Understanding cellular changes and mediators of airway remodelling in COPD

By

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Publications

Original research articles:

1. **Mathew Suji Eapen**, Phil M. Hansbro, Kielan McAlinden, Richard Y. Kim, Tillie-Louise Hackett, Eugene H Walters, Sukhwinder Singh Sohal (2017). Abnormal M1/M2 macrophage phenotype profiles in the small airway wall and lumen in smokers and chronic obstructive pulmonary disease (COPD). Scientific Reports Volume 7 Issue 1: Article number 13392. DOI: 10.1038/s41598-017-13888-x.
2. **Mathew Suji Eapen**, Kielan McAlinden, Daniel Tan, Chris Ward, Eugene Haydn Walters, Sukhwinder Singh Sohal (2017). Profiling cellular and inflammatory changes in the airway wall of mild to moderate COPD. Respirology Volume 22 Issue 6: pages 1125-1132. DOI: 10.1111/resp.13021.
3. Ling Chen, **Mathew Suji Eapen**, Graeme R. Zosky. Vitamin D both facilitates and attenuates the cellular response to lipopolysaccharide. (2017) Scientific Reports Volume 7, Article number: 45172. DOI: 10.1038/srep45172
4. Madhur D. Shastri, Niall Stewart, **Mathew Suji Eapen**, Gregory M. Peterson, Nuri Guven, Sukhwinder Singh Sohal and Rahul P. Patel (2015) Opposing Effects of Low Molecular Weight Heparins on the Release of Inflammatory Cytokines from Peripheral Blood Mononuclear Cells of Asthmatics. Plos one Volume 10 Issue 3: pages e0118798.DOI: 10.1371/journal.pone.0118798.

Review articles:

1. **Mathew Suji Eapen**, Eugene Haydn Walters, Sukhwinder Singh Sohal. (2017). Airway inflammation in chronic obstructive pulmonary disease (COPD): a true paradox. Expert Review in Respiratory Medicine Volume 11 Issue 10: pages 827-839. DOI:10.1080/17476348.2017.1360769.

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Abstract/conference presentations:

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3. **Mathew Suji Eapen,** Philip Hansbro, Kielan McAlinden, Richard Kim, Tillie. L. Hackett, Chris Ward, Eugene H Walters, Sukhwinder Singh Sohal. Abnormal M1/M2 macrophage phenotype switching occurs differentially in the small airway wall and lumen in smokers and chronic

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6. **Mathew Suji Eapen,** Philip Hansbro, Kielan McAlinden, Richard Kim, Tillie. L. Hackett, Chris Ward, E H Walters, Sukhwinder Singh Sohal. Abnormal M1/M2 macrophage switching in small airway wall and lumen of smokers and COPD patients. **Poster Discussion; ERS Congress 2017.**

7. **Mathew Suji Eapen**, Elizabeth Tan, Chris Ward, Eugene H Walters, Sukhwinder Singh Sohal. Lysosomal-Associated Membrane Protein 1 (LAMP1) Upregulated in Small Airway Epithelium of Smokers and COPD. Role in autophagy. **Poster Discussion ATS Congress 2017.** (ATS/APSR travel grant award)

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9. **Mathew Suji Eapen**, Kielan McAlinden, Chris Ward, Eugene H Walters, Sukhwinder Singh Sohal. M1 and M2 macrophage populations in the airways of smokers and COPD patients: insights from both airway tissue

and lumen **TSANZ Poster Presentations. Respirology. 2017; Volume 22: page 101.**

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17. **Mathew Suji Eapen**, Kielan McAllinden, Malik Mahmood, Muller H, Sohal SS, Walters E. - Macrophage phenotype changes within the small airway wall in smokers and Chronic Obstructive Pulmonary Disease. **Poster Presented at ERS- Lung Science conference 2016** (ERS travel bursary)

18. **Mathew Suji Eapen**, Kielan McAllinden, Tan D, Muller H, Sohal SS, Walters E. Inflammation paradox - A detailed analysis of inflammatory profile in the airway wall of mild to moderate COPD- **Poster Presented at ERS-Lung Science conference 2016** (ERS travel bursary)

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Abstract

Background and Aims: Chronic obstructive pulmonary disease (COPD) is emerging as a substantial global health problem, with an estimated annual mortality of over 3 million people, which is the third largest disease cause worldwide. COPD is an irreversible chronic, slowly progressive airway obstructive respiratory disease. Cigarette smoking mainly causes it, primarily through small airway fibrosis, narrowing and ultimately obliteration, accompanied by more generalized but non-obstructive “chronic bronchitis” throughout the airways, and in some individuals complicated by the later development of lung parenchymal destruction (emphysema). Approximately 50% of smokers develop COPD eventually.

