Bicester, UK). A digitised image of each section was displayed on a computer screen. The submucosa was manually outlined, excluding all areas of damaged tissue, smooth muscle, bronchial glands or cartrilage. The area was then automatically calculated. The epithelium was similarly outlined and measured.

2:6:6 Quantification of epithelial immunostaining

Quantification of epithelial staining was carried out, according to a previously described protocol (185) with the assistance of computerised image analysis (Image associates, Bicester, UK). For each biopsy specimen, the entire intact epithelium in two non-serial sections was systematically assessed based on red, blue, green (RGB) colour balance. The digitised image of the section was sampled interactively and the colour balance adjusted so that areas of positive staining were highlighted by the program. The system was then allowed to select all the pixels of the same RGB colour balance (ie positive staining) within the image. The area of the epithelium was then delineated interactively and the percentage of positive staining within the epithelium was determined; All measurements were performed on two separate occasions by the same observer, who was unaware of the clinical group from which the biopsy section was derived.

2:7 Cytokine measurement by ELISA

2:7:1 ELISA Kits

The cytokine levels in the PBS-processed sputum supernatants were determined using the following commercially available Enzyme Linked Immunosorbance Assay (ELISA) kits in accordance with the manufacturers' instructions: IL-8 (Minimum detectable concentration < 1pg/ml; Pelikine®, Sanquin Research, Amsterdam, Netherlands); GRO-α (Minimum detectable concentration < 10pg/ml; Quantikine®, R&D Systems, Abingdon, UK.)

2:7:2 ELISA protocol for IL-8

All reagents were brought to room temperature before use. Samples were diluted 1:100 with assay diluent and added in 100µl aliquots to a 96-well micotitre plate precoated with monoclonal anti-IL-8 antibody. Parallel samples were 'spiked' with 30-pg/ml recombinant human IL-8. Standards were made by serial dilution in accordance with the manufacturer's instructions. Following a 1-hour incubation at room temperature, samples were aspirated, the plates were washed and 100µl of biotinylated secondary polyclonal anti-IL-8 antibody was added to each well. The plate was sealed and incubated at room temperature for 1 hour. After a further wash, 100µl of streptavidin-horseradish peroxidase conjugate was added to each well and incubated for an additional 30 minutes at room temperature, followed by washing. Finally, 100µl of peroxidase substrate solution (H_2O_2 0.003% plus 3,3',5,5'-tetramethylbenzidine [TMB] 470 µM in 0.11-M acetate buffer, pH 5.5) was added to each well and colour development stopped after 30 minutes incubation by the addition of 100 µl of 1.8-M sulphuric acid. The colour reaction was quantified using a microplate reader at 450 nm.

A standard curve was plotted and cytokine concentrations of the samples determined by linear interpolation. Percent recovery of exogenous IL-8 was calculated from the difference in measured IL-8 concentration between spiked and unspiked samples and amounted to 58 ± 10 %.

2:7:3 ELISA protocol for GRO-α

All reagents were brought to room temperature. Samples were diluted 1:50 with assay diluent and added to a 96 well micotitre plate precoated with monoclonal anti-GRO-α antibody. Parallel samples were spiked with 80-pg/ml recombinant human GRO-α. Standards were made by serial dilution in accordance with the manufacturer's instructions. Following a 2-hour incubation at room temperature, samples were aspirated, the plates were washed and 200µl of GRO-α conjugate (polyclonal antibody against GRO-α, conjugated to horseradish peroxidase) was added to each well, the plate was sealed and incubated at for 2 hours at 4°C followed by 4 washes. Finally, 200µl of substrate solution (H_2O_2/TMB, supplied with the kit) was added to each well and colour development stopped after 15 minutes incubation by the addition

of 50μl of 2-M sulphuric acid. The colour reaction was quantified using an ELISA plate reader at 450 nm.

A standard curve was plotted and cytokine concentrations of the samples determined by linear interpolation. Percent recovery of exogenous GRO-α was calculated from the difference in measured GRO-α concentration between spiked and unspiked samples and amounted to 107 ± 26 %.

2: 8 Database creation and management

To allow effective management of the large amount of data gathered, a Microsoft Access database was created. This was utilised mainly in spreadsheet format. Each individual subject was assigned a row with columns pertaining to every variable. Columns were grouped into clinical (demographics, symptoms, signs, clinical groups etc.), lung function, radiographic, histological and sputum resulting in a total of 151 columns per subject. The program allowed versatile organisation and rearrangement of data to access and focus on parameters of interest. For statistical analysis the data was tranferrable to SPSS.

2:9 Statistical analysis

The statistical package SPSS 10.1 for windows was employed. Normally distributed data were compared between multiple groups by ANOVA, followed by the Scheffe examination for individual comparisons. Non-normally distributed data was analysed using the Kruskal-Wallis test followed by the Mann-Whitney test. Correlations were sought by Pearson's test and Spearman's test for normally and non-normally distributed data respectively. Correction for multiple correlations was carried out using the Holm method (287). Trend analysis for the GOLD groups was performed using the Jonckheere-Terpstra test. P values <0.05 were regarded as significant.

Chapter 3

Clinical characterisation of subjects with and without COPD

3:1 Introduction

COPD is characterised by both remodelling changes in the peripheral airways, and destruction of the lung parenchyma, both of which may contribute toward impairment of lung function. In clinical practice, however, the definition and classification of COPD have, in general, been based simply upon airflow obstruction severity (1, 2, 26, 33) while the exact mechanisms underlying the obstruction remain unclear. Controversy still exists as to the relative contribution of collapsibility and/or remodelling of the small airways (29, 42, 43) and the amount of emphysematous destruction of the alveoli *per se* (44-46). Accurate quantification of peripheral lung pathology by analysis of surgical or post mortem specimens (29, 44, 46, 73, 84, 93) is impractical for most clinical or research settings and less invasive methods are required.

High resolution computed tomography (HRCT) can clarify the relationship between disease phenotype and lung function impairment in COPD. This technique allows non-invasive, *in vivo* diagnosis of emphysema even when plain chest radiography or lung function tests are non-diagnostic (15, 288, 289). Assessment of emphysema severity can be performed both by visual scoring of CT films (31, 47, 48) and computerised analysis of lung density (14, 49, 50, 52, 55, 97), which has the advantage of negating inter- and intra-observer variability (14) and allows non-invasive quantitation of emphysema extent, that correlates well with pathology (52, 56). However, the clinical importance of HRCT analysis of emphysema remains uncertain as, although emphysema severity has been shown in some studies to be closely associated with airflow limitation (44-46), its presence does not always result in functional impairment (37, 38). HRCT measurement of peripheral airways dysfunction, by calculation of the ratio of the mean lung density on expiration to inspiration (E/I ratio) is associated with the severity of airflow obstruction (53, 60) but is less pathologically specific as it may be affected by either peripheral airway remodelling or emphysema (44, 73, 86). Impaired carbon monoxide gas transfer (TLCO), also used as an indicator of emphysema, is non-specific, and values may be impaired by separate mechanisms as a result of current cigarette smoking (16, 39-41).

Using a comprehensive approach I have examined the relationship between the clinical severity of COPD and both peripheral airways dysfunction and the overall

extent of emphysematous destruction of the lungs as quantified by HRCT. For this purpose I recruited and carefully characterised a cohort of COPD subjects, in addition to both non-smoking and healthy smoking controls. In these subjects I correlated lung function measurements, as an index of disease severity, with expiratory HRCT densitometry measurements and the expiratory/inspiratory mean lung density ratio (E/I ratio), which are affected by peripheral airway obstruction and air trapping (49, 53-55, 57, 60, 97), and inspiratory HRCT measurements, which reflect the extent of emphysematous destruction (52, 56). In addition, I have examined the relationship between COPD severity and the extent of respiratory debility, measured by the widely used St George's respiratory disease questionnaire (SGRQ) (269, 270).

3:2 Methods

Poster and newspaper advertisements were used inviting the participation of both current smokers and lifelong non-smokers in the Southampton area. No volunteer had any history of allergy, asthma, cardiovascular disease or chest disease other than COPD. All were free of chest infections and corticosteroid therapy for at least 3 months prior to study entry. The study was approved by the Southampton University and General hospital Ethics committee and volunteers gave their informed consent in writing. A detailed database was created of all subjects approved for entry into the study into which all of their subsequently recorded clinical, physiological and radiological parameters were entered.

3:2:1 Clinical assessment

E ach subject gave a detailed clinical history and underwent a physical examination. Symptom scores and quality of life were assessed using the st. Georges' respiratory disease questionnaire (269, 270).

Bronchodilator responsiveness was measured, and histamine bronchial provocation challenge was carried out by a method modified from that of Chai and colleagues (271). No subject showed significant (>15%) bronchodilator reversibility. Five subjects with more severe COPD did not undergo histamine bronchial provocation challenge as the baseline FEV_1 was too low (< 50%). All of the other subjects had normal responsiveness to histamine. Skin tests to a panel of common aero-allergens were negative in all subjects.

Spirometry and flow-volume loop was carried out according to ATS guidelines and posy-bronchodilator values recorded (272). The FEV_1 and FVC were expressed as percent predicted, and the FEV_1/FVC ratio was calculated. Flow rate measurements at 50% (MEF 50) expiration were also noted. The TLCO was measured by the single breath technique. RV and TLC were calculated by the helium dilution method. The ratio of RV to TLC (RV/TLC ratio), an indicator of expiratory air trapping, was calculated.

3:2:2 High resolution computed Tomography

HRCT scans were obtained at 10mm intervals with a collimation (slice thickness) of 1mm. All scans were carried out in the supine, feet first position, on full inspiration and expiration, from the lung apices to the costophrenic angles. Scanning voltage was 140kV and current was 250mA. Hard copy images were photographed at a window level of –650HU and a window width of 1500HU, which is appropriate for viewing lung parenchyma. The scans were evaluated for the presence of emphysema both qualitatively by two radiologists independently of the remaining research and quantitatively by computerised density mask analysis. Two density mask parameters were measured; the %LAA and the mean lung density (MLD). The %LAA is the percentage of lung area less than -950 Hounsfield units in density, which has been shown to correlate well with morphological emphysema severity (52, 56). The MLD is the average attenuation value of all pixels in the scan. As lung tissue density is decreased in areas of emphysema this measurement may provide and indication of emphysema severity (57, 97), but is also influenced by the effects of gas trapping and hyperinflation (50, 51). The average ratio of the MLD on expiration to inspiration (E/I ratio) was calculated also, as an indirect measurement of peripheral airways dysfunction (53, 60).

3:2:3 Statistical analysis

Statistical analysis was performed using SPSS version 10.1. Normally distributed data (lung function and HRCT) were compared between multiple groups by ANOVA, followed by the Scheffe examination for individual comparisons. Correlations were sought by Pearson's test. Correction for multiple correlations was carried out using the Holm method (287). Trend analysis for the GOLD groups was performed using the Jonckheere-Terpstra test. P values <0.05 were regarded as significant.

3:3 Results

3:3:1 Clinical groups

105 subjects were recruited to the study. Of these 74 completed all the above clinical tests. The remaining subjects were omitted due either to withdrawal from the study prior to completion, or failure to complete HRCT scanning satisfactorily.

Subjects were allocated into clinical groups based on symptoms, the presence or absence of emphysema on HRCT as detected by the radiologists, and application of the GOLD criteria for COPD disease severity (2). Non-smokers and healthy smokers were asymptomatic, with normal spirometry, and no evidence of emphysema by radiologist appraisal of CT. Subjects with COPD were divided according to the GOLD criteria depending on the presence of a reduced FEV_1, FEV_1/FVC ratio or both (2).

5 clinical groups were defined.

1. Healthy non-smokers
2. Healthy smokers, with no evidence of bronchitis or emphysema.
3. Stage 0 COPD; smokers with cough and sputum production but normal spirometry and normal HRCT
4. Stage I: mild COPD: smokers with mild airflow limitation (FEV1 > 80% predicted, FEV_1 /FVC ratio reduced < 70%).
5. Stage II/III: Moderate COPD: The majority of these were stage II ie. smokers with FEV_1< 80% but >30% of predicted and FEV_1/FVC<70%. Only 2 subjects had FEV_1 values of just under 30% (25% and 29% respectively). These were included, for simplicity, under the heading of stage II COPD.

The clinical characteristics of the 5 subject groups are summarised in table 1.

The 5 subject groups were matched for age. Although the three COPD groups were matched for the number of pack years, smokers with stage II COPD had a significantly greater pack year history than healthy smokers (table 1). FEV_1 was significantly lower in Stage II COPD when compared to all the other subject groups. The FEV_1/FVC ratio was significantly reduced in both Stage I and Stage II COPD when compared with the other three groups (table 1). When analysing for smoking-related trends using the Jonckheere-Terpstra test, significant trends were observed for

the RV/TLC to rise and for the MEF50 to fall with increasing disease stage ($p<0.001$ in all cases) (table 3:1). Although TLCO did not differ significantly between stages 0, I and II COPD, it was significantly reduced in all three groups when compared to non-smokers, and the trend for fall with advancing disease stage was highly significant ($p<0.001$) (table 3:1,figure 3:2).

3:3:2 Symptom scores

The mean total SGRQ scores displayed a significant upward trend with increasing disease stage ($p<0.001$); the scores were significantly elevated in both stage I ($p<0.05$) and stage II ($p<0.05$) COPD when compared to non-smokers and healthy smokers (figure 3:1). SGRQ scores correlated significantly with both lung function and HRCT measurements, the best correlation being with the FEV_1/FVC ratio ($r = -0.6$, $p<0.001$) (table 3:2). Significant correlations were observed between SGRQ scores and the E/I ratio and both %LAA and MLD on expiration but not on inspiration (table 3:2).

	Non smokers	Healthy smokers	Stage 0 COPD	Stage I COPD	Stage II COPD
N	12	17	17	10	18
Age	51 ± 7	49 ± 9.9	47 ± 5.9	55 ± 8	55 ± 7
Pack years	0	37 ± 6.7	46 ± 18	41± 9.9	52 ± 16.8§
FEV_1%	111 ± 12.2	102 ± 7.3	99 ± 13.9	90 ± 7.5	53 ± 13.1*
FEV_1/FVC	77 ± 5.4	78 ± 4.6	74 ± 3.8	66 ± 2.8 ll	56 ± 9.6 ll
MEF50%	85 ± 17.5	85± 23	76 ± 22.1	48 ± 9 †	24 ± 12.6*
RV/TLC	30 ± 3.5	28.7±7.1	35±7.7	38± 5.2	49±7.9 ll
TLCO%	84 ± 12.1	74 ± 12	64 ± 10.0 ll	60 ± 15.1 ll	57 ± 15.0 ll
Total SGRQ	4.7 ± 12.2	7.1± 5.0	20.4 ± 16.1	27.2±12.9 ‡	35.3±19.7 ‡

Table 3:1: table comparing the clinical characteristics of the 5 subject groups. All values are shown as mean ± SD.

*: $p < 0.001$ vs all groups,

†: $p < 0.05$ v NS, HS and stage 0 COPD,

‡: $p < 0.05$ vs NS and HS,

§: $p < 0.05$ vs HS

ll: $p < 0.05$ vs NS

Clinical variable	Correlation coefficient (r) with SGRQ
FEV_1%	-0.54, $p < 0.001$
FEV_1/FVC	-0.6, $p < 0.001$
RV/TLC	0.56, $p < 0.001$
TLCO%	-0.42, $p < 0.003$
Exp %LAA	0.49, $p < 0.01$
Insp %LAA	No correlation
Exp MLD	-0.536, $p < 0.001$
Insp MLD	No correlation
E/I ratio	0.54, $p < 0.001$

Table 3:2: Correlations between the St George's Respiratory Questionnaire score (SGRQ) and lung function and HRCT measurements.

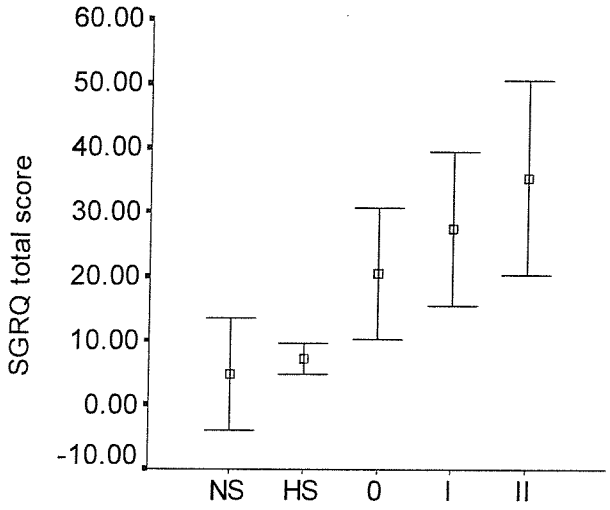


Figure 3:1. SGRQ scores in non-smokers (NS), healthy smokers (HS), stage 0 COPD (0), stage I COPD (I), and stage II COPD (II). Values are expressed as mean ± SD.

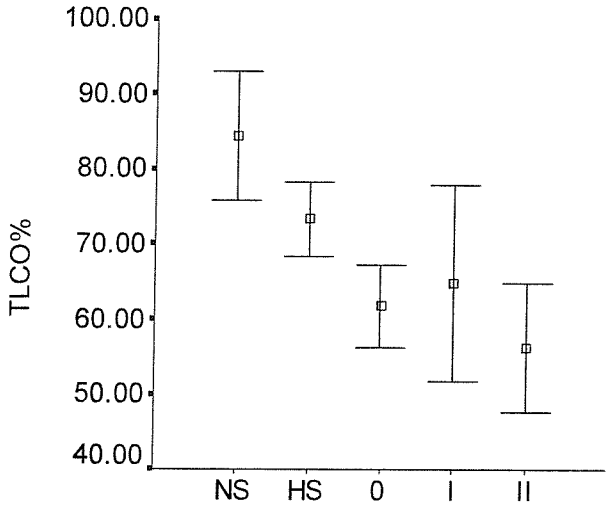


Figure 3:2. Comparison of TLCO% in the 5 clinical groups. Values are expressed as mean ± SD.

3:3:3 Radiologist's appraisal of CT

Four asymptomatic smokers with normal lung function but with evidence of emphysema on HRCT could not be classified according to the GOLD criteria and were, therefore, classified as a separate, "emphysema only" group that was too small for statistical comparisons. None of the healthy non-smokers and, by definition, the healthy smokers had emphysema on HRCT as assessed by the radiologist. In contrast, 11/17 subjects with stage 0 COPD, 7/10 subjects with stage 1 COPD and 12/18 subjects with stage II COPD had emphysema detectable on CT. The amount of emphysema in all 11 cases of stage 0 COPD was described as minimal (approximately < 5% of total lung area), while in stage I and II disease the extent of emphysema ranged from minimal to severe (approximately > 30% of total lung area).

3:3:4 Computerised density mask analysis of HRCT

HRCT densitometry values for the clinical groups are given in table 3:3.