In COPD research, there has been a long-held belief that airway disease progression is due to “inflammation.” Although this may be true in the airway lumen with innate immunity activated by the effect of smoke or secondary to infection, the accurate picture for inflammatory cells in the airway wall, where the pathophysiological COPD remodeling occurs, is uncertain and especially so in mild to moderate COPD patient’s, i.e., earlier disease. This study follows up on a previous finding from our group of possibly decreased cellularity in the airway wall of patients with COPD. Thus, in the current studies, along with total airway wall cellularity, I evaluated the contribution of the main immune cells such as neutrophils, macrophages, mast cells, CD4 and CD8 cells in the large and small airway wall of mild-moderate COPD current (CS) and ex-smokers (ES), plus normal lung function smokers (NLFS), and compared them to non-smoker controls (NC). My studies further delved into the macrophage sub-populations in smokers/COPD and ascertained whether abnormal differential switching occurs in the macrophage population in the small airway wall compared to the lumen. I also measured macrophage-related cytokine profiles in airway luminal bronchoalveolar lavage fluid (BALF) and investigated whether the microenvironment dictated these changes. I evaluated cell dysfunction in small airway wall cells by assessing their degranulating potential via expression of a degranulating-marker lysosome-associated membrane protein -1 (LAMP-1).

While analyzing the CD68+ population of macrophages, I noticed that many cells staining strongly were not macrophages as expected, but spindle-shaped cells likely to be a population of fibroblasts; indeed the literature strongly suggests that CD68 can also stain fibroblasts, but this has until now been ignored in respiratory research.

I further focused on the non-inflammatory cell components in the airway wall, investigated the relationship between alpha-smooth muscle actin+ myofibroblasts with an expression of epithelial-mesenchymal transition markers (a focus of our group for several years), key extracellular matrix proteins (collagen I and fibronectin) and airway wall thickness. I also related abnormalities to lung function disturbance in COPD.

In the final phase of my Ph.D. studies, I also managed to complete some preliminary investigations on abnormal lysosomal accumulation and catabolic changes in the airway epithelium of COPD patients and related these to indices of airway remodeling.

Methodology: I evaluated both large airway endobronchial biopsies (ebb) and small airway lung resected tissues (RT) from COPD-CS and ES, NLFS and NC. I immune-stained with human anti-CD68+ for macrophages, neutrophil elastase for neutrophils, mast cell tryptase for mast cells, CD4+ and CD8+ for T cell populations. I conducted total cell counts as well as differential counts in the airway wall lamina propria (LP), epithelium and reticular basement membrane. For the large airways, I standardized cell counts in the LP by taking the depth of 150 for LA and up to 100 microns deep SA while excluding smooth muscle layers. Further, subpopulations of macrophages were evaluated in the SA wall (epithelium and LP) and BAL samples from the same cohort of patients and were stained with CD163 and Arginase-1 antibodies for detection of M2 macrophages and dual stained with anti-CD68+ and anti-iNOS antibodies for M1 populations (CD68+ only cells were designated M0). Cells were quantified and normalized as per mm^2 of SA wall and as per mm of length for the epithelium, respectively. Bronchoalveolar lavage (BAL) cytospin macrophages were similarly stained and enumerated by randomly selecting 12 fields and quantified as per ml of original BAL fluid extracted. BAL cytokines to profile for both M1/Th1 and M2/Th2 cytokines used FACS multiplexing strategies.

The degranulation capacity of mast cells and total degranulating cells in the small airway wall were measured by dual staining mast cells with anti-mast cell tryptase and LAMP-1. Epithelial and sub-epithelial LAMP-1 were assessed per area and represented as percent degranulation. I measured the total airway wall thickness of SA and divided it into the epithelium, reticular basement membrane (Rbm) LP, smooth muscle layer, and sub-mucosal adventitia, which meets the alveolar tissue. Further, I quantitated the α-SMA+ cells present in the SA lamina propria and adventitia, while the ECM markers (collagen-1 and fibronectin) were quantitated as a percentage of area staining in the same areas.

Results: I confirmed hypo-cellularity in the airway wall in both large and small airways in smokers and in COPD; with LA wall cellularity least in the COPD-current smoker (CS), while SA cellularity was similar across smoker/COPD groups. LA neutrophils were decreased in COPD-CS, while SA neutrophil counts were unchanged. In contrast, a small but significant increase was observed in SA CD8+ cells in both normal smokers and COPD-CS but not in LA. Ratiometric analysis of CD4+ and CD8+ T cells showed a dominance of the CD8+ phenotype in the LP area of LA, but not SA. Compared to controls, LA macrophage numbers in COPD were significantly lower, with SA macrophage numbers unchanged. Further evaluation of the SA macrophage subpopulations showed a significant increase in pro-inflammatory M1s in the small airway walls of NLFS and COPD compared to controls with a reciprocal decrease in M2 macrophages, which remained unchanged among pathological groups. However, luminal macrophages went the other way, with a dominant M2 phenotype in both NLFS and COPD subjects. BAL cytokine profiles were skewed towards M2 with an increase in CCL22, IL-4, IL-13, and IL-10 in both NLFS and COPD.

A decrease in degranulating mast cell and total degranulating cells was observed in the SA wall via monitoring intracellular LAMP-1 expression. Further, and unexpectedly, this lysosomal LAMP-1 expression was also found to be markedly increased in the epithelium of small airways in COPD. This increase in LAMP-1+ lysosomes significantly correlated with a decrease in lung function increased airway obstruction and increased in airway thickness.

Among the non-inflammatory cell populations characterized, I found a significant and marked increase in αSMA+ myofibroblast numbers in the small airway tissue in both smokers and COPD compared to normal controls, which directly correlated with decreased airway caliber measures in COPD patients. A significant increase was observed in the ECM proteins collagen-1 and fibronectin in the small airway wall of smoker and COPD groups. The proliferation of myofibroblast directly co-related to this increased collagen and fibronectin deposition and airway wall thickening, which suggested an active role in airway remodeling. The increase in the myofibroblast population also correlated with increases in expression of the EMT markers S100A4 and vimentin and lung function.

Conclusions: These current studies confirmed our group`s novel finding of hypocellularity in the airway wall of COPD patients, both in large and small airways. Analysis of the literature suggests that this has hardly been looked previously and probably not as rigorously as this investigation. These changes corresponded to decreases in most airway inflammatory and immune cells. Overall, the contribution of inflammatory/immune cells to total airway wall cells was small, again something else never previously documented, with the majority likely to be stromal cells. Macrophage subpopulation showed abnormal differential switching in both the airway wall and lumen, though in different directions. Lack of degranulation activity in the cells of the airway wall in COPD indicated dysfunctionality, which could be crucial in viral and bacterial infections known to be essential disease exacerbations in particular. A proliferation of myofibroblast was present in small airways and strongly associated with both remodelling, active EMT and loss of airflow in COPD.

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ACOS	Asthma COPD overlap syndrome
AECOPD	Acute exacerbations of COPD
AIHW	Australian Institute for Health and Welfare
AMs	Alveolar macrophages
ANOVA	Analysis of variance
ATS	American Thoracic Society
ATT	α1-antitrypsin deficiency
BAL	Bronchoalveolar Lavage
BOLD	Burden of Obstructive Lung Disease
CCL22	C-C motif chemokine 22
CD	Cluster of differentiation
CFU	Colony forming unit
COPD	Chronic Obstructive Pulmonary Disease
DAMPs	Damage associated molecular patterns
DALYs	Disability-adjusted life years
DLco	Diffusing capacity of carbon monoxide
DNA	Deoxyribonucleic Acid
DAB	Diaminobenzidine
EBB	Endobronchial biopsy
ERS	European Respiratory Society
FEV1	Forced expiratory volume in one second
FEF_{25-75}	Force mid-expiratory flow rate
FER	Forced expiratory ratio
FVC	Forced Vital Capacity
EMT	Epithelial-Mesenchymal Transition
ECM	Extracellular Matrix
GOLD	Global Initiative for Chronic Obstructive Pulmonary Lung Disease
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GPCR	G-protein coupled receptor
H_2O_2	Hydrogen peroxide
H & E	Hematoxylin and Eosin
HRP	Horseradish Peroxidase
ICS	Inhaled Corticosteroids
IL	Interleukins

IFN-γ	Interferon γ
Ig	Immunoglobulin
LA	Large airways
iNOS	Inducible Nitric oxide synthase
LLN	Lower limit of normal
LP	Lamina Propria
LRTI	Lower respiratory tract infections
MCP	Monocyte chemotactic protein
MHC	Major-histocompatibility-complex
MIP	Macrophage inflammatory protein
MMPs	Matrix metalloproteinases
MPO	Myeloperoxidase
mRNA	Messenger Ribonucleic Acid
NC	Normal control
NE	Neutrophil Elastase
NF-κB	Nuclear factor kappa B
NLFS	Normal lung function smoker
NLRs	Nod-like receptors
NO	Nitric Oxide
O2-	Superoxide Anions
OR	Odds ratio
PAMPs	Pathogen-associated molecular patterns
PEF	Peak expiratory flow
PM	Particulate matter
PRRs	Pattern recognition receptors
Rbm	Reticular Basement Membrane
ROS	Reactive oxygen species
RR	Relative risk
RT	Resected tissue
SA	Small airways
SAD	Small airway disease
SE	Standard error
SES	Socioeconomic status
SHS	Second-hand smoke
TAHS	Tasmanian Longitudinal Health Study
TGF-β1	Transforming growth factor-Beta1

TLRs	Toll-like receptors
TNF-α	Tumour Necrosis Factor-Alpha
WHO	World Health Organization

Chapter 1

COPD: An introduction

1.1 Respiratory Tract: Normal anatomy and histology

The respiratory system does the critical function of acting as a portal for oxygen in the air and exporter of carbon dioxide, so facilitating the trillions of cells in the human body to carry out the fundamentally vital function of respiration. It consists of conducting airways, which extend from the nose to lung parenchymal alveoli. Thus, the nose, pharynx, larynx, trachea, bronchi, and bronchioles in series functionally connect the external environment with the internal extensive thin and delicate alveolar surface. The whole neuro-respiratory system coordinates four vital processes: pulmonary ventilation (breathing), gas exchange in the alveoli requiring optimal ventilation/circulatory matching, blood transport of respiratory gases (lung to tissue/cells and back), and finally internal cellular respiration at a mitochondrial level (Scanlon and Sanders, 2007). The intricate details of all these processes and their control and integration are beyond the scope of this review, and I will focus initially mainly on the structure of the respiratory system as it relates to function.