Comparison of the %LAA and MLD by ANOVA, allowing for multiple comparisons, showed significant differences between the groups only when comparing expiratory HRCT measurements (table 3:3). Post-hoc analysis using the Scheffe test showed no differences between non-smokers and any of the smoker groups for expiratory HRCT measurement (%LAA and MLD) (table 3:3), whilst the E/I ratio was significantly increased in stage II COPD when compared to non-smokers, healthy smokers and stage 0 COPD (table 3:3 and figure 3:3). Further comparison of %LAA on expiration showed a significant increase in both stages I and II COPD when compared to both healthy smokers or stage 0 COPD (Table 3:3 and figure 3:4). Moreover, MLD on expiration was significantly reduced in stage II COPD when compared to healthy smokers and stage 0 COPD, and in stage I COPD when compared to healthy smokers (table 3:3 and figure 3:5). When analysing for smoking-related trends in HRCT densitometry amongst the four smoking groups in isolation using the Jonckheere-Terpstra test, significant trends were observed for %LAA to rise and MLD to fall with advancing disease stage on both inspiration and expiration ($p<0.001$ in all cases). There was also a significant rising trend for the E/I ratio with advancing disease stage ($p<0.001$).

3:3:5 Correlations between HRCT and lung function

Lung function correlated moderately strongly with HRCT parameters (table 3:4). The strongest correlations seen in smokers were between the E/I ratio on the one hand and airway obstruction (FEV_1, FEV_1/FVC), mid-expiratory flow (MEF 50%) and gas trapping (RV/TLC) on the other, with only a very poor correlation being seen with TLCO (Figure 3:6). Strong and highly significant correlations were also observed between MLD and measurements of airway obstruction, mid-expiratory flow and gas trapping but not gas transfer. These correlations were stronger on expiration than inspiration. Both expiratory and inspiratory %LAA correlated weakly to moderately strongly with measurements of airway obstruction and gas trapping but also, albeit weakly, with TLCO. These correlations were only marginally stronger on expiration.

	Non smokers	Healthy smokers	Stage 0 COPD	Stage I COPD	Stage II COPD
Inspiration					
MLD (HU)	-840 ± 27	-825 ± 25	-824 ± 19	-852 ± 23	-846 ± 25
%LAA	8.9 ± 4.3	7.0 ± 3.2	5.8 ± 2.7	12.6 ± 8.1	11.4 ± 7.4
Expiration					
MLD (HU)	-755 ± 44	-727 ± 45	-732 ± 35	-783 ± 36 *	-801 ± 38 * †
%LAA	3.3 ± 2.3	2.7 ± 1.6	2.3 ± 1	7.1 ± 6.4 * †	7.3 ± 6.8 * †
E/I ratio	0.89 ± 0.03	0.88 ± 0.04	0.88 ± 0.03	0.91 ± 0.02	0.94 ± 0.02 ‡* †

Table 3:3: HRCT densitometry data for the 5 subject groups. All values given as mean ± SD.

* $p<0.05$ vs. healthy smokers.

† $p<0.05$ vs. stage 0 COPD.

‡ $p<0.05$ vs. non-smokers.

Functional variable	Correlation co-efficient (r)				
	Exp. %LAA	**Insp. %LAA**	**Exp. MLD**	**Insp. MLD**	**E/I ratio**
FEV_1%	-0.52	-0.45 ($p<0.01$)	0.62	0.38 ($p<0.01$)	-0.66
FEV_1/FVC	-0.57	-0.53	0.77	0.58	-0.69
RV/TLC	0.47	0.38	-0.67	-0.43 ($p<0.01$)	0.71
MEF 25%	-0.4	-0.41	0.66	0.46	-0.62
MEF50%	-0.51	-0.46	0.72	0.46	-0.72
TLCO%	-0.42	-0.36	No correlation	No correlation	-0.28 ($p<0.05$)

Table 3:4: Correlation between HRCT measurements, performed on inspiration (Insp.) and expiration (Exp.), and lung function and symptom scores. $p<0.001$ unless otherwise stated.

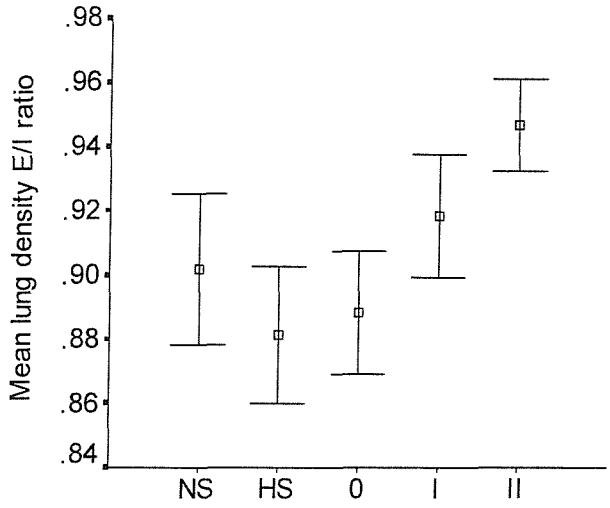
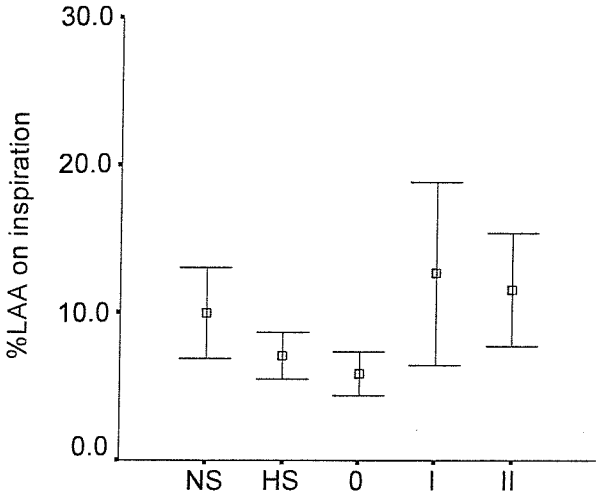


Figure 3:3: Lung density E/I ratio. The ratio was significantly higher in smokers with airflow obstruction when compared to healthy smokers, stage 0 COPD and non-smokers. Values are expressed as mean $\pm$ SD.

A



B

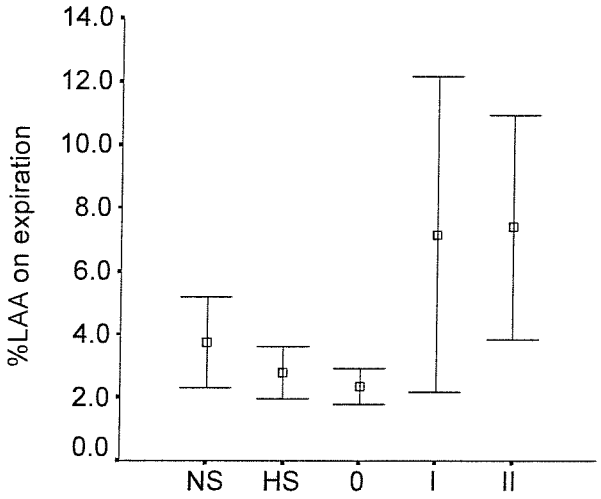


Figure 3:4: %LAA values for the clinical groups on inspiration (A) and expiration (B). Values are expressed as mean $\pm$ SD.

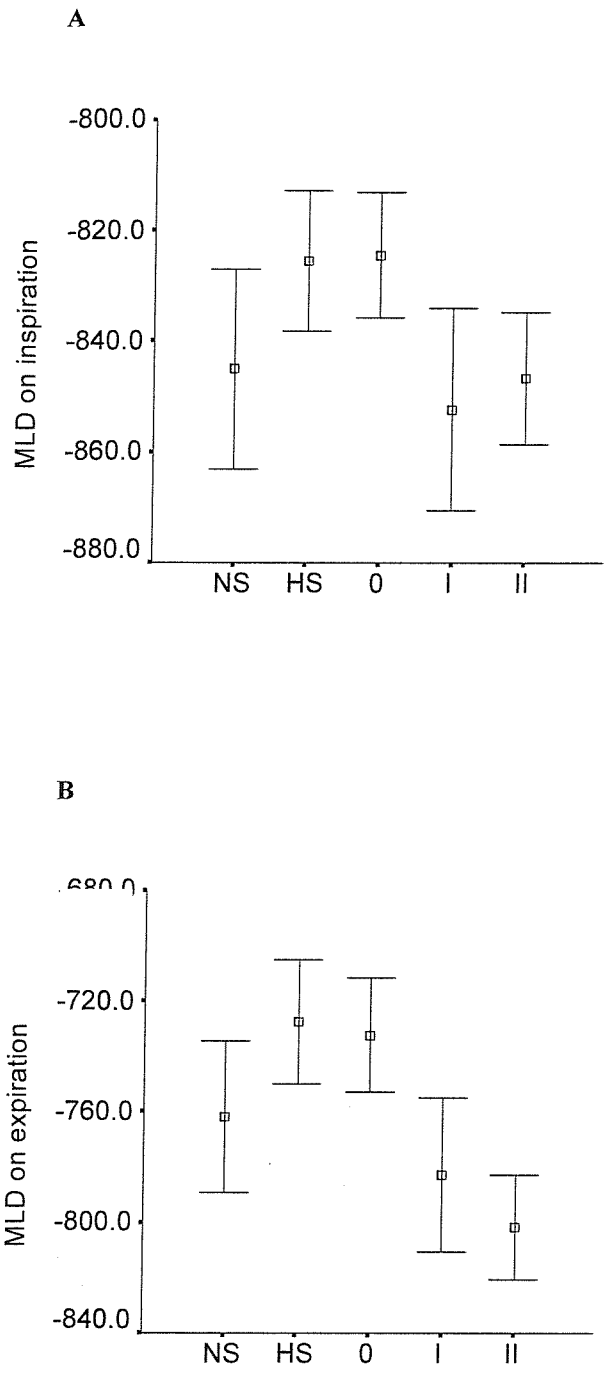
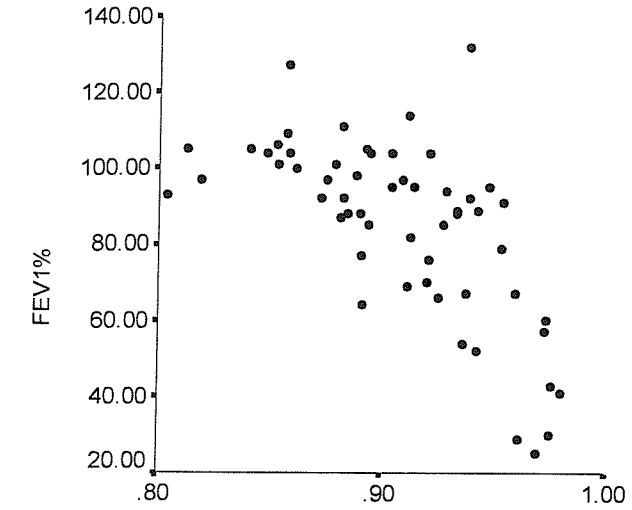
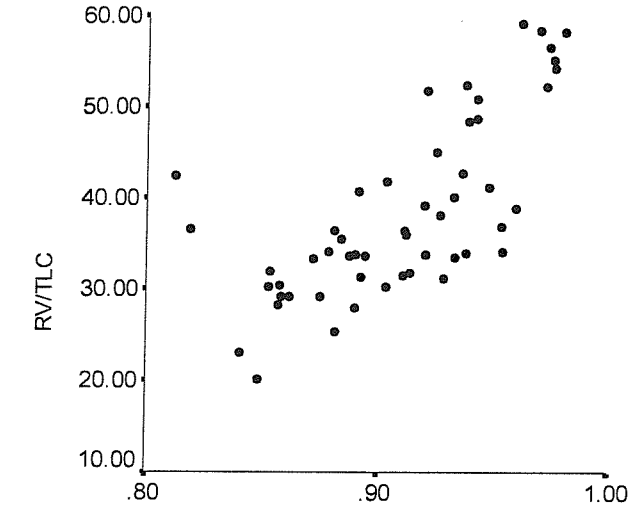


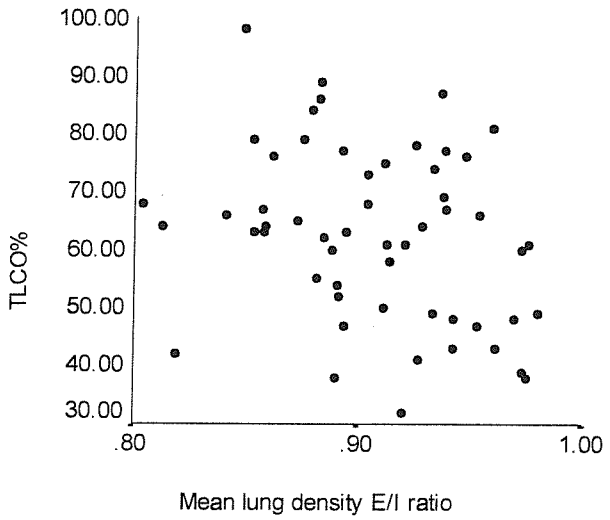
Figure 3:5: MLD values for the clinical groups on inspiration (A) and expiration (B). Values are expressed as mean ± SD.



r = -0.66, p < 0.001



r = 0.71, p <0.001



r =-0.28, p <0.05

Figure 3:6: Correlations between the E/I density ratio and lung function. In smokers the E/I ratio correlated strongly with FEV_1% and RV/TLC but poorly with TLCO%.

3:4 Discussion

In this study I have investigated in detail the relationship between clinical severity of COPD and HRCT measurements of peripheral airway dysfunction and emphysema. The results show that peripheral airway dysfunction is significantly associated with airflow limitation and impaired quality of life in COPD, although it does not elucidate whether this is due to small airway remodelling or loss of elastic recoil caused by emphysema. Moreover, HRCT indicators of peripheral airways dysfunction are more reliable than measurements of emphysema in discriminating smokers with COPD from healthy smokers. Finally, although there were strong associations between reduced quality of life, as judged by SGRQ scores, HRCT measurements and GOLD stages of COPD, the finding that the majority of patients with Stage 0 COPD had HRCT evidence of emphysema and that emphysema can be present in the absence of both symptoms and airway obstruction suggest that, at least for the purposes of research, the separate category of sub-clinical emphysema, not recognised by the GOLD staging criteria, should be investigated for comparison.

Airflow limitation in COPD can result from airway wall remodelling, emphysematous destruction of the parenchyma, or both, although there is controversy over their relative importance (29, 44, 89, 94, 97). Although some CT and pathology studies have shown that severity of emphysema correlates with airway obstruction (44, 97), others have found that a significant proportion of smokers who have airflow obstruction have little or no emphysema, while some smokers with marked emphysema have relatively good lung function (42, 43, 95). It is, therefore, apparent that for some patients with COPD, emphysema *per se*, i.e. without associated small airway collapsibility, is not a critical determinant of airflow limitation. Although HRCT is a useful non-invasive tool to assess emphysema (14), its interpretation depends on whether scans are taken on inspiration or expiration. Previous investigators have found that both MLD and the %LAA may be altered in smokers, due either to actual tissue loss or peripheral air trapping (49-52). Initial studies showed good correlations between visually scored CT, performed on inspiration, and morphological measures of emphysema (14, 98). Subsequent investigations, using %LAA obtained by density mask HRCT analysis, supported these findings by demonstrating that not only does the % LAA at full inspiration provide an objective measure of the extent of macroscopic emphysema (52) and a reflection of microscopic

emphysema (56) but also that the correlation with pathology is better on inspiration than expiration (56). These studies have, therefore, strongly suggested a link between inspiratory CT measurements and the extent of emphysematous destruction. Conversely, both MLD and %LAA correlate more closely with functional impairment (airflow obstruction and gas transfer) when analysed during expiration (54, 55) and CT on expiration differentiates better COPD subjects from healthy controls (97). Furthermore, calculation of the ratio of expiratory to inspiratory MLD (E/I ratio) also allows indirect evaluation of gas trapping and small airway dysfunction (53, 60). These differences have been explained by the fact that inspiratory CT densitometry does not detect peripheral airways dysfunction, which, as suggested by a number of studies, may be the principal source of airflow limitation in these patients (42, 43). Expiratory CT, on the other hand, also reflects small airway dysfunction that causes air trapping (53, 56, 59). Such air filled areas will appear less dense on HRCT. By contributing to both airway obstruction and gas trapping, peripheral airway dysfunction is believed to underlie the stronger correlation between spirometric and expiratory HRCT parameters (53, 55, 290). It has also been proposed that the same peripheral airway abnormalities could, by their effect on functional inhomogeneities, contribute to TLCO impairment (55).

My study shows that both expiratory HRCT measurements and the MLD E/I ratio correlate more closely with lung function impairment and are more effective in distinguishing smokers with and without airflow obstruction than either inspiratory HRCT or TLCO. Thus my study has exploited the differences in interpretation between inspiratory and expiratory HRCT to provide evidence that small airway dysfunction is a major determinant of reduced lung function in mild to moderate COPD, and a greater predictor of functional impairment than the percentage volume of emphysema as measured by inspiratory %LAA.

Whether peripheral airways dysfunction is principally determined by emphysematous destruction of the lung or small airways remodelling cannot be determined by HRCT because it is not as sensitive as histological analysis of lung tissue and overlooks early or mild emphysematous changes that can be detected by pathological examination (14, 291). Intrinsic airways inflammation and remodelling changes, including fibrosis, oedema, smooth muscle thickening and mucus overproduction all have the potential to narrow or block airway the peripheral airway lumen (77, 92-94). In emphysema,

loss of elastic recoil, with loss of supporting radial traction forces applied by surrounding alveoalar attachments, causes increased small airway collapsibility and impaired airflow (86). Subjects with significant airflow limitation may have more emphysema than can be appreciated by HRCT, because, whether analysed visually or by density mask, HRCT overlooks early or mild emphysematous changes that can be detected by pathological examination (14, 291). Even histological assessment has limitations; peripheral airway instability and loss of elastic recoil, linked to destruction of alveolar attachments (292), is a feature not only of established disease (89), but may be present in smokers without emphysema being detected even by pathological examination (293). Thus, subjects with significant airflow limitation may have more parenchymal destruction than can be appreciated by HRCT but which nonetheless is contributing to peripheral airway dysfunction. Nevertheless, this study shows that HRCT is a valuable method for assessing the extent of peripheral airways dysfunction *in vivo* in relation to reduced lung function regardless of the underlying cause.

This study has attempted to shed more light on the interpretation of MLD as an approach to the analysis of emphysema. Previous pathology studies have shown that MLD is influenced by air trapping and may be altered in the absence of emphysema (51) while %LAA reflects actual emphysema (52, 56). Moreover MLD has been shown in some studies to correlate poorly, and less efficiently than % LAA, with gas transfer impairment but well with lung volume measurements in smokers (50, 57). My study provides further evidence to support this view; while the MLD and E/I measurements predominantly correlated with changes in airflow, the increased %LAA correlated with both airflow limitation and TLCO impairment. However, this has to be taken in the context of mild to moderate disease since studies by Miniati and collleagues have shown that TLCO correlates with MLD in severe COPD (97). Final conclusions regarding the superiority of %LAA over MLD in detecting emphysema cannot be drawn from the data of the current study since TLCO is affected not only by emphysema alone, but also by distribution of ventilation, a factor influenced by small airways dysfunction (55). This may explain the weak correlation observed between the TLCO and the E/I ratio. Moreover TLCO can be influenced by other factors in current smokers such as circulating carboxyhaemoglobin (40) and ventilation-perfusion mismatch (16).

When comparing healthy smokers and non-smokers, HRCT density readings are mildly, albeit statistically non-significantly, increased in the former relative to non-smokers. This is in contrast to the strong and significant downward trend seen among smokers with increasing COPD severity. I speculate that the mild increase in density in healthy smokers is due to oedema and inflammation of the distal lung caused by smoking, a speculation that is based on observations of bronchiolitis and alveolitis in smokers. These appear as micronodules and areas of ground glass attenuation on HRCT and are found more commonly in current smokers than ex-smokers (108, 294). Follow up over a 5-year period has shown that MLD increases in persistent smokers in contrast to ex-smokers in whom it reduces (55). These findings suggest that it may be necessary to interpret HRCT measurements in COPD as a sum of the effects of inflammation, which increases density, and destruction and air trapping, which reduce it.