A respiratory system is functionally a single unit that can be divided into upper and lower respiratory tracts. As outlined above, the gas-conducting upper respiratory tract consists of parts outside the chest cavity - nasal air passages, nasal cavities, pharynx, larynx, and upper trachea, while the lower respiratory tract consists of lower trachea, approx 23 generations of branching bronchial tree (large airways defined as >2mm in diameter, and small airways <2 mm in diameter). The final bronchioles being tiny airways gradually merge with the gas-exchanging clusters (acini) of the alveoli (Kerr, 2010).

Histologically, the mucosa of the upper respiratory tract consists of, from the outside to inside, a luminal/superficial ciliated epithelium lining in contact with the atmosphere and attached to a basement membrane deeply, a denser connective tissue reticular basement membrane beneath that which fuses into

a less dense connective tissue lamina propria, then a circumferential smooth muscle bundle layer, and below this the adventitia of looser connective tissue containing supporting cartilage structures, and mucous glands containing mucus secretory cells which empty into ducts which join to find their way out to the lumen through the other more superficial structures. The adventitia itself in the lower airways gradually fuses with the lung parenchyma and provides basic anchoring points for the alveoli, which provide elastic and supporting interactions between the parenchyma and airways for optimal airflow.

The structural details of the airways change as they transition into the smaller airways - cartilage, goblet cells, smooth muscles and connective tissue gradually diminish, and are completely absent in the terminal gas-exchanging respiratory bronchioles and alveoli (Figure 1.1).

Ciliated epithelial cells persist as far down the bronchial tree as the bronchioles, while goblet cells are replaced by non-ciliated dome-shaped club cells, present intermittently along the bronchiole epithelial lining. The club cell carries out the vital function of secreting surfactant proteins (Kerr, 2010).

The connective tissue across the airway wall have large numbers of resident interstitial stromal cells, mainly comprised of fibroblasts. Fibroblasts produce extracellular matrix protein (ECM) such as collagen, fibronectin and various varieties of proteoglycans which includes versican, desmin among other, which are vital to maintaining airway stiffness/ elasticity for their mechanical function (Burgess et al., 2016). Thus, there lies a huge potential for variability in all these structural and cellular composition across the airways (and lung parenchyma) that has enormous implications for disease states, which underlies the core interest of my thesis work. For instance, thickening of reticular basement membrane – one of the two structural layers of the bronchial epithelial basement membrane – is usually observed in asthma characteristically though our research group has also shown significant changes in COPD (Figure 1.1).

Figure 1.1 Pictorial illustrations of the central compartment and their sub-divisions of the human lung.