TLCO values were reduced in a number of healthy smokers in my study, an expected finding as all were current smokers. There is much evidence that current cigarette smoking reduces the TLCO independently of emphysema (41, 295) and that this effect can occur early in the smoking history. Smokers less than 30 yrs of age, some as young as 15, have been shown to have reduced TLCO values when compared to non-smokers (296). Moreover, cross-sectional studies have suggested that ex-smokers have values similar to never smokers (296) and that the TLCO actually increases in smokers once they have stopped smoking (41). Thus TLCO reduction in current smokers can be rapid in onset, reversible and independent, therefore, of emphysema, a permanent, slowly developing disease. A number of mechanisms have been suggested. The presence of circulating carboxyhaemoglobin may lead to reduced driving pressure for CO uptake by the alveolar capillaries, reduction of available haemoglobin for binding CO, and a possible increase in Hb affinity for oxygen (40). Increased airspace size, without actual alveolar and capillary destruction could be a forerunner to emphysema in apparently healthy smokers, resulting in compromised diffusion efficiency of alveolar gases (39). Pulmonary vasoconstriction occurring as a direct response to nicotine, or perhaps due to cigarette smoke induced inflammatory mediators, may possibly lead to reduced lung perfusion (high V/Q ratio) and restricted exposure of circulating haemoglobin to inhaled carbon monoxide (296). Uneven

distribution of inspired gas, due to peripheral airways disease, may result in areas with a disproportionately low V/Q ratio and vascular shunting (102). Although my study cannot cast further light on the relative importance of these mechanisms my results support the view that TLCO impairment in current smokers is either unrelated to structural lung alteration or due to alveolar destruction too mild to detect by the HRCT imaging technique.

My results underline the importance of detailed subject characterisation for COPD research. Many studies of airways inflammation in COPD have used the FEV_1 % predicted alone as a marker for COPD (78, 110, 115, 123). My study subjects were categorised according to the GOLD criteria, which recognises the presence of mild airflow limitation as detected by an abnormal FEV_1/FVC ratio in the presence of a normal FEV_1 %; the stage I group. In this group, mean HRCT densitometry results were altered compared to healthy smokers. Classification of these individuals according to FEV_1 % alone would therefore have resulted in smokers with significant pathology being labelled as healthy smokers. However, although the GOLD criteria may be more sensitive in terms of airflow obstruction, they do not take the underlying pathology into account. Four subjects in my study did not fit into any GOLD group because they had emphysema on HRCT but no symptoms of chronic bronchitis and no airflow obstruction. Also, the majority of patients with Stage 0 COPD had HRCT evidence of emphysema. I suggest therefore that, at least for the purposes of research, a separate category of sub-clinical emphysema in the absence of airflow limitation should be created.

In conclusion the results of this chapter suggests that peripheral airway dysfunction, caused either by small airway remodelling or loss of elastic recoil due to emphysema, is a key contributor to airflow limitation and impaired quality of life in COPD patients. When utilising objective HRCT analysis to assess subjects with COPD, measurements of peripheral airways dysfunction are of more clinical relevance than the percentage volume of emphysema. Finally, I have shown that the international GOLD criteria, although useful for clinical staging of subjects with COPD do not recognise emphysema in the absence of airflow obstruction.

Chapter 4

Characterisation of cellular airway inflammation in COPD

4:1 Introduction

COPD is characterised by airway inflammation, documented in early pathological studies of the disease (84), and confirmed in later analyses of resected or post-mortem lung tissue (44, 73, 93, 187, 297). More recent studies of resected lungs have confirmed associations *in vivo* between specific inflammatory cells in the peripheral airways and parenchyma on the one hand and reduced lung function on the other (167, 298). In recent years the less invasive techniques of bronchial biopsy, BAL and induced sputum have been used to describe inflammatory features in the proximal airways in COPD consisting of infiltration with neutrophils (109-111), macrophages (78, 112, 113) and CD8+ T cells (114, 115).

Whilst knowledge of the pathogenesis of COPD is considerable, a number of gaps have remained. None of the studies to date has included a wide spectrum of COPD, which would enable the identification of the most relevant determinants of disease severity, and none has combined several research tools that provide insight into different components of the disease process. Thus, there have been no attempts to combine bronchoscopic biopsy and/or induced sputum (to analyse large airway changes) and HRCT (which provides measurements of emphysema and peripheral airways dysfunction). As a consequence, it is unclear whether there is any relationship between proximal and distal airway changes. A clear profile of proximal airways inflammation in COPD has yet to emerge as prominent studies have had conflicting results. Prominent observations, for example that CD8+ T lymphocyte infiltration of the airways occurs in COPD (114, 115) have been balanced by other studies (110, 112) that have failed to produce similar findings. Macrophage infiltration has similarly been both confirmed (78, 122) and refuted (115, 123) while studies measuring neutrophils, eosinophils and mast cells in airway wall and sputum, have, in like fashion, produced inconsistent results.

I set out to relate the type and intensity of large airway inflammation in COPD to disease severity on the one hand and small airways dysfunction and emphysema (as determined by HRCT) on the other. For this purpose I performed both bronchial biopsy and sputum induction on COPD subjects, clinically characterised as described in chapter 1, in addition to non-smoking and healthy smoking controls. Inflammatory cell numbers were quantified in bronchial submucosa and epithelium and in sputum.

The results were compared between subject groups and correlated with symptoms, lung function and HRCT measurements.

4:2 Methods

4:2:1 Subjects

As described in chapter 1, subjects underwent detailed clinical assessment, including lung function and HRCT, and were allocated into groups of non-smokers, healthy smokers and COPD subjects. Only subjects aged between 40 and 65 were included, and who had smoked at least 20 pack years of cigarettes.

4:2:2 Bronchoscopy

Bronchoscopy was conducted, according to approved international guidelines (275). Subjects abstained from smoking for four hours prior to the procedure. Pre-medication consisted of nebulised salbutamol 2.5mg and ipratropium bromide 500μg. Fentanyl 50μg and atropine 600μg were given intravenously. Local anaesthesia was achieved using topical 10% lignocaine. A flexible fibreoptic bronchoscope (Olympus BF Type XT20) was used. Endobronchial biopsies were taken from the middle lobe and right lower lobe sub-carinae.

4:2:3 Immunohistochemistry

Endobronchial biopsies were processed into GMA resin for immunohistochemical staining according to Britten and co-workers (276). Briefly the GMA sections were cut at 2μm in thickness and floated onto ammonia water (1:500), picked onto 0.01% poly-l-lysine glass slides, and allowed to dry at room temperature for 1h. The sections were treated with 0.1% sodium azide and 0.3% hydrogen peroxide in distilled water to block endogenous peroxidase. Nonspecific antibody binding was blocked with undiluted culture supernatant for 30 min followed by the primary monoclonal antibody (mAb). Mouse monoclonal antibodies to the following inflammatory cell populations were applied; CD4+ cells (clone leu-3a + leu-3b Dako, Denmark) CD3+ cells (Clone UCHT1 Dako, Denmark) CD8+ cells (Clone DK25, Dako, Demark), neutrophils (neutrophil elastase, clone NP57, Dako, Denmark), macrophages (CD 68+ cells, clone PG-M1 Dako, Denmark), eosinophils (EG2 pharmacia, Sweden), mast cells (AA1, Dako, Denmark) and natural killer cells (CD56 clone moc-1 Dako,

Denmark). After rinsing in TRIS-buffered saline (TBS), biotinylated rabbit anti-mouse IgG Fab (Dako Ltd., High Wycombe, UK) was applied for 2h, followed by the streptavidin-biotin horseradish peroxide complex (Dako Ltd.) for another 2h. After rinsing in TBS, sections were developed with AEC. All sections were counterstained with Mayer's haematoxylin.

Positively stained inflammatory cells were counted in the submucosa using a light microscope. Computerised image analysis (Image associates, Bicester, UK) was employed to measure submucosal area and epithelial length. Cell counts were expressed per mm^2 submucosal area. All measurements were performed without the knowledge of the clinical group from which the biopsy section was derived.

4:2:4 Sputum induction

Sputum induction was carried out, according to departmental protocol, via the inhalation of aerosolized hypertonic saline (4.5%) for a period of 20 minutes. The induction period was kept constant for all subjects regardless of the quality of the sample obtained. Cytospins were obtained and differential cell counting was performed by counting 600 cells in each cytospin in a blinded fashion. The mean of the two scores was used for analysis. Absolute inflammatory cell numbers per 20 minutes induction were calculated by multiplying the cell percentage with the total (non-squamous) cell number in the sputum.

4:2:5 Statistical analysis

The statistical package SPSS 10.1 for windows was employed. For group comparisons the Kruskal-Wallis test was used followed by the Mann-Whitney test. Correlations were carried out using the spearman equation. A p value of less than 0.05 was regarded as statistically significant.

4:3 Results

4:3:1 Clinical groups

67 subjects were included in this part of the study. The remainder were omitted because either they withdrew from the study prior to bronchoscopy, or because bronchial biopsies taken from them were not adequate for analysis. Subjects were classified, as described in chapter 1, into the following groups; healthy non-smoking controls (n=12), healthy smokers (n=13), stage 0 COPD (n=16), stage I COPD (n=9),

and stage II COPD (n=17). Sputum induction was successful in 10 non-smokers, 13 healthy smokers, 14 stage 0 COPD, 9 stage 1 COPD and 17 stage II COPD subjects. The clinical characteristics of the 5 subject groups are summarised in table 4:1.

	Non smokers	Healthy smokers	Stage 0 COPD	Stage I COPD	Stage II COPD
N	12	13	16	9	17
Age	51 ± 7	49 ± 8	49 ± 7	58 ± 7	55 ± 7
Pack years	0	37 ± 12	50 ± 34	44 ± 12	55 ± 16
FEV_1%	111 ± 12.2	101 ± 8	100 ± 14	92± 6	56 ± 17
FEV_1/FVC	77 ± 5.4	79 ± 4	76 ± 3	66 ± 3	56 ± 12
TLCO%	84 ± 12.1	72 ± 11	64 ± 10	65 ± 16	56 ± 16

Table 4:1: table comparing the clinical characteristics of the 5 subject groups. All values are shown as mean ± SD.

4:3:2 Sputum cell counts

Median (range) sputum cell counts are given in table 4:2. No significant differences were observed between any of the groups with regard to the total numbers of cells in induced sputum. The only cell counts that were different between the groups were the relative counts of neutrophils and macrophages. Relative neutrophil counts were significantly higher in stage II COPD when compared to all other groups (Table 4:2, Figure 4:1). The relative macrophage counts followed a reciprocal pattern to the neutrophils, being significantly reduced in stage II COPD when compared to all the other groups (figure 4:2). Absolute neutrophil counts displayed a non-significant tendency to be elevated in stage II COPD compared to the groups without airflow obstruction. However, despite the absence of significantly elevated absolute neutrophil counts, significant trends were observed using the Jonckheere-Terpstra tests for both relative ($p=0.001$) and absolute ($p=0.007$) neutrophil counts to rise with advancing disease stage.

Correlations between neutrophil counts and functional and HRCT parameters are provided in table 4: 3. Relative neutrophil counts correlated significantly with measurements of airflow obstruction and gas trapping and with the E/I ratio (figure 4:3). Relative neutrophil counts correlated more strongly with expiratory MLD than with inspiratory MLD. No significant correlations were observed between sputum neutrophils and either inspiratory %LAA or TLCO%.

4:3:3 Submucosal cell counts

The inflammatory cell counts in the bronchial submucosa are given in in table 4:4. Although the Kuskal-Wallis p value was non-significant (0.07), there was a tendency for CD8+ cell numbers to be elevated in the submucosa in stage II COPD compared to non-smokers, stage 0 and stage I COPD (Figure 4:4). Trend analysis confirmed that CD8+ve cell numbers tend to rise with advancing disease stage ($p<0.05$). The CD4+/CD8+ ratio was significantly reduced in all the smoking groups when compared to non-smokers (Figure 4:4). Submucosal CD8+ve cell counts correlated weakly, but significantly, with the FEV_1% ($r=-0.41$, $p = 0.002$), FEV_1/FVC ratio ($r = -0.34$, $p = 0.01$) and inspiratory %LAA ($r=0.31$, $p<0.05$), but not with other lung function or HRCT parameters.

No significant differences were observed between the subject groups with regard to any other inflammatory cell type in the submucosa. With regard to inflammatory cell counts in the epithelium, there were also no differences between any of the subject groups, and there were no associations with disease severity (data not shown).

	Non smokers	Healthy smokers	Stage 0 COPD	Stage I COPD	Stage II COPD
Total inflammatory cells ($x10^6$)	1.75 (0.9-6)	1.65 (0.8-24.7)	2.0 (0.95-7.5)	2.8 (1.2-5.1)	2.6 (1.3-10.6)
Neutrophils (%)	44.3 (16.1-54.5)	46.0 (30.7-67.9)	50.9 (25.1-66.9)	53.9 (26.9-70.7)	67.8 (44.6-78.1) * † ‡ §
Neutrophils ($x10^6$)	776 (103-2774)	546 (217-4358)	830 (233-3692)	1387 (482-1986)	1416 (789-6485)
Macrophages (%)	51.9 (41.9-75.1)	49.5 (24.5-68.9)	42.4 (30 –70.4)	43.4 (17.6-59.2)	27.5 (10.1-41.5) * † ‡ §
Macrophages ($x10^6$)	618 (335-3330)	513 (91-4195)	871 (298-2840)	806 (298-2646)	722 (203-1941)

Table 4:2: Relative (percentage of non-squamous cells) and absolute (total number of cells $x10^6$, expectorated over 20 mins) neutrophil and macrophage counts in induced sputum. Data are expressed as median (range).

* $p < 0.005$ vs. non-smokers.

† $p < 0.05$ vs. healthy smokers.

‡ $p < 0.001$ vs. stage 0 COPD.

§ < 0.05 vs. stage 1 COPD.

Clinical variable	Correlation coefficient (r) with neutrophils %
SGRQ	0.53, $p<0.001$
FEV_1%	-0.54, $p<0.001$
FEV_1/FVC	-0.52 $p<0.001$
RV/TLC	0.56 $p<0.001$
TLCO%	No correlation
Exp %LAA	0.34, $p<0.05$
Insp %LAA	No correlation
Exp MLD	-0.59, $p<0.001$
Insp MLD	-0.42, $p< 0.005$
E/I ratio	0.55, $p<0.001$

Table 4:3: Correlations between relative sputum neutrophil counts (expressed as a percentage of total non–squamous cells) and lung function and HRCT measurements.

	Non smokers	Healthy smokers	Stage 0 COPD	Stage I COPD	Stage II COPD
Neutros	17.5 (6.3-49.6)	14.6 (1.8-27.1)	15.7 (3.9-28.2)	13.9 (1.3-38.3)	17.7 (3.5-91.8)
CD68+	1.5 (0.0-11.9)	5.1 (0.0-13.5)	3.1 (0.2-13.6)	9.8 (0.6-20.1)	4.7 (0-37.3)
EG2+	0.23 (0-6.46)	27 (0-2.8)	1.0 (0 – 4.2)	0 (0-1.1)	0.9 (0-8.5)
AA1+	41.2 (20.3-97.7)	0.0 (6.2-100.7)	31 (0.6 – 69.5)	45.9 (11.3-78.1)	41.8 (12.9-88.6)
CD3+	30.5 (0.3-195.2)	26.7 (4.7-43.5)	13.6 (2.6-40.1)	23.2 (5.6-59.8)	37.3 (4.4-157.6)
CD4+	25.8 (2.7-198.4)	6.4 (2.1-32.7)	5.8 (0.0-27.1)	15.9 (3.6-34.1)	10.2 (0-65.8)
CD8+	7.8 (0.7-62.3)	11.2 (0.0-34)	5.8 (0-28.8)	17.4 (3.0-29.3)	18.1 (1.9-100)
CD4+/CD8+	3.0 (0.9-6.1) *	0.8 (0.3-3.5)	0.7 (0-20.6)	0.9 (0.4-2.5)	0.6 (0-1.8)

Table 4:4: Inflammatory cell counts in the bronchial submucosa in the clinical groups. No differences were observed in the counts of any cell type in the bronchial submucosa. The CD4/CD8 ratio was significantly higher in non-smokers compared to smokers.

Values expressed as median (range) /mm^2 submucosal area.

* $p < 0.05$ vs. all smoking groups.

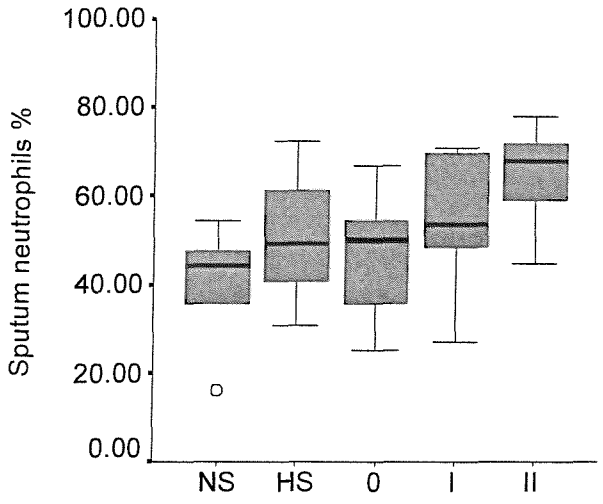


Figure 4:1. Sputum relative neutrophil counts shown as percentages of total non-squamous cells. Data are expressed as median, interquartile range and range. Open circles represent outliers.

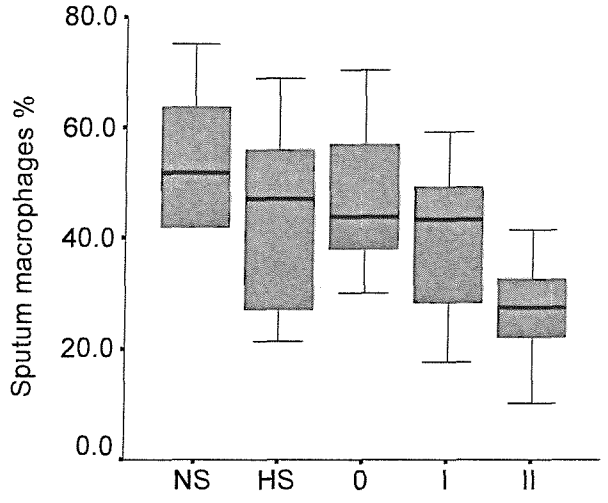


Figure 4:2: Sputum relative macrophage counts shown as percentages of total non-squamous cells. Data are expressed as median, interquartile range and range.