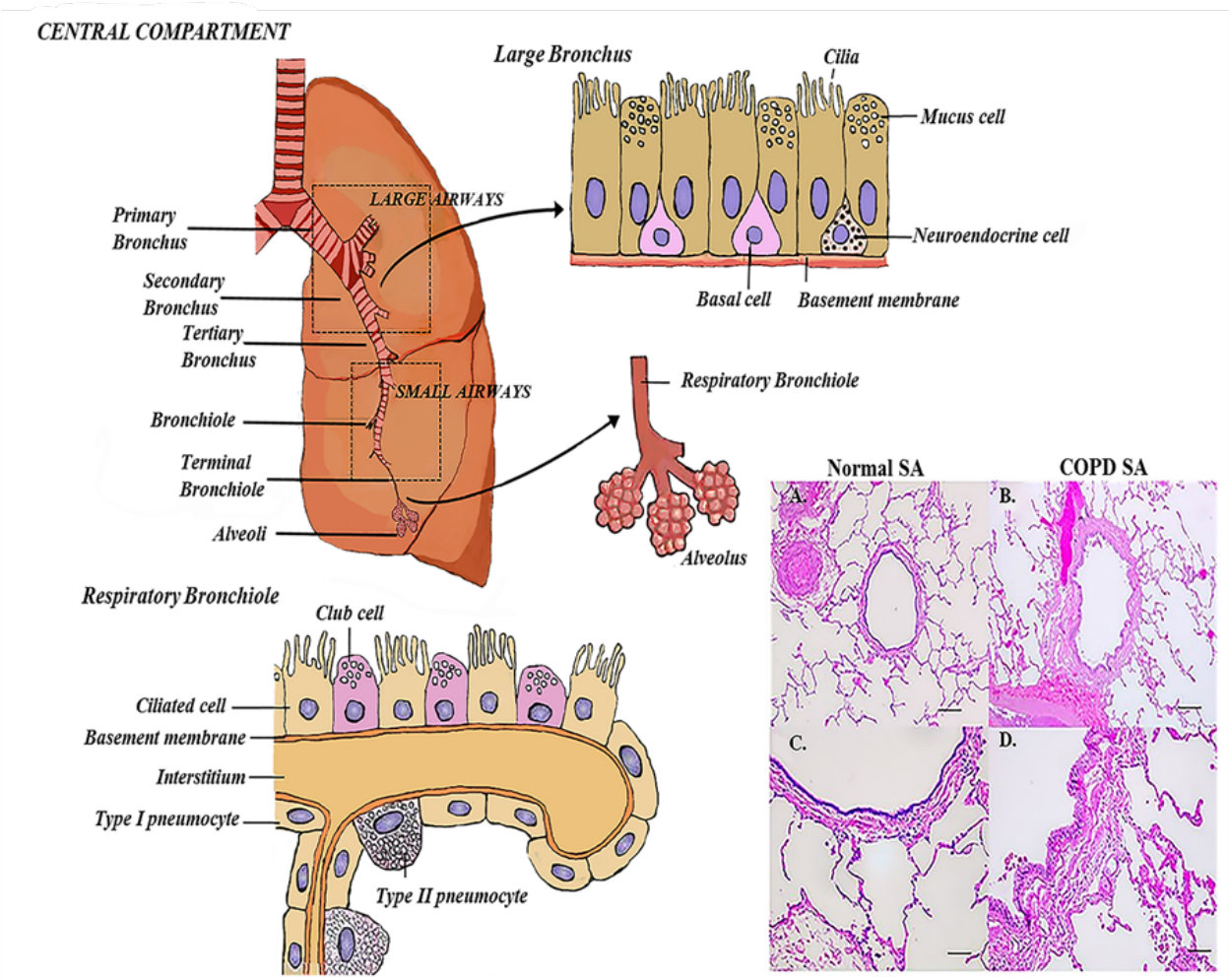


Figure 1.2 Schematic adapted and modified from Sun et al. Nat Rev Cancer 2007 titled 'Lung cancer in never smokers: a different disease' shows the central compartment (top left), and structure of large bronchus (top right) and respiratory bronchioles (bottom left). Histological representation of thin-walled small airways (SA) (A and C) from a normal non-smoker, thickened in COPD (B and D) (bottom right). A, B at 50X, and C, D. is at 200X magnification.

1.2 Characteristics of COPD

1.2.1 Clinical

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) has defined COPD as a disease state characterized by airflow limitation (obstruction) that is not entirely reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the `lungs` to noxious particles or gases" (GOLD 2015) (Vestbo et al., 2013), by far the most common of these in Western countries being cigarette smoke. This "abnormal inflammatory response" is instead an ambiguous and vague term, which in fact is a matter that I have dealt with in depth in this thesis, at least as far as the airway wall is concerned.

Common signs and symptoms of COPD include especially progressive shortness of breath, but also commonly chronic cough and mucus hypersecretion. The major pathologic changes of COPD are observed in the airways, at least in its early stages in most cases. Lung parenchyma and pulmonary vasculature also go on to show significant pathological manifestations in many patients at a later stage in COPD natural history (Thurlbeck et al., 1968).

The landmark study by James Hogg, working as a young researcher in Peter Macklem`s lab in Montreal, from the late 1960s, suggested that the earliest pulmonary changes relating to airflow obstruction in smoking-related COPD occur mainly at the level of the small conducting airways (Hogg, Macklem & Thurlbeck 1968). Briefly, what have been again somewhat vaguely termed "various cellular and biochemical processes" result in progressive fibrosis and thickening of the airway wall in the small airway with luminal narrowing, progressing to small airway obliteration (~50% gone before conventional lung function deteriorates) (Belperio et al. 2003; Hogg et al. 2004; Hogg, McDonough & Suzuki 2013). These changes gradually increase airway resistance and eventually show up as classic airflow obstruction. Subsequent experimental measurements suggested that as high as 30-40% of airway

resistance could be attributed to small airways (Takishima, Yanai & Sasaki 1991), which is considerably higher than what had been estimated earlier (~20%) (Hogg, Macklem & Thurlbeck 1968).

More generalized throughout the airway wall in COPD, but also in smokers without COPD, is remodelling in the form of epithelial goblet cell hyperplasia ("bronchitis"), epithelial squamous metaplasia and submucosal gland hyperplasia in a majority of patients. This aspect of COPD was very much the focus of clinical and epidemiology research in the 1950s and 1960s with some excellent researchers such as Prof Lynne Reid in London, but it took some time to realize that these changes are not central to functional abnormalities on spirometry, although they may have a symptomatic impact.

Emphysema is the destruction of the lung parenchyma, i.e., the alveolar lung compartment, leading to loss of elastic recoil forces and so airway tractional support and luminal pressure during expiration, so adding to airflow obstruction due to pre-existing intrinsic airway fibrosis and luminal narrowing. This develops only in a subset of individuals, usually as a secondary phenomenon. Thus, the dilatation and destruction of the gas-exchanging tissue beyond the terminal bronchioles are termed "emphysema" (McDonough et al., 2011). Emphysema is defined in pathologic terms as "alveolar wall destruction with irreversible enlargement of the air spaces distal to the terminal bronchioles and without evidence of fibrosis" (Snider et al., 1985), and in general, occurs a decade later than airway narrowing (Dunnill et al., 1969). Morphologically, there are two main subtypes of emphysema (Kim et al., 1991). The centrilobular (or centriacinar) emphysema is more closely associated with cigarette smoking, is usually most marked in upper lobes. The emphysema subtype causes more severe small airways obstruction, because of loss of alveolar-airway support points. Panlobular (or panacinar) emphysema is mainly associated with α1-antitrypsin deficiency, resulting in an even dilatation and destruction of the entire acinus, and especially in the lower lobes (Litmanovich et al., 2009), which leads especially to early loss of lung gas diffusion capacity and ventilation/perfusion mismatching with exercise-related breathlessness being the primary symptom as a result.