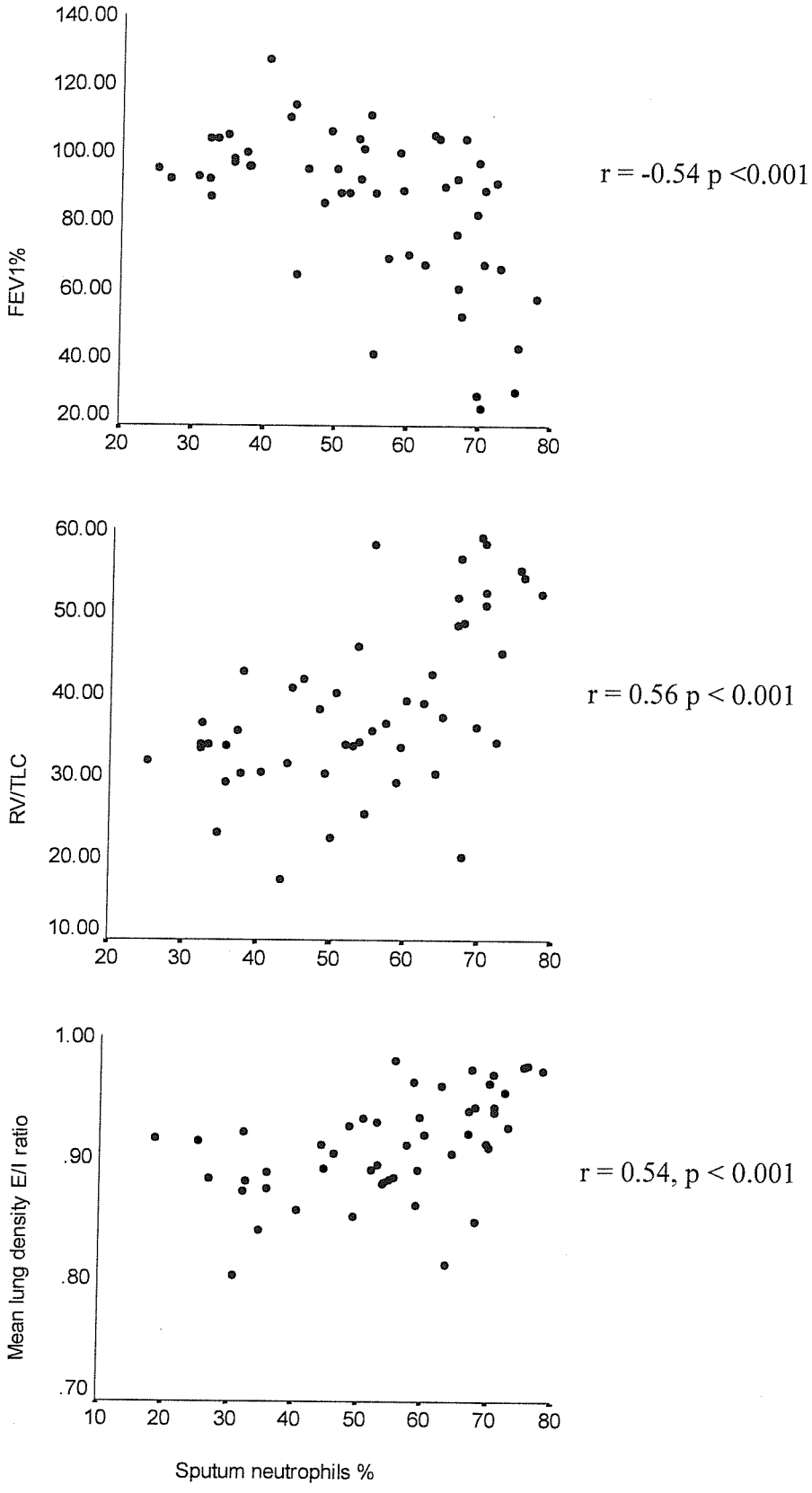


Figure 4:3: Correlation between sputum neutrophils and measurements of airflow obstruction and gas trapping.

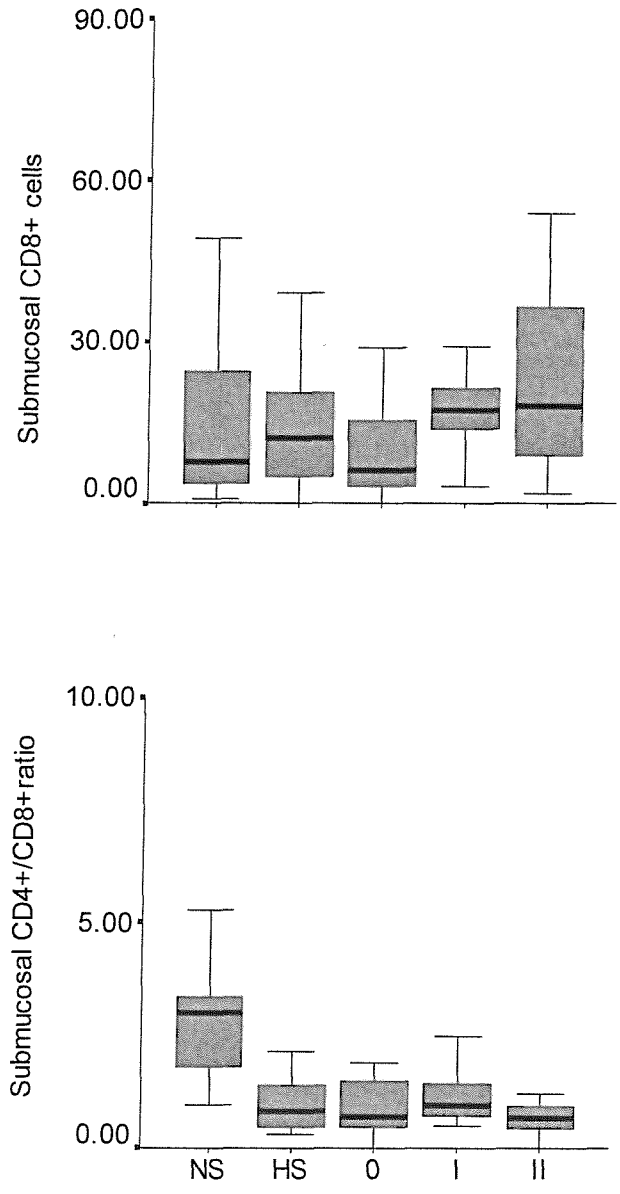


Figure 4:4: Submucosal CD8+ve cell numbers (top) and CD4+/CD8+ cell ratios in healthy non-smokers, healthy smokers, and smokers with COPD stages 0-II. A trend was observed for submucosal CD8+ve cell numbers to rise with increasing GOLD stage of COPD severity ($p<0.05$). The CD4+/CD8+ T lymphocyte ratio in the bronchial submucosa was significantly reduced in all smoking groups compared to non-smokers ($p<0.05$).

4:4 Discussion

To my knowledge this is the first study to investigate in detail the relationship between proximal airway inflammation, as judged by induced sputum and bronchial biopsy and both clinical severity of COPD on the one hand and HRCT measurements of peripheral airway dysfunction and emphysema on the other. Subjects were studied from across a wide spectrum of COPD severity. Of the numerous read-outs studied, only sputum neutrophil counts and CD8+ T cell counts in bronchial biopsies were significantly related to COPD severity. However, the results show, for the first time, that neutrophil counts are significantly associated with HRCT indices of peripheral airways dysfunction, more so than with the severity of emphysema. The number of CD8+ T cells in the submucosa was only weakly related to disease severity and the extent of emphysema.

Consistent with other studies (110, 111, 148) an increase in neutrophil counts was observed in sputum taken from COPD subjects, and these were associated with airflow obstruction, supporting the notion that COPD is associated with an influx of neutrophils into the airway lumen. Interestingly, although the counts correlated strongly with measurements of peripheral airway obstruction and gas trapping, ie. the RV/TLC ratio, MLD on expiration and the E/I ratio, no correlation was observed with either %LAA or TLCO%, both of which are more closely associated with emphysema. Moreover, only subjects with stage II COPD had significantly raised neutrophil counts. In keeping with these results, it has been demonstrated previously that, although smokers with very severe emphysema have prominent neutrophilic inflammation in the alveolar walls and air spaces (167), smokers with mild emphysema without airflow obstruction have neither BAL (124) nor alveolar neutrophilia (167). My results suggest, therefore, that sputum neutrophilia in smokers is of greater clinical relevance as an indicator of peripheral airways dysfunction than of emphysema detected by HRCT. I speculate that sputum neutrophilia reflects best the more distal neutrophilic inflammation that results in destruction of peripheral airway alveolar attachments.

Although the potentially confounding effects of airway sampling upon cell recovery in sputum must be considered they are unlikely to account for my results. Neutrophils are more numerous in the proximal airways, while, in the distal airways and alveoli,

macrophages predominate. In both induced sputum and BAL, increasingly peripheral luminal sampling, achieved by either a longer sputum induction duration or by taking a later BALF aliquot, results in progressive lowering of neutrophil and elevation of macrophage percentages respectively (119, 121). Martin and colleagues illustrated this fact by showing that smokers with severe airflow limitation had significantly lower percentages of macrophages and higher percentages of neutrophils in early BALF aliquots compared to those with less severe obstruction ($FEV_1 > 60\%$) but that with successive aliquots, and therefore likely more peripheral airway sampling, in both groups this disparity in macrophage % and neutrophil % retrieved became less marked (119). Belda and colleagues, moreover, showed that increasing the duration of sputum induction, with, it is likely, increasingly peripheral airway sampling, resulted in progressive lowering of sputum neutrophil counts (121). Peripheral airways dysfunction is a major contributor to airflow obstruction in our subjects. It is possible therefore that, with sputum induction in subjects with airflow obstruction, a degree of preferential sampling of the proximal airways occurs, because peripheral airways narrowing restricts the passage of the more macrophage rich peripheral airway sputum into the large airways. However, the importance of this effect must not be over estimated. There is abundant evidence from other studies that neutrophil activity is increased in luminal secretions in COPD. Absolute sputum counts (as well as percentages) of both neutrophils and macrophages have been shown to be elevated in COPD (110, 148), a tendency observed in our subjects also, although not statistically significant. Myeloperoxidase (MPO), the neutrophil activation marker, is found in increased quantities in both sputum (148, 153) and BALF (152) in COPD subjects. Moreover, although sputum neutrophils were not found to be raised in emphysema without airflow obstruction, elevated levels of the neutrophil degranulation products, neutrophil elastase, human neutrophil lipocalin (HNL), MMP-8 and MMP-9, and the neutrophil chemokine IL-8, have been observed in these subjects compared to healthy smokers (124, 232). It is likely, therefore, that although raised sputum neutrophil percentages may be influenced by sampling effects in COPD, disease related neutrophilic airway inflammation is genuinely present also.

As previously noted in studies of central airways (110, 114, 115) and in a recent study of surgical specimens (298), which also did not show raised neutrophil counts in the mucosa of small airways, I have not shown elevated neutrophil numbers in the airway

wall. Taken together all the studies to date point to neutrophils in the lumen as opposed to tissue as an important inflammatory factor that is related to airflow limitation in COPD. The explanation for the lack of tissue neutrophilia in COPD is unknown. One possibility is that immunostaining for neutrophil elastase identifies only non-degranulated cells, allowing activated neutrophils that have released their granular contents to go undetected. It has also been suggested that in COPD neutrophil migration from blood vessel to airway lumen is swift, defying their detection in the airway wall itself.

CD8+ cells are believed to play a key role in COPD pathogenesis, particularly with regard to emphysema. Postulated mechanisms of CD8+ cell induced lung injury include the release of lytic substances, such as perforin and granzyme, that damage the lung interstitium (164) and the induction of structural cell apoptosis (116). Although the stimulus for CD8+ cell infiltration in emphysema is unknown, both structurally altered self-antigens (116) and persistant intracellular adenoviral proteins (167) have been suggested as candidates. Speculation that CD8+ cells are involved in the development of emphysema has been fuelled by recent findings that CD8+ cell numbers are increased in alveolar walls (116) and small airways (298) of patients with this disease. One of the limitations of the current study is the lack of BAL or transbronchial biopsies that probably reflect better the distal airway and parenchymal changes. BAL was not conducted because bronchial brushings were taken as part of another study and it was not deemed safe or ethically acceptable to take transbronchial biopsies. Limitations in extrapolating large airway to distal pathology notwithstanding, the association between CD8+ve cell numbers and airflow limitation and inspiratory density mask readings provides some support for a role for these cells in emphysema. However, the correlations are weak and the differences between COPD smokers and controls fails to reach significance. Whilst one study (114) has demonstrated significantly increased CD8+ve cell numbers in the proximal airway submucosa of COPD smokers, others have not (110, 112). Differences in sample processing, laboratory analysis or type of airway sampled may have contributed to the failure of my study to demonstrate significantly increased CD8+ cell numbers in COPD. Samples in my study were embedded in GMA, as compared to paraffin (115, 298) and OCT in the other studies (114). In their studies Saetta and colleagues and Turato and colleagues (115, 298) concentrated on the peripheral airways whereas I

examined biopsies taken from the proximal airways. O' Shaughnessy and colleagues also measured inflammation in the proximal airways, but restricted their cell counting to an area 100μm deep to the reticular basement membrane (114), whereas I counted cells in the entire submucosa.

A more significant finding in the current study is the reduction in the CD4+/CD8+ cell ratio in all smokers, including those with no disease. This finding extends previous observations in patients with COPD (114) but not yet reported in healthy smokers. O'Shaughnessy and colleagues noted that the submucosal CD4+/CD8+ cell ratio was reduced, not only in subjects with COPD, but also in subjects with chronic bronchitis without airflow obstruction, even though in this group absolute CD8+ numbers were not significantly elevated (114). My finding that the submucosal CD4+/CD8+ T cell ratio is reduced in all smoking groups extends the observation of O'Shaughnessy et al (114) to include asymptomatic smokers and is similar to findings in the small airway submucosa (123). Rutgers and colleagues, on the other hand, found no decrease in CD4+/CD8+ ratio in COPD airways (110). These investigators, however, included only ex-smokers in their study, whilst all the smokers in our study were current heavy smokers. Smoking is associated with an increased number of CD8+ cells in peripheral blood that falls back to normal after smoking cessation (299). Rutgers and colleagues speculated that current smoking affects T cell subsets in the airway wall in a similar fashion (110), a theory confirmed by my results.

In this study airway wall macrophage numbers, like neutrophils, did not differ significantly between COPD subjects and either smoking or non-smoking controls. This finding, although in agreement with some reports (113, 115), is contrary to the majority of recent studies. Elevated submucosal macrophage counts has arguably been the most consistent inflammatory cell finding in the airways of COPD subjects (78, 112, 114, 157). A possible explanation for the discrepancy between my results and those of others may rest with the choice of anti-macrophage antibody, clone PGM1, used in my study. This antibody is more specific to the macrophage than that used in other studies (clone KP1) which may also detect mast cells (300).

With regard to numbers of the other inflammatory cells studied (eosinophils, mast cell and natural killer cells) no differences were observed in the airway wall between any

of the subject groups. Sputum eosinophil counts, also, were similar in the groups and consistently normal, at levels beneath those found in eosinophilic airway diseases such as asthma or eosinophilic bronchitis (301). Reports have conflicted with regard to eosinophil levels in COPD airways. Although some authors have found that COPD is associated with increased eosinophil numbers in BALF (110) and the submucosa of both large (117) and small airways (123), the importance of these cells in COPD remains uncertain. In some cases these studies pointed to the conclusion that the elevation in eosinophil numbers in the airways in COPD is not accompanied by increased eosinophilic activity (110, 117). There has been some speculation, however, that increased eosinophil numbers in COPD airways could be associated with clinical features similar to asthma. Recent investigations have suggested that raised eosinophil levels in sputum or BALF in COPD is predictive of a clinical response to corticosteroid therapy (79, 125, 177). Whether such subjects have asthma as a co-morbid state or an eosinophilic variant of COPD is a matter for debate. In our study subjects were carefully screened to exclude asthma and allergy. This may account for the low eosinophil counts in biopsies and sputum.

In conclusion, this chapter provides evidence that, in mild or moderate COPD, luminal neutrophilic airway inflammation is significantly associated with peripheral airways dysfunction, which, as shown in chapter 1, is a key determinant of COPD severity. Only a limited role for mucosal CD8+ T cells in determining the severity of COPD has been found. Further studies, focussing on mediators released by neutrophils and indicators of oxidative stress, are needed to shed more light on what determines disease progression.

Chapter 5

Expression of ErbB Receptors and Mucins in the Airways of Smokers with and without COPD; relationship with neutrophilic Inflammation

5:1 Introduction

In chronic smokers bronchial epithelial cells, as the front line recipients of constant injury, undergo significant alterations in both morphology and phenotype, including focal squamous cell metaplasia (29, 65), goblet cell metaplasia (44, 188) and pro-inflammatory functional changes including enhanced adhesion molecule (193, 194) and chemokine (128, 190) expression. Goblet cell hyperplasia and metaplasia, in particular, are well-established hallmarks of the airways of cigarette smokers with and without COPD (44, 45, 94, 188). Goblet cells, as a result, become more numerous and more evenly distributed across the spectrum of airway size (72, 73). These changes, in combination with submucosal gland hypertrophy, lead to pathological mucus overproduction.

Three gel forming mucins (MUC 2, MUC5AC and MUC5B) are found in the lung. Of these MUC5AC is the predominant secreted mucin of the bronchial epithelium while MUC2 is expressed in lower amounts, and MUC 5B, a product mainly of submucosal glands, has only very limited epithelial expression (248, 249, 302). Enhanced expression and transcription of gel forming mucins is believed to be the rate-limiting step for goblet cell metaplasia (68). Experimental evidence suggests that bronchial epithelial expression of both MUC2 (252, 303-305) and MUC5AC (304, 306) may be augmented by inflammatory stimuli. Enhanced MUC5AC expression, in particular, has been linked with the effects of smoking (251, 267), supporting the view that MUC5AC upregulation leads to epithelial goblet metaplasia in smokers' lungs. Activated neutrophils have the ability to induce epithelial MUC 5AC expression (134, 135) suggesting a possible link between neutrophilic airway inflammation and epithelial goblet metaplasia in COPD. However, to date, no quantitative comparisons have been performed of airway mucin protein expression in non-smokers and smokers with or without COPD, nor of their relationship to airway neutrophilia.

In vitro and *in vivo* models of oxidant-induced airway epithelial injury have demonstrated a link between the epidermal growth factor receptor (EGFR, ErbB1) and mucin expression (134, 267). The EGFR is the prototype member of a structurally homologous family of transmembrane receptor tyrosine kinases that also comprises ErbB2, ErbB3 and ErbB4. Ligand binding stabilises the EGFR in a dimeric form comprising either EGFR homodimers or EGFR/ErbB heterodimers involving one of

the other members of the ErbB family (255). The phenomenon of heterodimerisation allows recruitment of the intracellular signalling moieties utilised by both receptor types involved thus increasing the repertoire of activated intracellular messengers and helping to explain the pleiotropic effects of the EGF family (256). There is much *in vitro* and *in vivo* evidence that EGFR activation plays a key role in stimulation of cell motility, migration, proliferation and differentiation, all processes important in the process of epithelial repair, however its dysregulation leads to induction of hyperproliferative and neoplastic conditions (255). In asthmatic airway epithelium EGFR expression is elevated, suggesting the presence of a "stressed" epithelium exhibiting an abnormal, heightened repair response (184, 185).