Changes in the pulmonary vasculature include intimal hyperplasia and smooth muscle hypertrophy/hyperplasia, likely attributed to be chronic hypoxic vasoconstriction of the small pulmonary arteries (Harkness et al., 2014), although this is likely an over-simplification with loss of pulmonary capillary capacity and also endothelial activation occurring. Thus, emphysema especially can lead to loss of the associated areas of the pulmonary capillary bed, and some have said that this is a primary pathology in causing emphysema.

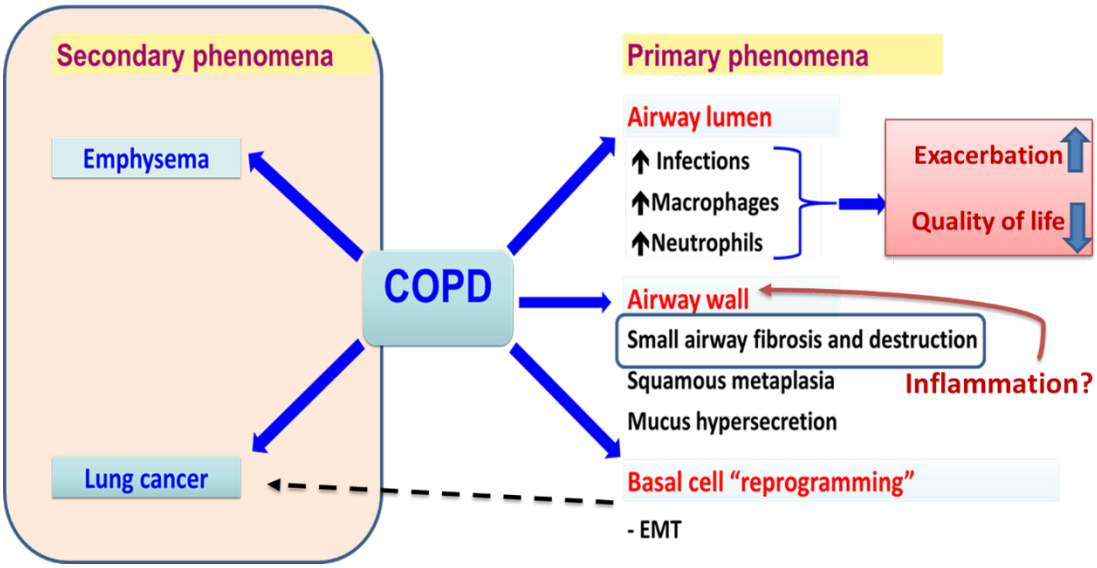


Figure 1.2 Clinical characteristics of COPD.

In summary, the primary pathophysiology in smoking-related COPD, including fibrosis and obliteration of small airways, associated temporally with other airway remodelling changes such as squamous metaplasia, mucus hypersecretion and goblet cell hyperplasia in large airways. Basal cells in the airway epithelium are increasingly regarded as extremely important in all of these aspects of airway remodelling, one manifestation of which is a feature that our group described and had focused on, namely epithelial-mesenchymal transition (EMT), type II or III, categorized on the basis of presence (type III) or absence (type II) of angiogenesis. We feel that this is a fundamental process in

airway fibrosis and obstruction and I will overview the literature on this later, and come back to it repeatedly in the experimental sections of my thesis.

I have already mentioned that there is a dogma reflected in international position papers and initiatives, e.g., GOLD definitions, that airway "inflammation" is a core feature of COPD. I will be dealing with this aspect also in great detail later, but Fig 1.2 summarises what my research group felt was the provisional truth about inflammation and airway pathology in general when I began my work, and it has been a model that I have been much guided by. It emphasizes that innate inflammatory activation is really a proven feature only of the airway lumen in COPD, with marked increases in macrophages and to a lesser extent neutrophil (and sometimes eosinophils) in this compartment, perhaps driven by oxidant stimulation and chronic infection. The high vulnerability of the airways to infection and chronicity of infection with particular viral and bacterial organisms have been the subject of the thesis and published papers from a co-PhD student, Dr. Shakti Shukla, within the CRE (Shukla et al., 2015). I am not going to deal with this further, except to say that these infective manifestations of COPD, and the significant associated exacerbations of COPD clinically which tend to occur increasingly frequently as the disease progresses. All these manifestations are the consequence of abnormally activated epithelium that induces infection and inflammation in the epithelial and luminal airways, thus forming a major feature of this complex disease. However, my work has dealt with the nature of cellularity in the airway wall itself, rather than the airway lumen, and this will remain the almost exclusive focus of my discussions.