EGFR, ErbB2 and ErbB3 are all expressed in the bronchial epithelium (246, 261). Although previous reports have shown that cigarette smoking augments EGFR expression in human bronchial epithelium (262) and that, *in vitro,* EGFR activation mediates increased epithelial expression of MUC 5AC in response to cigarette smoke or products of activated neutrophils (134, 267), the expression of other ErbB receptors in current smokers with or without COPD has never been investigated. Therefore I hypothesised that increased airway expression of several members of the ErbB receptor family occurs in response to chronic smoking and that this is associated with increased mucin expression. Secondly, I set out to prove *in vivo* that bronchial epithelial goblet cell metaplasia in smokers is primarily due to enhanced MUC5AC expression. To test these hypotheses, I compared bronchial epithelial expression of immunoreactive EGFR, ErbB2, ErbB3, MUC 2, MUC 5AC and MUC 5B in a cohort of current smokers with and without COPD, and non-smoking controls. By examining the relationship between ErbB receptor expression and mucin expression I looked for evidence to support the role of EGFR/ErbB receptors in the development of epithelial goblet cell metaplasia *in vivo*. I also looked for evidence to support a role for neutrophilic inflammation in the development of epithelial goblet cell metaplasia, by examining the relationship between ErbB receptors, mucins and neutrophil numbers *in vivo*.

5:2 Methods

5:2:1 Subjects

Clinically characterised subjects were studied, allocated into groups of non smokers, healthy smokers, and COPD subjects as discussed in chapter 1.

5:2:2 Bronchoscopy

Bronchoscopy was conducted according to approved international guidelines (275) as described in chapter 4.

5:2:3 Immunohistochemistry

Endobronchial biopsies were processed into GMA (276). Two micron sections of the resin embedded tissue were cut, coded, and immunostained using the following antibodies: anti-EGFR, 1:800, anti-ErbB2 1:20 and anti ErbB3 1:20 (246, 261), anti-MUC5AC, 1:400 (a gift from Professor J.M.Bara (284)), anti-MUC2, 1:20 (285) and anti-MUC5B (1:20 hybridoma culture supernatant, prepared in the London laboratory (286)). Neutrophils were detected using an anti-neutrophil elastase antibody (280). Isotype-matched antibody controls were routinely employed and were negative in all cases. In addition, sections were cut and stained with Periodic acid-schiff (PAS) to quantify goblet cell numbers. Finally, double staining for ErbB3 and PAS was performed to determine whether ErbB3 expression was present on goblet cells. This involved following the standard immunohistochemistry protocol for ErbB3, but, instead of the final counterstaining step, the PAS technique was applied. Quantification of ErbB and MUC immunostaining in the epithelium was performed according to a previously described protocol (185) with the assistance of computerised image analysis (Image associates, Bicester, UK) and expressed as a percentage of the total epithelial area. Epithelial cells staining positively for PAS were counted and expressed as a percentage of the total number of epithelial cells.

5:2:4 Measurement of neutrophilic airway inflammation

Neutrophilic inflammation in both the airway wall and lumen was quantified as described previously. Sputum induction was carried out as previously reported (274). Cytospins were made, coded and differential cell counting performed on 600 cells. Airway wall neutrophil counts were expressed per submucosal surface area and length of epithelium measured by computerised image analysis (Image associates, Bicester, UK).

5:2:5 Statistical analysis

Statistical analysis was performed using SPSS version 10.1 software for Windows. Data for ErbB receptors and mucin immunoreactivity was expressed as medians and

ranges. Comparisons between groups were made by the Kruskal-Wallis test followed by the Mann-Whitney test. Correlations were sought by using Spearman's test. A p value of less than 0.05 was regarded as statistically significant.

5:3 Results

5:3:1 Clinical groups

Of the subjects recruited, 62 were included in this part of the study. The remainder were omitted due to either their withdrawal from the study prior to bronchoscopy, or because bronchial biopsies taken from them did not have adequate epithelium present for analysis. Subject numbers in the clinical groups were as follows; healthy non-smoking controls (n=12), healthy smokers (n=11), stage 0 COPD (n=16), stage I COPD (n=9), and stage II COPD (n=14). Of the 23 smokers with either stage I or II COPD, 17 had symptoms of chronic bronchitis. The clinical characteristics of the 5 subject groups are summarised in table 5:1.

	Non smokers	Healthy smokers	Stage 0 COPD	Stage I COPD	Stage II COPD
N	12	11	16	9	14
Age (yr)	54 ± 8	45 ± 12	50 ± 7	57 ± 7	55 ± 7
Pack years	0	40 ± 15	43± 19	44± 13	52 ± 14 §
Current smoking (cigs/day)	0	18 ± 6	24 ± 11	18 ± 5	23 ± 12
FEV_1%	107 ± 14.7	102 ± 8	99 ± 14	91 ± 6	56 ± 16 *
FEV_1/FVC	73 ± 7.6	80± 5	75 ± 3	66 ± 3 †	55 ± 12 †
TLCO%	84 ± 11.9	74 ± 12	63 ± 10.0 ll	65 ± 18 ll	56 ± 16 ‡

Table 5:1: Clinical characteristics of the 5 subject groups. All values are shown as mean ± SD.

*: $p < 0.001$ vs all groups,

†: $p < 0.05$ v NS, HS and stage 0 COPD,

‡: $p < 0.05$ vs NS and HS,

§: $p < 0.05$ vs HS

ll: $p < 0.05$ vs NS

5:3:2 Epithelial ErbB receptor expression

Immunohistochemical analysis of normal airway mucosa revealed positive staining for EGFR, ErbB2 and ErbB3 in bronchial epithelium, submucosal glands and vascular endothelium. Within the bronchial epithelium immunostaining was associated with the epithelial cell membranes and was most marked at the lower lateral junctions between columnar cells and their junctions with basal cells (Figure 5:1a-c); immunostaining for ErbB2 was weaker than that for EGFR or ErbB3, as previously described (246). A similar pattern of ErbB immunostaining was observed in morphologically normal epithelium of smokers' biopsies except that staining tended to extend closer to the apical surface (Figure 5:1d-f). Both ciliated and goblet cells were positive for EGFR and ErbB3 immunostaining. In 48 of the 62 subjects, additional intra-cytoplasmic immunostaining for the EGFR was also observed in some epithelial goblet cells; this did not follow any pattern of disease severity and was observed in 6 of the 12 non-smokers. The biopsies of some smokers contained areas of squamous metaplasia where uniform membrane immunostaining was observed for all three ErbB receptors (Figure 5:1g-i). There were also regions of damaged epithelium that lacked columnar cells; in these areas, ErbB staining was present at similar intensity to that of neighbouring intact epithelium (data not shown).

Epithelial immunostaining for the various receptors was quantified (ie the percentage epithelial area showing expression) by computer aided image analysis. This involved measurements of immunostaining in areas of intact epithelium. Metaplastic or damaged epithelium were excluded from the analysis. Significantly increased immunostaining for the EGFR ($p<0.05$) and ErbB3 ($p<0.001$) was observed in biopsies of smokers compared to non-smokers (Table 5:2). Neither the percentage expression nor staining pattern of ErbB2 differed significantly between smokers and non-smokers. Within the smoker group, comparison of healthy smokers and COPD smokers showed no significant difference in expression of any of the ErbB receptors, irrespective of disease severity (Figure 5:2). Moreover, when smokers were divided into those with and without chronic bronchitis, there were no differences in ErbB receptor expression between either of the groups. No significant correlations were observed between epithelial ErbB receptor expression and pack year history, symptom scores or lung function measurements of COPD severity. Current smoking habit, however, correlated weakly, but significantly, with expression of ErbB3 (r =

0.37, $p < 0.05$); no correlations were observed between current smoking and the expression of the EGFR or ErbB2.

5:3:3 Epithelial mucin expression

In normal airway mucosa positive immunostaining was observed with the MUC5AC, MUC5B and MUC2 antibodies. In all cases, staining was predominantly intracytoplasmic and localised in goblet cells; no staining was observed in basal cells or ciliated cells. On visual inspection, MUC5AC staining was the most prominent, present in the majority of goblet cells. In contrast, MUC5B and MUC2 staining was found in fewer goblet cells and was of lower intensity. In the smokers, there was a marked increase in goblet cells staining positively for MUC5AC compared to non-smokers ($p<0.001$)(Figure 5:1j,m). Image analysis confirmed a large and highly significant increase in epithelial MUC5AC staining in smokers ($p<0.001$)(Table 5:2). The slight differences observed between smokers and non-smokers for MUC2 and MUC5B were not statistically significant. As had been observed for the ErbB receptors, there were no significant differences in the expression of any of the three mucins studied between healthy smokers and COPD smokers (Figure 5:3), and there were no differences in ErbB receptor expression between smokers with and without symptoms of chronic bronchitis. No correlations were found between epithelial mucin expression and current smoking habit, pack year history, symptom scores, or lung function measurements of COPD severity. PAS staining confirmed that goblet cell numbers were significantly elevated in smokers compared to non-smokers ($p<0.01$) but there were no significant differences between healthy smokers and COPD smokers or between smokers with and without chronic bronchitis. Significant correlations were observed between MUC5AC percentage epithelial area staining and the numbers of epithelial cells staining positively for both MUC5AC ($r=0.527$, $p<0.001$) and PAS ($r=0.43$, $p<0.05$).

The submucosal glands were strongly stained for MUC5B in all subjects, irrespective of disease. In contrast MUC5AC and MUC2 showed very little glandular staining. No attempt was made to quantify this immunostaining because glands were either only partially present, completely absent or distorted by crush artefact, in the majority of the biopsies.

5:3:4 Correlation between ErbB and mucin expression

Epithelial MUC5AC expression correlated significantly with ErbB3 expression either when all subjects were considered together ($r=0.58$), $p<0.001$) or when smokers were analysed on their own ($r=0.38$, $p<0.01$) (figure 5:4). No other correlations were observed between any of the mucins and the other ErbB receptors.

5:3:5 Co-localisation of ErbB3 and mucin staining

Using the dual staining technique, staining for ErbB3 and PAS was found to co-localise to epithelial cells. Thus, cells postive for intracellular mucin demonstrated membrane expression of ErbB3, most marked at the lower lateral junctions of these cells (figure 5:5).

Epithelial marker	Non smokers	Smokers
N	12	50
EGFR	2.8 (0.8–7.0)	5.8 (0.3–22.3) *
ErbB2	1.3 (0–5.4)	2.5 (0.1–9)
ErbB3	5.5 (1.0–19.0)	14.7 (4–37.3) ‡
MUC2	0.1 (0–1.1)	0.7 (0–7.6)
MUC5B	1.2 (0–8.1)	0.9 (0–13.4)
MUC5AC	3.3 (1.1–11.7)	24.7 (3.5–49.3) ‡
MUC 5AC %+ve cells	7.7 (0.4-21.4)	32.5(0.4-59.6) ‡
PAS %+ve cells	13.9 (0.5-19.2)	26.8 (0 – 64.1) †

Table 5:2: Comparison of epithelial immunostaining for ErbB receptors, mucins and PAS in smokers vs non-smokers. The data show percentage epithelial immunostaining for ErbB receptor and mucin expression and percentage epithelial cells positive for MUC5AC and PAS in bronchial biopsies of non-smokers and smokers. All data expressed as median (range).

*: $p<0.05$.

†: $p<0.01$

‡ $p<0.001$

Epithelial marker	Healthy smokers	Stage 0 COPD	Stage I COPD	Stage II COPD
N	11	16	9	14
EGFR	5.1 (1.1-20.4)	3.3 (0.3-16.5)	6.8 (0.6-12.1)	7.6 (1.6-22.3)
ErbB2	3.6 (0.2-7.6)	2.7 (0.1-9)	1.8 (0.1-1.9)	1.9 (0.1-6.1)
ErbB3	13.6 (4-26.8)	16.8 (4.3-23.2)	13.8 (8.5-25.9)	18.1 (5.3-37.3)
MUC 5AC	26.5 (4.8-31.5)	21 (3.5-40.2)	21.6 (13.3-35)	32.2 (13.7-49.3)
MUC 2	0.9 (0-7.6)	0.8 (0-6)	0.2 (0-1.4)	0.8 (0-5.9)
MUC 5B	1.2 (0-5)	0.7 (0-5.2)	0.7 (0-13.4)	0.9 (0-12.3)

Table 5:3: Comparison of epithelial immunostaining for ErbB receptors and mucins in each of the smoking groups. All values are expressed as median (range).

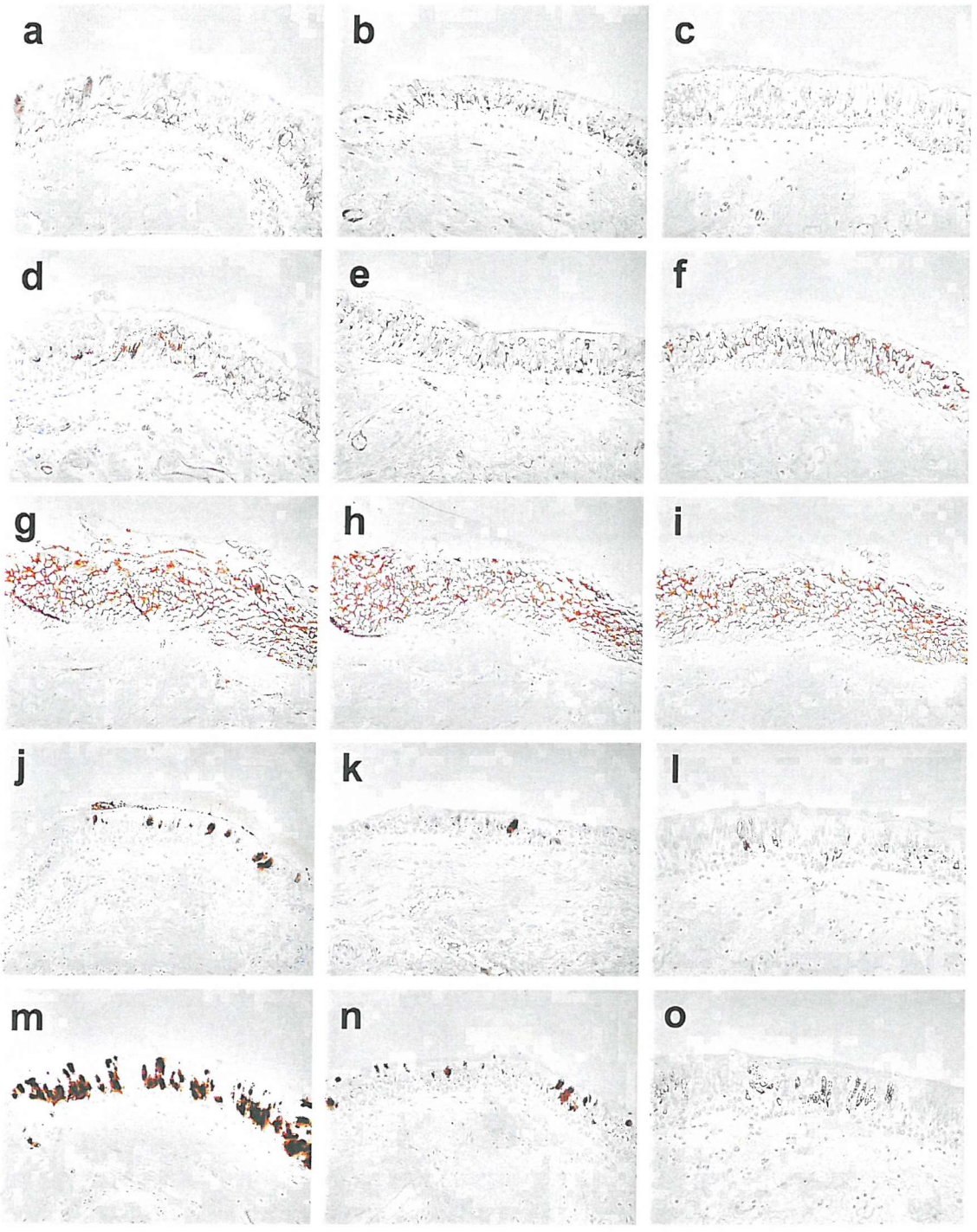


Figure 5:1: Typical patterns of EGFR (a,d), ErbB2 (b,e) and ErbB3 (c,f) staining in the bronchial epithelium of non smokers (a-c) and COPD subjects (d-f). Patterns of EGFR (g), ErbB2 (h) and ErbB3 (i) immunostaining in biopsies of smokers showing squamous metaplasia (g-i) are also illustrated. Patterns of MUC5AC (j, m), MUC5B (k,n) and MUC2 (l,o) is shown in non-smokers (j-l) and COPD subjects (m-o). In each plate, the lumen is at the top of the section. Magnification is x 20.

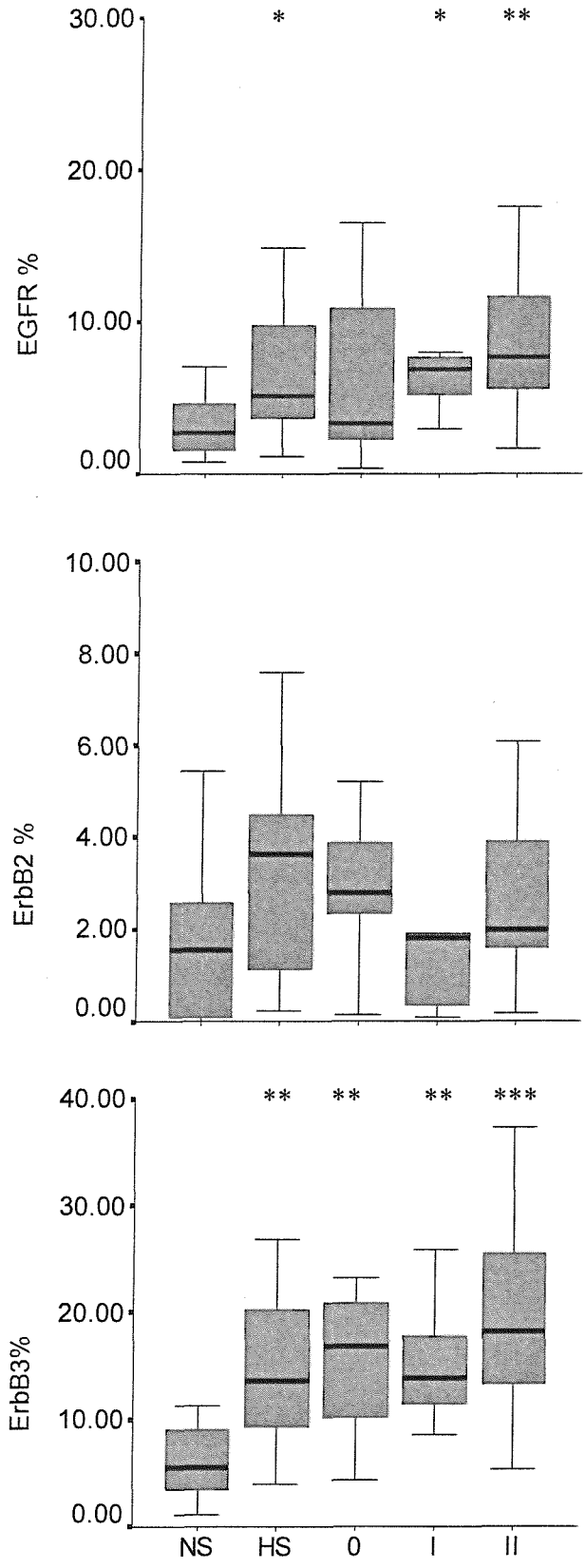


Figure 5:2: Analysis of ErbB receptor expression in bronchial epithelium of non-smokers *versus* smokers without or with COPD.

*: $p<0.05$ vs NS.

**: $p < 0.01$ vs NS.

***: $p < 0.001$ vs NS.

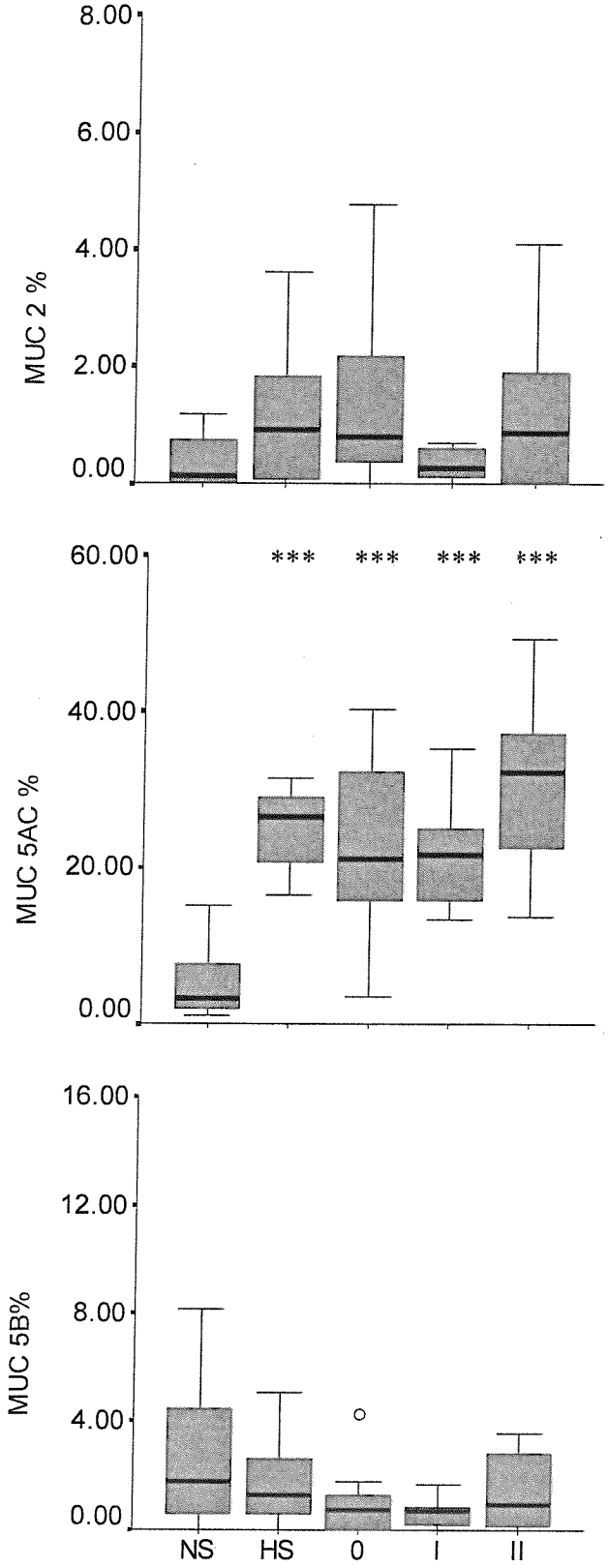


Figure 5:3: Comparison of the percentage of area expressing MUC2, MUC5AC and MUC5B in the bronchial epithelium of non-smokers and smokers.

***: $p<0.001$

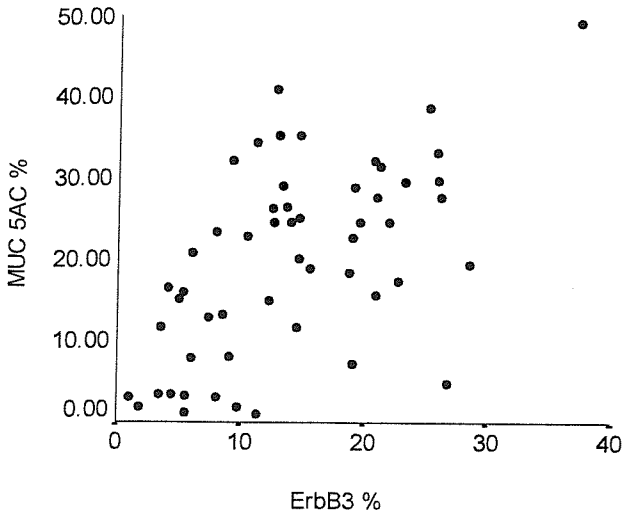


Figure 5:4: Correlation between MUC5AC and ErbB3 expression in bronchial epithelium of smokers without or with COPD.

Figure 5:5: Co-localisation of ErbB3 and intracellular PAS in bronchial epithelium. ErbB3 staining is brown and confined to the epithelial cell membrane. PAS staining is pink and intracellular.

5:3:5 Neutrophil counts

Sputum, submucosal and epithelial neutrophil counts are given in table 5:3. Relative sputum neutrophil counts were significantly higher in stage II COPD when compared to all other groups except stage I COPD and correlated significantly with the severity of airflow obstruction. Absolute neutrophil counts displayed a non-significant tendency to be elevated in stage II COPD compared to the groups without airflow obstruction. No differences were observed between the clinical groups with regard to neutrophil counts in either the bronchial submucosa or epithelium. No correlations were observed between sputum, epithelial or submucosal neutrophil counts and the % epithelial expression of any of the mucins or ErbB receptors studied.

	Non smokers	Healthy smokers	Stage 0 COPD	Stage I COPD	Stage II COPD
Sputum (%)	44.3 (16.1-54.5)	47.2 (30.7-67.9)	50.9 (25.1-66.9)	53.9 (26.9-70.7)	66.1 (44.6-78.1) * † ‡
Epithelium	0.4 (0-1.6)	1.6 (0-5.3)	0.2 (0-3.7)	0.3 (0-3.1)	0.6 (0-5.6)
Submucosa	19.3 (6.3-49.6)	16.1 (1.8-25.9)	13.4 (3.9-28.2)	14.4 (7.9-38.3)	16.7 (3.5-61)

Table 5:4: Relative (median (range)) neutrophil counts in sputum, bronchial epithelium and submucosa.

* $p < 0.005$ v non-smokers

† $p < 0.05$ v healthy smokers

‡ $p < 0.001$ v stage 0 COPD

5:4 Discussion

To my knowledge this study is the first to demonstrate *in vivo* that current smoking increases MUC5AC expression in human bronchial epithelium and that epithelial ErbB3 expression is augmented in parallel, and co-localised to mucin producing cells, thus suggesting a novel association between epithelial mucin expression and ErbB3 expression in chronic smokers. In addition, while I have confirmed previous reports (262, 267) that smoking enhances bronchial epithelial EGFR expression, my study is the first to carry out such an analysis in an extremely well characterised cohort of smokers, both with and without COPD. However, the results suggest that, in current smokers, enhanced expression of ErbB receptors occurs as a response to smoking, with no detectable relationship with disease severity. Whether the effects of current smoking obscure COPD related changes in ErbB receptor expression, or whether there is genuinely no association between simple upregulation of these receptors and the pathophysiology of airflow obstruction in COPD remains uncertain. The increase in MUC5AC (but not MUC5B) that I have observed is consistent with the known increase in goblet cell numbers in the airways of smokers (45). However, similarly to ErbB receptor expression, the extent of epithelial MUC5AC expression was similar in healthy smokers and COPD subjects, consistent with previous studies of goblet cell numbers in peripheral airways of symptomatic and asymptomatic smokers (307). Finally, the extent of MUC5AC expression showed no association with the severity of neutrophilic inflammation, suggesting that neutrophilic inflammation *per se* is not a key determinant of proximal airway goblet cell hyperplasia in cigarette smokers.

The relative importance of goblet cell derived MUC5AC, compared to submucosal gland MUC5B, to mucus overproduction in COPD is uncertain. In a recent analysis, MUC5AC and MUC5B were found to be the predominant mucins in normal airway secretions, with a trend for levels of MUC5B to be proportionally higher than MUC5AC in COPD patients compared to healthy subjects (308). MUC2, on the other hand, has been estimated to account for less than 2.5% of total sputum mucin content (308). Epithelial expression of both MUC2 and MUC5AC may be stimulated by both noxious environmental stimuli and altered host factors. MUC2 expression is augmented by exposure to TNF-α in cultured epithelial cells, while constitutive MUC2 expression is elevated in cystic fibrosis (CF) patient-derived bronchial explant

cultures compared to non-CF derived explants, and is augmented by exposure to bacterial exo-products (250, 305). In contrast, in smokers, as shown in my study, epithelial expression of both MUC2 and MUC5B was low grade and did not differ significantly to that of non-smokers. MUC5AC expression has been linked experimentally with goblet cell metaplasia in COPD. In rat models, exposure to the cigarette smoke constituent, acrolein, or to cigarette smoke itself, enhances airway epithelial MUC5AC expression (251, 267). *In vitro* both cigarette smoke itself and products of activated neutrophils augment MUC5AC expression by cultured epithelial cells (134-136, 267). This chapter provides *in vivo* confirmation that chronic smoking is associated with augmented bronchial epithelial expression of MUC5AC. These findings, coupled to the fact that epithelial expression of both MUC2 and MUC5B was low grade and did not differ significantly between smokers and non-smokers, support the view that, in smokers, goblet cell hyperplasia is dependant on upregulation of epithelial MUC5AC expression. Although there was no correlation with disease severity, the current study's findings are confined to the proximal airways of current smokers, where enhanced mucin expression may be secondary to the acute effects of smoking and less likely to impact upon airflow. Whether or not enhanced MUC5AC expression in peripheral airways, an important site of airflow obstruction in COPD (29), is significantly associated with severity of airflow limitation remains to be determined.

Augmented epithelial expression of the EGFR has been demonstrated previously in smokers (262) and in asthma where it has been shown to be significantly associated with both disease severity and the extent of subepithelial remodelling changes (184, 185, 261). In contrast, in my study, epithelial expression of EGFR showed no corresponding association with clinical markers of COPD disease severity, nor was there any difference between healthy smokers and COPD subjects. Furthermore, unlike asthma (261), epithelial expression of ErbB3 was also elevated in the airways of smokers. Whilst asthma and COPD are both obstructive lung diseases characterised by airways inflammation, remodelling and airflow obstruction, the nature of these changes differs considerably between the two diseases. Asthma is associated with epithelial shedding (309), CD4+ cell TH2 dominated inflammation and characteristic subepithelial basement membrane thickening (310). Distinguishing features of COPD include focal epithelial squamous metaplasia (29, 65) and inflammation typified by an

influx of CD8+ cells, neutrophils and macrophages (78, 109, 114). Although fibrosis is found in the peripheral airways in COPD, large airway sub-epithelial basement membrane thickening is not a usual feature (64, 78). As the biological responses of normal cells correlate with the pattern of ErbB receptor expression (311) some of the differences in epithelial function in asthma and COPD may reflect differences in ErbB expression between smokers, observed in this study, and asthmatics as described previously (185, 246). However, my study's findings are limited to current smokers, therefore I cannot exclude the possibility that the effects of persistent cigarette smoke exposure on the epithelium obscure a disease relationship in COPD. Moreover, as my immunohistochemical study provides only a snapshot view of EGFR protein expression, it remains possible that there is an underlying disease-related difference in the extent of activation of these receptors. Further studies are needed comparing ErbB receptor expression of current smokers with that of ex-smokers with and without COPD.

In both asthmatics and smokers elevated EGFR expression was observed on morphologically intact epithelium, supporting the view that epithelial functional alterations persist despite apparent structural integrity (261). However, in asthma, the increased EGFR expression was observed throughout the epithelial layer (185, 261), and, in disease damaged epithelium, EGFR immunoreactivity was markedly elevated on all surfaces (185, 261). In contrast, although staining in smokers tended to extend closer to the apical surface, the pattern of EGFR expression was more similar to control subjects, localised predominantly at the lower lateral junctions between columnar cells and their junctions with basal cells. Moreover, staining on damaged epithelium was present only at a similar intensity to that of neighbouring intact epithelium. It has been hypothesised that, in asthma, the epithelial response to injury is fundamentally abnormal (182). My study provides indirect evidence to support this view, by showing that changes in EGFR expression in chronic heavy smokers are less profound than previously described in asthma, where epithelial injury occurs as a result of relatively innocuous stimuli.

Elevated epithelial EGFR expression in asthma does not appear to be coupled to a corresponding proliferative response, as suggested by the low levels of proliferating cell nuclear antigen (PCNA) in basal epithelial cells (312). Rather a significant

positive correlation has been observed between EGFR expression in asthmatic epithelium and the extent of sub-epithelial fibrosis (185). A dynamic relationship exists, in repairing tissue, between epithelial cell activity and the activity of underlying fibroblasts and myofibroblasts. It has been suggested therefore that, in asthma, an imbalance exists between parallel pro-proliferative pathways, regulated by the EGFR, and pro-fibrotic signalling in repairing epithelial cells. Factors interfering with EGFR activity such as, for example, ligand damage caused by proteolysis or high levels of anti-proliferative growth factors such as TGF-β could effectively prolong the process of epithelial repair. This, it has been proposed, would also result in prolongation of the duration of the parallel, EGFR independent, profibrogenic pathways that are associated with remodelling (185), leading to the generation of the sub-epithelial basement membrane thickening that is characteristic of asthma (310). In contrast, in chronic bronchitis, PCNA levels are increased in basal cells (312) suggesting that in smokers the airway epithelium mounts an adequate proliferative response. This is pronounced in areas of squamous cell metaplasia, a hyper-proliferative pre-malignant condition, prevalent in smokers' epithelium (263, 313). Previous studies have shown that EGFR expression is elevated in areas of squamous metaplasia, and therefore potentially plays a role in regulation of proliferation of these cells (263). In my study, there was uniform expression of all three ErbB receptors in squamous metaplasia. This data supports the view that proliferative repair responses to cigarette smoking are not being countered by the same anti-proliferative responses found in asthma, an observation in keeping with findings that, in smokers with COPD, subepithelial basement membrane thickening is not a recognised feature.

Previous studies, both in animal models and *in vitro,* have suggested the involvement of the EGFR in the regulation of mucin gene expression. In animal models, foreign body-induced upregulation of epithelial MUC5AC expression has been shown to be EGFR-dependent (264). *In vitro* the EGFR ligands, EGF and TGFα, induce mucin synthesis in airway epithelial cells (314). In asthmatic subjects both the EGFR and its EGF ligand are expressed in increased amounts in bronchial *glandular* tissue compared with controls (184). In epithelial goblet cells, EGFR expression is co-localised with MUC5AC protein in both asthmatics and normal subjects (137). Even though EGFR expression is known to be elevated in the epithelium of smokers, it is

noteworthy that, in my study, I have found that ErbB3 expression, rather than EGFR, correlated strongly with MUC5AC expression. Moreover, ErbB3 expression was demonstrated by co-localisation to be present on mucin producing goblet cells. As epithelial expression of ErbB3 is unchanged in asthma (261) this may also indicate a functionally relevant difference between the epithelium in asthma compared to smokers or it may simply reflect the use of different upstream mechanisms that converge on a common downstream signalling pathway leading to mucin gene expression. An example of such a common link may be phosphatidyl inositol 3-kinase (PI 3-kinase) which can be activated either by ErbB3 or by IL-13 which is strongly implicated in goblet cell hyperplasia in asthma (315).

My *in vivo* observations suggest, for the first time, that regulation of mucin expression may involve more than one member of the ErbB family and/or that activation of ErbB heterodimers may play a role in induction of goblet cell hyperplasia and/or metaplasia. Receptor heterodimerisation allows recruitment of the intracellular signalling apparatus utilised by both receptor types involved, increasing the repertoire of ErbB receptor activation (256). Thus a specific ErbB receptor family member can be activated in the absence of its cognate ligand. For example EGF can induce tyrosine phosphorylation of ErbB3 through formation of EGFR/ErbB3 heterodimers (316). Similarly, EGFR can become activated by heregulin, a ligand for ErbB3 and ErbB4, through the formation of EGFR/ErbB4 heterodimers (317). Variations in the ErbB receptor complement of different cell types will influence the pattern of heterodimerisation, the extent of activation of different intracellular signal transduction pathways and downstream functional responses such as survival, proliferation, transformation and differentiation (311). Given the increase in both EGFR and ErbB3, it is likely that EGFR/ErbB3 heterodimers are the favoured pairing. However even though expression of ErbB2 was unchanged, ErbB2/ErbB3 interactions may also be of importance, particularly as ErbB2 is the preferred dimerisation partner for the ErbB family (318). As signalling via EGFR/ErbB3 or ErbB2/ErbB3 heterodimers is likely to involve activation of PI 3-kinase (316, 319), it is significant that this enzyme has previously been implicated in goblet cell hyperplasia in response to IL-13 (315).

While correlations provide useful indicators of potential mechanisms, they do not necessarily imply cause and effect. For example, as PI 3-kinase is also strongly linked to cell survival, I cannot exclude the possibility that increased ErbB3 expression is unconnected to enhanced mucin expression, rather that EGFR and ErbB3 each provide a distinct cytoprotective mechanism against noxious stimuli. Induction of a pro-survival response following exposure to cigarette smoke has been suggested by *in vitro* studies where cytotoxicity measurements have shown that low doses of cigarette smoke extract cause lactate dehydrogenase activity to *fall* below that observed in untreated cells (242). Furthermore, in a recent study of mucin gene expression induced by *Haemophilus influenzae*, activation of the PI 3-kinase/Akt pathway leads to *down*-regulation of MUC5AC transcription (320). As my study investigated only ErbB3 protein expression, it will be important to determine whether or not smoking promotes ErbB3 activation. Moreover, as protein expression may outlast receptor activation any temporal relationship between growth factor activation and mucin gene transcription remains uncertain. Functional *in vitro* investigation is needed, not only to assess the effects of cigarette smoke on ErbB3 activity, but also to elucidate whether inhibition of ErbB3 signalling decreases mucin mRNA expression and goblet cell hyperplasia in response to cigarette smoke. Moreover, other potential mechanisms of cigarette smoke induced goblet cell hyperplasia exist also. Oxidant stress can occur directly as a result of smoking; In cultured cell lines exogenous hydrogen peroxide augments both EGFR tyrosine phosphorylation and MUC5AC expression (134). Meanwhile, inhibition of reactive oxygen species partially inhibits the elevation in MUC5AC expression induced directly by cigarette smoke (267). In addition, cigarette smoke can directly induce ligand independant EGFR tyrosine phosphorylation *in vitro* (267). Blockage of this reaction with a tyrosine kinase inhibitor prevents cigarette smoke induced epithelial MUC 5AC synthesis both *in vitro* (H292 cells) and in rat models (267). The expression and release of several EGFR ligands, including TGF- α and AR, is stimulated by cigarette smoke extract (CSE) in a dose dependant manner, supporting the possibility that autocrine ligand release and binding are also potential stimulants of MUC5AC expression (242).

Of the three ErbB receptors that are expressed by airway epithelial cells, only expression of ErbB2 was not different from normal in the epithelium of the smokers

studied. However, its expression in the smoking groups showed more variability than either EGFR or ErbB3. The reason for this is unknown, but given its strong association with malignant transformation (321), it may be speculated that higher ErbB2 expression in a subset of smokers might reflect other smoke-related processes linked to development of lung cancer. Expression of ErbB4, the final member of the receptor family was not examined as part of this study as previous investigations have failed to detect ErbB4 expression in either normal human bronchial mucosa or in cultured epithelial cells at the mRNA level (246). The possibility remains, however, that cigarette smoking induces ErbB4 expression by bronchial epithelium.