1.2.2 Historical developments in understanding COPD

Some early clinical finding relating to COPD were reviewed by Thomas PL Petty in 2006. The most prominent observations were: Badham C in 1814 was the first to provide a clinical description on "chronic bronchitis," a major symptomatic component in COPD patients. He described the presence of "bronchiolitis" as well as chronic bronchitis as disabling disorders (Badham, 1814, Petty, 2006). Laënnec (1821) in his book titled "Treatise on the diseases

of the chest" was the first to describe the emphysematous lung along with the presence of chronic bronchitis (Laënnec, 1821). The Spirometer as the key instrument in the physiological diagnosis and management of COPD was first invented by John Hutchinson in 1846 and further developed a hundred years later by Tiffeneau in 1947 (Tiffeneau,1947). Although there were considerable clinical advances in the late 19th and early 20th century, it was not until the mid-20th century that COPD was more clearly defined in modern terms

Two symposia played a critical role in defining the disease, the CIBA Guest Symposium in 1959 (Donald, 1971) and the American Thoracic Society (ATS) meeting on diagnostic standards in 1962. While the former delved into defining and classifying emphysema, the latter provided a key understanding of the components of COPD with "chronic bronchitis" defined in clinical terms as a persistent cough that last for three months for a two-year duration, and emphysema in anatomic terms, as enlarged alveolar and the loss of alveolar walls. Though there were no physiological parameters considered, these statements have provided strong foundations in how we now define the disease today, as I did earlier. William Briscoe first coined the term COPD (Chronic Obstructive Pulmonary Disease) itself in 1965 in a presentation at the 9th Aspen Emphysema Conference (Briscoe and Nash, 1965), although other variants such as Chronic Obstructive Airway or Lung Disease (COAD/COLD) have been in vogue at different times. The term COPD has the advantage of being deliberately open about where the obstruction is anatomically and how caused, i.e., instead presumes a mix of airway fibrotic obstruction and emphysema, but also suffers from not emphasizing the core airway component.

Lynne Reid in 1960 was the first to provide pathology measurement criteria for COPD called the Reid Index (Reid, 1960), which was a ratio of the mucous glands to the thickness of the bronchial wall. Later on, in a book authored by her, titled "The pathology of Emphysema" she makes a clear distinction between airway bronchitis and anatomic emphysema (Reid, 1966). However, she missed the central role of small airway narrowing/fibrosis rather than the cause of symptoms in her emphasis on mucus production. Dunhill,1969 further developed the field by emphasizing with the pan airway nature of the disease and its mixed pathology and suggested studying both airway and lung components to establish the pathogenesis of the disease

(Dunhill, 1969). Macklem and Mead were first to define the role of small airway pathology in COPD, and their study was based on a novel method developed using a specialized catheter to provide direct measurements of the resistance in peripheral airways with less than <2 mm in diameter (Macklem and Mead, 1967).

Since the 1960's research into COPD substantially lost out to interest in asthma, at least for 3-4 decades as the key airway disease. However the last decade (partly due to the increased smoking-related disease and co-morbidities) has seen a surge in focused research around COPD.

1.2.3 Clinical classification of COPD

1.2.3.1 *Measurement of Lung Function*

The symptoms of COPD are a cough, sputum production and chronic progressive dyspnoea as guided by the GOLD standards. Hyatt and Black introduced the obstructive index ratio also called forced expiratory ratio (FER), which essentially is described as the ratio of forced expiratory volume in 1 second (FEV_1) to the forced expiratory vital capacity (FVC) (Hyatt and Black, 1973a, Hyatt and Black, 1973b). Spirometry has been successfully used for the physiological assessment for determining lung function, and it is a safe, non-invasive, reproducible breathing test. Spirometry is an essential pulmonary function test (PFT) and can be performed to assess bronchodilators responses before and after their administration. The test can thus be used to confirm whether airflow limitation is wholly or partially reversible. An FER of <0.7 confirms the presence of airway obstruction, while FEV_1percentage predicted is used to classify COPD severity (mild ≥80 percentage, moderate 50-80%, severe 30-49%, very severe <30%) (Table 1).

Aging also affects FEV_1 and FER, which remains underdiagnosed using a fixed ratio method in COPD patients (Ito & Barnes, 2009). For smokers who are having more than ten pack-years the decline in FER is much more rapid and twice higher than for healthy non-smokers (Rennard, 1998). Further, in healthy people, the lower limit of normal (LLN) for FER is dependent on the age and thus requires to be accounted in COPD patients (Hankinson et al., 1999).

One other disadvantage of the traditional PFTs is that evaluation of patients are usually done at rest, and at best provide only baseline pulmonary performance. A more modern cardiopulmonary exercise testing (CPET) is a potentially alternative for PFT as the preferred predictor of actual lung function. CPET is primarily employed in sports physiology to analyze endurance capacity in elite athletes; they are sophisticated physiologic testing technique capable of measuring multiple physiological parameters in an exercise environment. The instruments measure maximal or peak oxygen consumption (VO2max), Respiratory exchange ratio (RER), minute ventilation (VE) and Anaerobic Threshold (AT) which is calculated from data collected from CPET gas analysis (Suji Eapen et al., 2016). These sophisticated instruments are highly priced and are less likely affordable in every hospital facility.