Neutrophils and their products have been strongly implicated in the pathological changes linked to mucus over production. Of particular relevance to the present study is the demonstration that neutrophil-derived enzymes and oxidants have the ability to induce both EGFR expression and tyrosine phosphorylation and to transactivate the EGFR leading to increased mucin gene expression (134-136). However, in my *in vivo* study of human volunteers, epithelial EGFR and MUC5AC expression showed no association with the severity of neutrophilic inflammation, suggesting that the presence of neutrophilic inflammation *per se* (as distinguished from activation of neutrophils) is not a key determinant of proximal airway goblet cell hyperplasia in cigarette smokers. Furthermore, studies in cystic fibrosis (322) have failed to show any significant change in EGFR levels even though this disease is characterised by neutrophilic inflammation. In the present study, the most striking differences in EGFR, ErbB3 and MUC5AC expression were observed between smokers and non-smokers, suggesting that cigarette smoke may have a direct effect on the bronchial epithelium to promote goblet cell hyperplasia. This does not exclude a role for neutrophils in other chronic lung diseases such as asthma and cystic fibrosis. Nor does it exclude a contributory role for neutrophils in COPD, where their actions in the proximal airways could possibly be overshadowed by the direct effects of cigarette smoke. Furthermore, as all subjects were free from respiratory infections for three months prior to the study, I cannot exclude the possibility that neutrophil-derived products released during an exacerbation may augment mucin gene expression and act as exocytotic stimuli for mucus hypersecretion from the goblet cells. Finally, my study's findings are applicable to the proximal airways only. Airways inflammation in COPD, however, may be most marked in the peripheral bronchioles, thus it is possible

that in these airways a different relationship exists between smoking, ErbB receptor expression and mucin production.

In conclusion, my study demonstrates that bronchial epithelial expression of the EGFR, ErbB3 and MUC5AC are augmented in current smokers with and without COPD, suggesting that ErbB receptor heterodimerisation may be an important determinant of epithelial responses to cigarette smoke. Although my results do not point to any differences in either EGFR or ErbB3 protein levels in differentiating current smokers who remain healthy from those who develop COPD, this does not exclude underlying disease-related differences in ErbB receptor activation. In addition, my results suggest that neutrophilic inflammation is not a key determinant of proximal airway goblet cell hyperplasia in cigarette smokers.

Chapter 6

Measurement of neutrophil chemokines in the sputum and bronchial epithelium of smokers with and without COPD

6:1 Introduction

COPD is characterised by neutrophilic airway inflammation (110, 111, 117, 148-150) due, at least in part, to enhanced neutrophil chemotaxis (147). Increased amounts of the neutrophil chemoattractants IL-8 and GRO-α have been found in airway secretions in COPD, supporting speculation that they play a contributory role (110, 234). However, their importance in determining disease severity, and to what extent the pro-inflammatory effects of smoking influence measurements, remain unknown. Uncertainty exists, also, as to the role played by the bronchial epithelium as a producer of either agent. Epithelial expression of both IL-8 and GRO-α are augmented by exposure to toxic stimuli (191, 237, 240) and the epithelium is the front line recipient of constant cigarette smoke injury. However, only bronchiolar epithelial IL-8 expression has been shown *in vivo* to be elevated in smokers with or without COPD (128, 323) while expression in the proximal airways has been found to be similar to that in non-smokers (128, 323). Epithelial expression of GRO-α has not, to date, been reported in smokers with or without COPD.

This chapter was designed to achieve two objectives. First, I set out to explore in detail the relationship between luminal IL-8 and GRO-α levels and both smoking habit and different aspects of COPD disease severity. To achieve this, sputum IL-8 and GRO-α levels were measured in the cohort of smokers with and without COPD and related to current and past smoking history, lung function impairment, HRCT determinants of peripheral airways dysfunction and emphysema extent, and neutrophilic airway inflammation. Second, by immunohistochemical analysis of bronchial biopsies, I sought to determine whether proximal airway epithelial expression of either chemokine was related either to disease or to sputum measurements.

6:2 Methods

6:2:1 Subjects

Clinically characterised subjects were studied, allocated into groups of non-smokers, healthy smokers and COPD subjects, as outlined in chapter 1.

6:2:2 Bronchoscopy

Bronchoscopy was conducted according to approved international guidelines (275) as described in chapter 4.

6:2:3 Immunohistochemistry

Endobronchial biopsies were processed into GMA (276). Two-μm thick sections were cut, coded, and immunostained using the following mouse monoclonal antibodies; anti-IL-8, 1:200 (Q-Biogene, Harefield, UK), anti-GRO-α, 1:20 (R&D Systems, Abingdon UK) and anti-neutrophil elastase (Dako, Cambridge, UK). Isotype-matched antibody controls were negative in all cases. Quantification of staining was performed in the epithelium with the assistance of computerised image analysis as described previously (185) and expressed as a percentage of the epithelial area with positive immunostaining as compared with the total epithelial area.

6:2:4 Sputum induction

Sputum induction was carried out as described previously (274). Cytospins were obtained and differential cell counting was performed by counting 600 cells in each cytospin in a blinded fashion. Sputum supernatants were stored for analysis of chemokines.

6:2:5 Measurement of neutrophilic airway inflammation

Neutrophilic inflammation in both the airway wall and lumen was quantified as described previously. Airway wall neutrophil counts were expressed per submucosal surface area and length of epithelium measured by computerised image analysis (Image associates, Bicester, UK). Sputum neutrophil counts were expressed as both relative and absolute counts.

6:2:6 Cytokine measurement by ELISA

Cytokine levels in the sputum supernatants were determined using commercially available Enzyme Linked Immuno-sorbent Assay (ELISA) kits for IL-8 (Minimum detectable concentration < 1pg/ml; Pelikine®, Sanquin Research, Amsterdam, Netherlands); GRO-α (Minimum detectable concentration < 10pg/ml; Quantikine®, R&D Systems, Abingdon, UK.) in accordance with the manufacturers' instruction.

6:2:7 Statistical analysis

Statistical analysis was performed using SPSS version 10.1 software for Windows. Data for neutrophil counts, sputum chemokine levels and epithelial chemokine immunoreactivity was expressed as medians and ranges. Comparisons between groups were made by the Kruskal-Wallis test followed by the Mann-Whitney test. Correlations were sought by using Spearman's test. A p value of less than 0.05 was regarded as statistically significant.

6:3 Results

6:3:1 Clinical groups

62 subjects were included for epithelial chemokine analysis, similar to the cohort studied in chapter 5. The remainder were omitted due to either their withdrawal from the study prior to bronchoscopy, or because bronchial biopsies taken from them did not have adequate epithelium present for analysis. Subject numbers in the clinical groups were as follows; healthy non-smoking controls (n=12), healthy smokers (n=11), stage 0 COPD (n=16), stage I COPD (n=9), and stage II COPD (n=14). The clinical characteristics of the 5 subject groups are as summarised in chapter 5, table 5:1. 46 of these subjects underwent successful sputum induction for chemokine analysis. Of these, 10 were non-smokers, 10 were healthy smokers, 10 were stage 0 COPD, only 4 were stage 1 COPD and 12 were stage II COPD. The clinical characteristics of this smaller cohort, in addition to HRCT measurements of emphysema severity (%LAA on inspiration) and peripheral airways dysfunction and gas trapping (MLD on expiration and E/I ratio) are summarised in table 6:1.

	Non smokers	Healthy smokers	Stage 0 COPD	Stage I COPD	Stage II COPD
N	10	10	10	4	12
Age (yr)	54 ± 8	46 ± 11	50 ± 6	56 ± 8	56 ± 6
Pack years	0	32 ± 13	39± 13	49± 14	56 ± 15
FEV_1%	112 ± 14	101 ± 7	97 ± 14	90 ± 7	51 ± 15
FEV_1/FVC	76 ± 5	79± 6	74 ± 3	66 ± 2	53 ± 12
TLCO%	79 ± 11	78 ± 12	56 ± 14 [II]	77 ± 12	56 ± 17
Exp. MLD (HU)	-749 ± 31	-728 ± 45	-741 ± 43	-756 ± 16	-803 ± 40
Insp. %LAA	8.6 ± 2.8	6.7 ± 2.2	6.5 ± 3.0	8.7 ± 2.4	12.9 ± 6.5
E/I ratio	0.88 ± 0.04	0.88 ± 0.04	0.89 ± 0.03	0.9 ± 0.02	0.94 ± 0.02

Table 6:1: Clinical characteristics of the 5 subject groups including only subjects who had sputum chemokine analysis. All values are shown as mean ± SD.

6:3:2 Neutrophil counts

Sputum, submucosal and epithelial neutrophil counts, including only those 46 subjects who also had sputum chemokine analysis, are given in table 6:2. Even with the inclusion of stage I COPD, which only contained 4 subjects, the Kruskall-Wallis test revealed significant overall group differences in sputum neutrophils ($p = 0.012$). Relative sputum neutrophil counts were significantly higher in stage II COPD when compared to all other groups except stage I COPD. Absolute neutrophil counts displayed a non-significant tendency to be elevated in stage II COPD compared to the groups without airflow obstruction. No differences were observed between the clinical groups with regard to neutrophil counts in either the bronchial submucosa or epithelium

6:3:3 Epithelial chemokine expression

Immunohistochemical analysis of biopsies from both smokers and non-smokers revealed positive staining for IL-8 and GRO-α in bronchial epithelium. There were no significant differences in the percentage expression of either chemokine between non-smokers, healthy smokers or COPD smokers (table 6:3). No significant associations were observed between the expression of either chemokine and smoking history, lung function or HRCT measurements of disease severity or with neutrophil counts or chemokine levels in sputum.

6:3:4 Sputum Chemokine analysis

The results of sputum chemokine analysis are given in table 6:3 and figure 6:1. The Kruskall-Wallis test revealed significant overall group differences in sputum levels of both chemokines ($p < 0.05$). Significantly increased levels of IL-8 were detected in sputum taken from smokers with both stage I and II COPD compared to non-smokers. Within the smoker group, comparison of healthy smokers and COPD smokers showed no significant differences in the level of IL-8, irrespective of disease severity. Significantly increased levels of GRO-α were detected in sputum taken from smokers with both stage I stage II COPD compared to both non-smokers and healthy smokers. No significant correlations were observed between sputum GRO-α levels and current smoking habit, pack year history, lung function or HRCT measurements, submucosal neutrophil counts or relative or absolute sputum neutrophil counts. IL-8 levels, on the

other hand, displayed a significant negative association with current smoking habit ($r = -0.48$, $p = 0.006$) (figure 6:2). As with GRO-α, no correlations were observed with pack year history, SGRQ total score, lung function or HRCT measurements, submucosal neutrophil counts or relative or absolute sputum neutrophil counts.

	Non smokers	Healthy smokers	Stage 0 COPD	Stage I COPD	Stage II COPD
Sputum absolute ($x10^6$)	776 (103-2774)	352 (217-4258)	474 (233-3692)	1297 (482-1392)	1459 (5789-6845)
Sputum (%)	44.3 (16.1-54.5)	46.3 (34.7-72)	37.8 (25.1-66.9)	52.1 (26.9-69.6)	70.5 (55.4-78.1) *†‡
Epithelium	0.0 (0-1.1)	2.0 (0.1-3.2)	0.3 (0-0.9)	1.0 (0-3.1)	1.2 (0-13.4)
Submucosa	17.8 (14.1-23.8)	17.2 (8.2-27.1)	19.9 (5.9-28.2)	12.9 (10.4-28.1)	18.4 (3.5-91.8)

Table 6:2: Relative and absolute neutrophil counts in sputum, bronchial epithelium and submucosa in those subjects who had sputum chemokine measurement. Data expressed as (median (range).

*: $p<0.05$ vs NS.

†: $p<0.05$ vs HS

‡: $p<0.001$ v stage 0 COPD.

	Non-smokers	Healthy smokers	Stage 0 COPD	Stage I COPD	Stage II COPD
IL-8 (sputum pg/ml)	545 (53-16667)	2001 (75-15011)	2414 (1510-19561)	6436 (649-17402) *	4092 (1510-19561) †
GRO-α (sputum ng/ml)	5.0 (0.6-32.1)	25.2 (0-333.1)	29.2 (0-477)	149.5 (72.3-467) †§	140 (6.6-2578) ‡§
IL-8 (epithelium)	5.1 (1.1-16.6)	5.8 (0-21.7)	5.7 (0.3-20.8)	6.8 (0.4-8.0)	7.9 (2.1-23.2)
GRO-α (epithelium)	3.2 (0-9.8)	4.5 (0.1-12.9)	3.5 (0-18.3)	7.7 (0.2-9.3)	4.4 (0.1-17.6)

Table 6:3: Sputum levels and percentage epithelial immunostaining for IL-8 and GRO-α in each of the clinical groups. Values are presented as median (range).

*: $p<0.05$ vs NS

†: $p<0.01$ vs NS

‡: $p<0.001$ vs NS

§: $p < 0.05$ vs HS

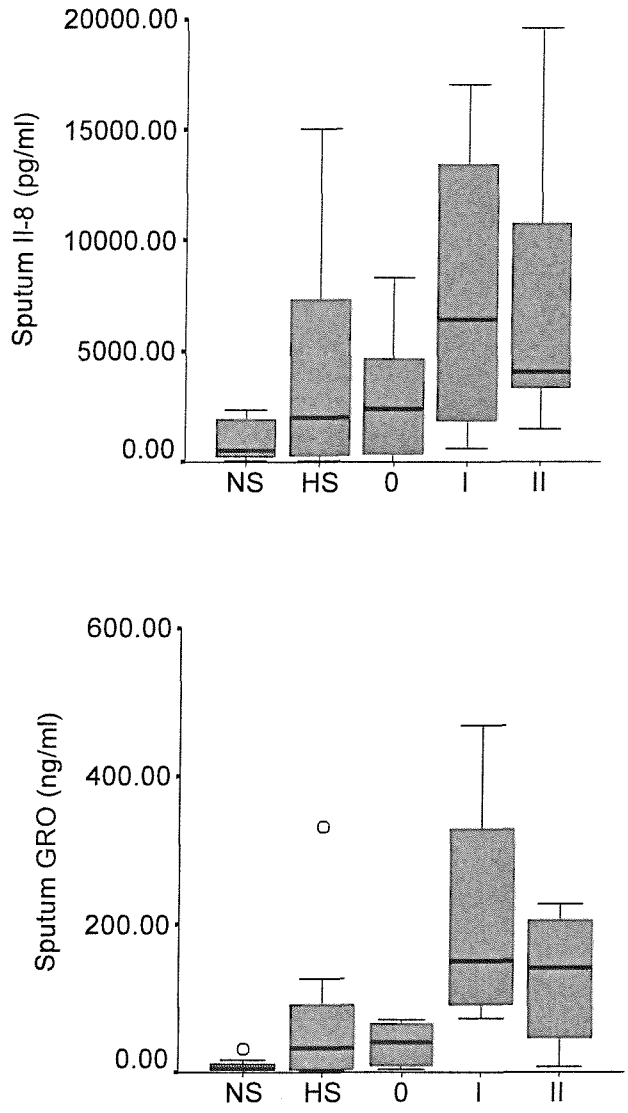


Figure 6:1: Analysis of IL-8 and GRO-α levels in the sputum of non-smokers versus smokers without or with COPD. The box and whisker plots show median, range and interquartile ranges.

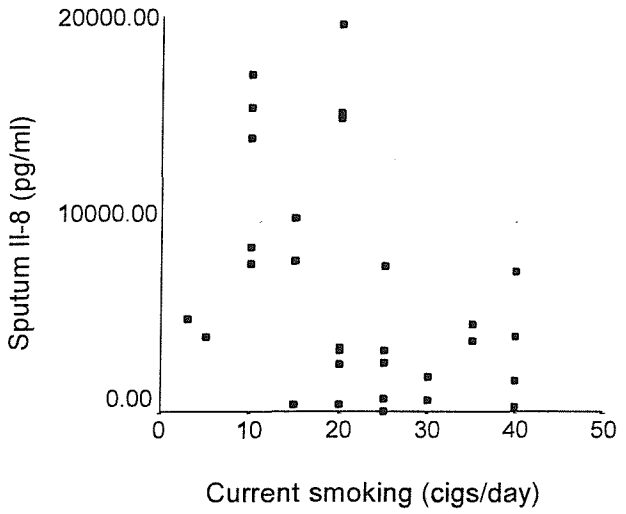


Figure 6:2: Relationship in smokers between between IL-8 levels measured in sputum and current smoking habit ($r = -0.48$, $p = 0.006$).

6:4 Discussion

In this chapter I investigated the importance of the neutrophil chemoattractants IL-8 and GRO-α, measured in induced sputum, as indicators of disease severity in current smokers with COPD and the potential role of the proximal airway epithelium as producers of both. This study is the first to examine either the luminal or epithelial expression of either chemokine in a cohort of current smokers that have been characterised clinically, physiologically and radiologically by quantitative HRCT. Also, it is the first to measure proximal airway epithelial expression of GRO-α in smokers with or without COPD. The results show that sputum IL-8 levels were significantly higher in smokers with COPD compared to non-smokers, but there were no differences compared to healthy smokers. GRO-α levels, on the other hand, were elevated in smokers with COPD compared to both non-smokers and healthy smokers. There were no associations observed, among smokers, between the sputum levels of either chemokine and disease severity. However, IL-8 levels were associated inversely with current smoking habit. Finally, no differences in the epithelial expression of either chemokine were observed in smokers with or without disease compared to non-smokers. My findings support the view that both chemokines play a role in the pathogenesis of COPD but suggest, also, that current smoking significantly influences the detection of IL-8 in sputum, and may reduce its value as an indicator of COPD severity in current smokers. Moreover, my results suggest that, in smokers, proximal airway epithelium does not contribute significantly to the enhanced production of either agent.

Airway neutrophilia in COPD is likely to be multifactorial, dependant on a complex interplay of chemokines, lipid mediators, adhesion molecules, components of the damaged extracellular matrix and neural activity (324). IL-8 and GRO-α, whose pro-inflammatory properties have been well documented both *in vitro* (212) and in animal models (221, 222) are logical candidates for study in COPD and other diseases associated with neutrophilic inflammation, and my demonstration that sputum levels are increased in COPD support the findings of others. Elevated luminal IL-8 levels have been shown to correlate with neutrophilic inflammation in bronchiectasis (325), severe asthma (227, 228), sarcoidosis (230) and ARDS (229). In COPD both airway remodelling and parenchymal destruction have been linked to neutrophilic

inflammation (124, 134, 151). Accordingly, studies have shown that IL-8 is increased in airway secretions from both healthy smokers (148), and smokers with disease, including simple chronic bronchitis (152, 231), subclinical emphysema (232) and airflow obstruction (171, 172). Although GRO-α has been less studied, a recent investigation demonstrated increased sputum levels in COPD that correlated with disease severity and sputum neutrophil counts ($x10^6$/ml) (234). The relative importance, however, of influences such as smoking history, infection and clinical phenotype as compared to airflow obstruction severity on the expression of either chemokine in COPD remains uncertain. In the case of IL-8, associations have been found between raised luminal levels and current smoking habit (122), bacterial infection of the airways (326) and the presence of emphysema on CT scan (232), and, although some investigators have shown that sputum IL-8 levels correlate strongly with airflow obstruction severity (172), others found similar levels in chronic bronchitis patients with and without airflow obstruction (152). In the current study, unlike previous investigations, smokers were comprehensively characterised with regard to both current and past smoking habit, lung function and HRCT measurements of peripheral airways dysfunction and emphysema extent. Moreover, all were current smokers and both exacerbation and medication free. The finding that current smoking habit, rather than disease severity or neutrophil counts, correlated with sputum IL-8 levels, is in contrast to the report by Yamamoto and colleagues, who also measured sputum IL-8 in COPD (172, 234). In this study, however, only 11 of the 33 COPD subjects were currently smoking. My results suggest that current smoking has an impact on the recovery of IL-8 that may confound associations with disease and suggest that measurements must be interpreted in the context of the amount of cigarettes currently being smoked.

The relationship between sputum IL-8 and current smoking amount was an inverse one. Thus, although previous reports have shown that smoking *per se* augments sputum IL-8 recovery (327), my results suggest that the increase in expression is dampened in the presence of higher current smoking levels. This unexpected, though highly significant, finding disagrees with the study by Kuschner and colleagues who stratified smokers into three groups according to whether they smoked less than, equal to or greater than one pack per day and whose results suggested that current smoking

augments IL-8 production in a dose dependant manner (122). However, as acknowledged by the authors, the size of this study was small (only 14 smokers in total) and there was only a weak ($r=0.31$) and barely significant ($p=0.04$) correlation between smoking intensity and BALF IL-8 concentration. Although *in vitro* cell stimulation studies have proven that cigarette smoke induces IL-8 secretion and that this may occur in a concentration dependant manner (190, 242, 328), some reports have suggested that the relationship between dose and response is more complex. IL-8 secretion by cultured alveolar macrophages is lower in cells taken from smokers compared to non-smokers (329), and current smokers compared to ex-smokers regardless of the presence of emphysema (232). CSE has been shown to modulate IL-8 release from a human monocyte/macrophage cell line in a bell-shaped manner, stimulating release at lower concentrations but inhibiting release when higher concentrations were used (330). If such responses are mirrored *in vivo* the mechanisms remain speculative. Evidence from primary epithelial explant cultures has shown that cigarette smoke induced IL-8 expression in healthy smokers is downregulated compared to COPD subjects suggesting that, in some smokers, inhibitory mechanisms protect against the development of airway inflammation (239). Alternatively, it is possible that some components of cigarette smoke inhibit IL-8 production at high concentrations. Nicotine, which is mainly associated with the particulate phase of cigarette smoke (331) is a possible candidate. Exposure of a cultured macrophage cell line to nicotine has been shown to inhibit expression of IL-8, possibly by blocking activation of the transcription factor nuclear factor κB (NF-κB) (332) an essential step in IL-8 gene transcription (213).

Whatever the mechanism linking current smoking habit with chemokine production, the results from the current study need to be interpreted with caution. Smoking estimates were subject dependent and not independantly verified and therefore may be imprecise. Moreover, the brand or type of cigarettes smoked was not recorded, nor was smoking technique examined. Thus, although this study highlights the potential importance of current smoking, a further prospective study is needed to examine the relationship between current smoking and IL-8 expression *in vivo* in smokers matched for disease severity with careful monitoring of all aspects of smoking habit.

The results of this chapter suggest that proximal airway epithelium is not a significant contributor to raised levels of either IL-8 or GRO-α in the lungs of current smokers. Although epithelial expression of GRO-α in smokers' lungs has not been reported previously, the results with regard to IL-8 are in accordance with a previous study of the proximal airways (323). In contrast, studies of the peripheral airways, using both RT-PCR analysis of brushed bronchiolar epithelial cells (323) and *in situ* analysis of resected lung tissue (128), have suggested that *bronchiolar* IL-8 mRNA expression is increased in smokers. It has been speculated that, in the proximal airways, greater exposure to other environmental compounds (eg. viral particles, bacteria, pollutants) may obscure differences in IL-8 expression between smokers and non-smokers (323). Another possibility is that native inflammatory factors, such as, for example, neutrophil elastase, a known inducer of IL-8 expression (223, 243), play a relatively more important role in the lung periphery, where COPD pathology is more marked. Alternatively, if enhanced epithelial chemokine expression is only detectable in the acute phase of smoking, as suggested by stimulation studies of primary epithelial explant cultures (239), immunohistochemistry, which provides only a snapshot view of epithelial chemokine production may have failed to capture it, as all smokers in the current study abstained for at least 4 hours prior to bronchoscopy. Whatever the explanation, the principal sources of sputum IL-8 and GRO-α, both of which are produced by a variety of resident and non-resident cell types in the lung, including monocytes/macrophages, neutrophils and airway epithelium (213), remain obscure in smokers.

In conclusion, the results of this chapter support the view that COPD is associated with enhanced production of neutrophil chemokines in the airways, but suggest that current smoking is an important determinant of sputum IL-8 levels in smokers, outweighing the impact of disease severity or neutrophilic inflammation, and that proximal airway epithelium does not contribute significantly to the enhanced production of either agent.

Chapter 7

Overall Summary and Discussion

My thesis is a detailed study of subjects from across a wide clinical spectrum of COPD. Using a comprehensive clinical approach, these were carefully characterised to record smoking history and identify key clinical determinants of disease severity. I went on to examine the relationship between these findings and inflammatory and pathological changes in the proximal airways.

Research into COPD has always been beset by a number of fundamental difficulties. To begin with, COPD is not a single disease but rather a heterogeneous syndrome unified by a common aetiology, smoking. Disease susceptibility, in terms of exposure to cigarette smoke, varies (11). The mechanisms linking smoking and recognised aspects of disease pathogenesis, such as airway inflammation or remodelling, are poorly understood. Moreover, to what degree such airway changes are involved in disease, as opposed to due to smoking *per se,* has not been explored in depth. Clinical assessment of COPD patients is not straightforward as the symptoms are often non-specific, and detection of physical signs is subject to considerable inter-observer variability (34, 35). Finally, the relationship between clinical severity, functional impairment and pathology is complex. The major pathological processes, airway remodelling and emphysema, may be present to greatly varying degrees and occur predominantly in the peripheral lung, hence descriptive studies of COPD pathology have required either lung resection or post-mortem specimens.

The first aim of my thesis, therefore, was to obtain a detailed profile of COPD disease phenotype and severity in a population of smokers. However, as this was to be done in a non-invasive manner, a comprehensive clinical approach was required. Each subject's assessment included careful medical screening, taking into account a detailed current and past smoking history in combination with both validated functional assessment and state of the art imaging techniques. The results, outlined in chapter 3, demonstrate not only the complimentary relationship between different modalities of COPD assessment but also highlight limitations of techniques and staging criteria that are in common clinical use. Thus, for example, my study supports the view that FEV_1 measurement is not sensitive to mild airways disease, that TLCO impairment is not specific to emphysema in current smokers and does not accurately reflect mild differences in airflow obstruction severity (333), and that staging criteria, that are based upon airflow obstruction, fail to recognise asymptomatic emphysema.

In addition, with regard to HRCT, my study, which is the first to evaluate the technique in subjects with a wide spectrum of COPD severity, has provided useful insights into its clinical importance. Although HRCT is not routinely required to diagnose COPD in clinical practice, its value as a surrogate for pathology, in detection and quantitation of emphysema (52, 55, 97) is well recognised, while more recent studies have defined a role for HRCT in evaluation of peripheral airways dysfunction (53, 60). The results of my study demonstrated that HRCT measurements of peripheral airways dysfunction are strongly associated with functional impairment while suggesting that HRCT measurements of emphysema extent have less clinical relevance as indicators of disease severity.

This clinical aspect of my thesis has important limitations. The findings must be interpreted in the context of mild/moderate COPD. In severe emphysema, not included in this study, it is possible that the functional and radiological significance of expiratory air-trapping may be outweighed by more extensive lung destruction and obliteration of conducting bronchioles. HRCT does not allow definite conclusions to be drawn regarding the relationship between disease severity and lung pathology. Although calculation of the %LAA has been reliably correlated with emphysema severity (52, 56), morphological examination of lung tissue is required for accurate measurement. HRCT evaluation of peripheral airways dysfunction has been validated only in comparison with clinical tests of small airways obstruction (53, 60). The dynamic nature of this abnormality, and the fact that both airway remodelling and emphysema contribute, makes even pathological evaluation difficult. On the other hand, structure/function data achieved non-invasively has its own particular merits in terms of its relevance to patients in clinical practice and in research. HRCT may be of practical value in future clinical trials in COPD, which are unlikely to utilise pathological samples from the peripheral lung. For, although measurement of airflow obstruction is the mainstay of disease assessment, and is used as an indicator of therapeutic response (334), its relationship with symptom severity is often poor (36). HRCT represents a safe, reproducible modality of disease quantification that may be useful when determining therapeutic responses. Results from this study and others may help in the design of therapeutic trials employing HRCT so that the most clinically relevant results are recorded.

The numerous results outlined in chapter 3 also allowed accurate interpretation of histological and sputum data obtained from the same individuals in the context of smoking habit and disease severity. Chapters 4, 5 and 6 report the analysis of specimens obtained by bronchoscopy and sputum induction, used to sample the proximal airways. Together, these chapters examine the relationship between smoking, disease severity, airways inflammation, chemokine expression and remodelling of the proximal airway epithelium.

Chapter 4 reports the study of proximal airways inflammation in COPD, by quantification of inflammatory cell counts in bronchial biopsies and induced sputum samples. The importance of inflammation, towards the generation of both airway remodelling and parenchymal destruction in COPD is widely accepted (65). In particular, neutrophils (109-111), macrophages (78, 112, 113) and CD8+ T lymphocytes (114-116) are believed to play key roles. Multiples studies of airways inflammation in COPD have been performed in recent years. No study, however, has included a wide spectrum of COPD disease severity, using the GOLD classification criteria, that differentiates smokers with mild COPD from healthy smokers. Moreover, no previous study has examined the relationship between inflammation and quantitative HRCT. My results provided limited support for the role of CD8+ cells in COPD with associations demonstrated between CD8+ cell numbers and both airflow obstruction and HRCT determined volume of emphysema. It may be speculated that these results reflect a relationship between proximal airways inflammation and emphysematous destruction of the distal lung, a finding that would support the use of bronchoscopy as an indicator of distal airway changes. For, although one prominent study has documented increased CD8+ cells in proximal airways in COPD (114), the bulk of the evidence implicating these cells has come from analyses of peripheral airways (115, 298) or alveoli (116). However, as the associations observed in my study were weak, and as HRCT is only a surrogate for pathological examination, firm conclusions cannot be drawn. Further studies, directly comparing proximal airways inflammation with both distal airways inflammation and pathologically quantified emphysema are required to explore this relationship.

Neutrophils, in induced sputum, were by far the most prominent inflammatory cells identified in the airways in COPD. Although many studies have documented the

association between luminal neutrophilic inflammation and COPD (110, 148), mine was the first to demonstrate that, not only are they strongly related to airflow obstruction, but also that the increased counts are associated with the severity of peripheral airways dysfunction rather than emphysema. As with CD8+ cells, these findings must be interpreted with caution in the absence of morphological data. Neutrophils have been implicated in both the generation of emphysema (21, 131) and airway remodelling, in particular goblet cell hyperplasia (132, 135, 136). As both emphysema and airway remodelling may contribute to peripheral airways dysfunction, the pathological significance of raised sputum counts remains uncertain. The lack of association with measurements of emphysema on HRCT may due to the fact that sputum induction samples the more proximal lung, particularly as other investigators have shown that severe emphysema is associated with a prominent neutrophilia in both alveolar walls and air spaces (167). I speculated that, in smokers, neutrophilic inflammation leads to peripheral airways dysfunction via destruction of alveolar attachments not detectable by HRCT. Comparison of sputum neutrophils with pathological examination of peripheral airway wall alveolar attachments is required to confirm this.

The dominance of the neutrophil among the inflammatory cells studied prompted the directions taken in both chapter 5 and 6 of the thesis. In chapter 5, one particular aspect of remodelling, goblet cell metaplasia, was examined in more detail. Goblet cell metaplasia has been linked *in vitro* and in animal models with neutrophilic inflammation, and occurs not only in distal but also proximal airways. Therefore I set out to determine whether a relationship could be identified *in vivo*, between neutrophilic inflammation and key factors in the development of goblet cell metaplasia. Using immunohistochemistry, I examined the expression of mucins in the proximal airway epithelium and their relationship with ErbB receptor expression, neutrophilic inflammation and overall disease severity.

This study is the first to confirm *in vivo* that smoking enhances epithelial MUC5AC expression, as had been suggested by previous reports from animal (251) and *in vitro* (267) models. These studies and others suggested that enhancement of MUC5AC expression is mediated via EGFR activation occurring either directly as a result of smoking (267) or by transactivation via the products of neutrophilic inflammation (134). No association was observed, however, in my study, between ErbB receptor or

MUC5AC expression and either neutrophilic inflammation or disease severity, suggesting that exposure to cigarette smoke is the principal determinant of enhanced mucin expression. These findings do not, however, discount a role for neutrophils in the generation of proximal airway goblet cell hyperplasia, as sputum neutrophil counts may not accurately reflect local inflammation at the sites biopsied or the degree of neutrophil activation. Moreover, these findings cannot be extrapolated to the distal airways where cigarette smoke exposure may be less and inflammation may be more pronounced. Analysis of a further control group, containing ex-smoking COPD subjects may help to clarify the issue as, although such subjects are no longer exposed to cigarette smoke, there is evidence that they have ongoing neutrophilic inflammation in their airways (110).

While it is not surprising, because of airway geometry, that proximal airway mucin expression is unrelated to airflow obstruction, the impact of enhanced expression may be greater in the peripheral airways, and merits exploration using resected lung specimens. Although, unlike in asthma (185), EGFR expression showed no correlation with disease severity, growth receptor presence does not imply activation and thus the potential of this receptor as a determinant of disease severity remains to be examined. My demonstration that in smokers not only is EGFR expression enhanced, as demonstrated previously (262), but that ErbB3, also, is upregulated, and correlates with MUC5AC expression and is expressed by mucin producing goblet cells, deserves further study. Although the findings are limited by their purely descriptive nature, it is possible, given the evidence supporting the involvement of the EGFR in mucin synthesis (134, 137, 184, 264, 267), that its family member ErbB3 plays a regulatory role also.

Chapter 6, the final chapter of my thesis, was also inspired by the observation that sputum neutrophils were the major inflammatory cell in the airways in COPD. However, in contrast to chapter 5, this chapter examined a potential cause rather than effect of neutrophilic inflammation. Thus the expression of two neutrophil chemokines, IL-8 and GRO-α, was studied, and related to disease severity and neutrophilic inflammation. Both chemokines were chosen not only for their known potent neutrophil-inflammatory properties (217, 222) but also because they

(particularly IL-8) had been highlighted by previous reports in the field as possible key candidates in COPD in particular (171, 172, 234). Mine is the first study to compare both luminal and epithelial expression of the two chemokines with smoking habit, functional and HRCT indicators of disease severity and neutrophilic inflammation. Unlike the markers studied in chapter 5, epithelial chemokine expression appeared unaffected by smoking. Sputum chemokine levels, meanwhile, were elevated only in smokers with COPD, but did not correlate significantly with any aspect of COPD severity, nor with sputum neutrophil counts. IL-8 levels, rather, were inversely associated with current smoking amount. A true reflection of the importance of either chemokine to COPD requires analysis of further control groups, including ex-smoking subjects, with and without COPD. Taken together, the results of chapters 5 and 6 highlight the fact that smoking *per se* influences the expression of mediators in the airways, whether cell surface receptors, mucins or chemokines. This effect may confound interpretation of assays designed to measure inflammatory/ remodelling effects in the airways in COPD and suggests the necessity of controlling for current smoking history.

In summary, by taking a detailed approach to the clinical evaluation of smokers with and without COPD, I have identified functional and radiological parameters of relevance to disease severity and airway inflammation. However, there were no associations observed between clinical parameters and the measured inflammatory and remodelling factors, chosen for their potential importance to disease pathogenesis and their relationship with neutrophilic inflammation. The impact of smoking itself as a potential concealer of such associations deserves further study.

Future directions

Further studies could be carried out to expand the work outlined in my thesis. As a straightforward initial step, further descriptive data could be collected involving comparison with further control groups, including ex-smokers with and without COPD. This would help clarify the role of current smoking as a potential confounder of relationships between clinical disease severity and large airway pathology. Peripheral lung pathological correlation with factors such as HRCT E/I ratio, proximal airways inflammation and epithelial marker expression could be achieved by

enrolling patients listed for lung resection in a prospective study. This would involve performance of HRCT and sputum induction prior to resection and detailed pathological examination of both the peripheral airway wall itself and its alveolar attachments. As many patients who have lung resection have bronchoscopy beforehand, a separate "research" biopsy could be taken, from a separate airway, for immunohistochemical evaluation. Such a study might firmly establish the validity of bronchoscopy as a surrogate for peripheral airway sampling. Less invasively, the use of ultrathin bronchoscopy might allow clarification of whether or not mediator expression by epithelium in the distal, as opposed to proximal, airways has a plausible cause/effect relationship with sputum inflammation and mediator expression. Descriptive studies alone will not suffice, however. For example, although my work supports the notion that neutrophils play a role in COPD pathogenesis, it illustrates also the deficiency of descriptive data in uniting inflammation demonstrated *in vivo* with its expected causes or consequences. *In vitro* studies are essential to explore theories in COPD pathogenesis, whether they are supported or not by descriptive work. Studies of neutrophil chemotaxis and activation have, for many years, been performed in COPD (147). Future studies might usefully employ such techniques to compare the behaviour of neutrophils taken from COPD subjects with and without emphysema to those taken from subjects with other neutrophilic airways diseases. Although there have only been a few bronchoscopic studies of severe asthma, a prominent finding is of an increase in neutrophil numbers in the large airway wall (140, 141) whereas this has not been found in the bulk of studies of COPD (78, 110, 114, 117, 157) or in my own study. As yet there is no proven explanation as to why submucosal neutrophil counts are frequently normal in biopsies of patients with COPD. Nor is it clear why, although neutrophils are strongly implicated in the pathogenesis of emphysema, similar pathology does not occur in the lungs of subjects with severe asthma, bronchiectasis (142) or cystic fibrosis (143), in which neutrophil influx is a recognised feature. *In vitro* comparison of the neutrophil chemotactic properties of sputum supernatants taken from subjects with each of these diseases, including current and ex-smokers with COPD, could be performed. Measurement of the responses of native neutrophils from such subjects to standard chemotactic stimuli could be carried out also. Such studies may help clarify whether neutrophil chemotaxis, as opposed to activity, is a discriminating factor and justify further

studies comparing levels of, not only IL-8 and GRO-α, but also other chemotactic factors in these diseases.

In vitro studies are likely, also, to provide further further insights into the intracellular pathways activated as part of the epithelial response to smoking. Stimulation of cultured epithelial cells with ErbB3 ligand, with measurement of MUC5AC production, could be performed, while selective inhibitors of ErbB3 activation could be used to examine further the role of this receptor in the generation of goblet cell metaplasia, in a similar manner to experiments using selective EGFR tyrosine kinase inhibitors (267). Such epithelial culture systems may also aid in the development of new treatments, as stimulation and blocking experiments may not only establish cause/effect relationships, but also allow testing of interventional strategies.

New treatments for COPD are much needed. Notwithstanding ever-stricter legislation on smoking, this is an activity that is unlikely to die out in the near future. Although our understanding of the pathogenesis grows, the key factors linking smoking with inflammation, remodelling and lung destruction remain uncertain and, thus, targeted therapies are not yet available. Hopefully my research will add significantly to the work of others in addressing this problem and improving our understanding of a disease that will continue to represent a major health burden for the future.

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