Table 1. Staging of disease severity in COPD patients

Disease Severity	(FEV_1 Predicted)			
	ATS	ERS	BTS	GOLD
Stage 0: at risk	—	—	—	Normal spirometry; Chronic symptoms (cough, sputum production)
Stage I: mild	≥50%	≥70%	>80%	≥80% with or without chronic symptoms
Stage II: moderate	35%-49%	50%-69%	31%-80%	50%-79% with or without chronic symptoms
Stage III: severe	<35%	<50%	≤30	30%-49% with or without chronic symptoms
Stage IV: very severe	Terminally ill			<30%

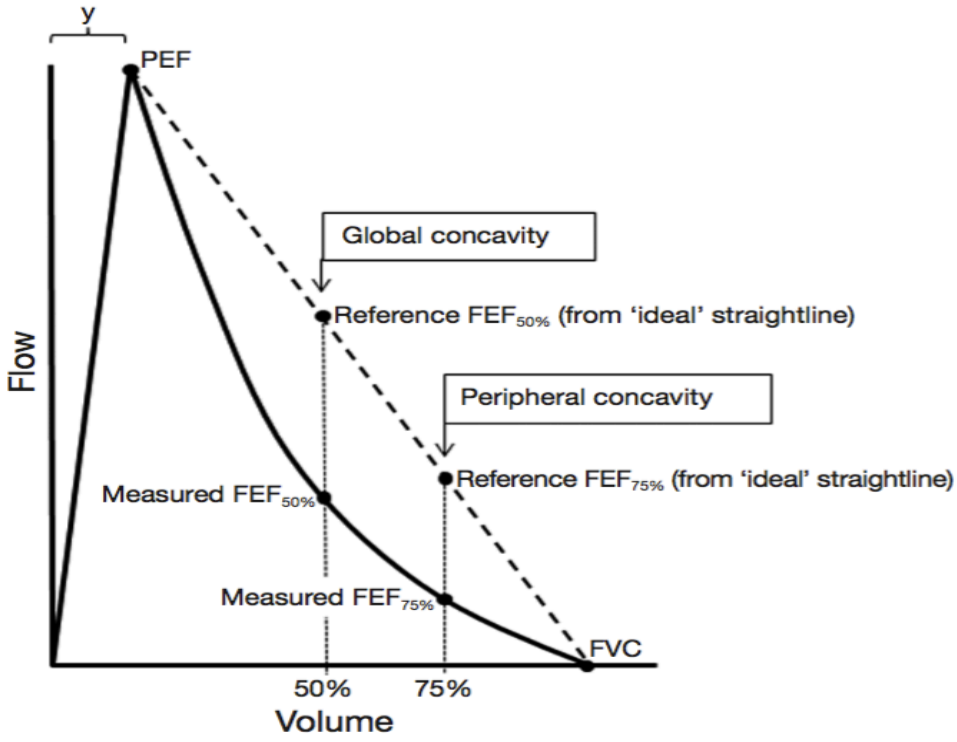
FER < 70%

FEV_1- forced expiratory volume in 1 second; ATS- American Thoracic Society; ERS- European Respiratory Society; BTS- British Thoracic Society; GOLD- Global Initiative for Chronic Obstructive Lung Disease.

1.2.3.2 *Measurement of Airway obstruction*

Although FER remains the standard measurement for measuring lung flow capacity, there has been increasing recognition that small airway represents a quiet zone in the normal lung where the disease can persist. This would possibly help predict early detection of the disease before symptoms are noticed and when routine test is abnormal. Currently, the measure of small airway dysfunction by spirometry is determined by measuring the average forced expiratory flow over the middle half (50%) of the FVC ($FEF_{25-75\%}$) and forced expiratory flow at 75% of the FVC ($FEF_{75\%}$) (McFadden and Linden, 1972). However, the clinical relevance of these measures has been debated recently by Quanjer et al., (2012) who found no difference in the convention indices to that of $FEF_{25-75\%}$ measurements. Recently, however, a new method for accurately analysing the concavity in the descending expiratory limb of the routinely used flow-volume curve. Such concavity is a hallmark of small airway obstruction, and this new analytical technique has been reported to improve the detection of fixed small airway obstruction by 25% in community-based samples (Figure 1.3) (Johns et al., 2014). Other recent technology developments have used forced oscillations of air at the mouth into the respiratory tract as a convenient way to detect small airway abnormalities. This method uses air pressure-wave impedance oscillometry , and includes a forced oscillation during tidal breathind and impulse oscillometry which are both now entering clinical practice (Hogg et al., 2017).

Figure 1.3. Variables used to quantify global and peripheral concavity.



A novel way of interpreting the expiratory limb of the F-V curve. Measured $FEF_{50\%}$ and measured $FEF_{75\%}$ are the forced expired flows when 50% and 75% of the FVC has been expired. Reference $FEF_{50\%}$ and Reference $FEF_{75\%}$ are the references flows that would be obtained if the flow-volume curve had zero curvature, i.e., a linear descending limb (dotted line). The variable, y, is the volume of peak expiratory flow (PEF); a value of 0.6 L can be assumed for this. In this example, the global concavity is approximately 50 Units, and the peripheral concavity is approximately 65 Units. Such an analysis may markedly improve the detection of early COPD (by perhaps 25%).

The graph and the legends are reproduced from Johns, Walters, and Walters (Johns et al., 2014).

1.3 Epidemiology of COPD

1.3.1 Burden of COPD (Global)

COPD, which was ranked the sixth leading primary cause of death in the 1990s, increased rapidly in prevalence to gain the fourth position by 2000 and is now being estimated to rise to be in third place by 2020 (Mathers et al., 2006, Lopez et al., 2006).

The Burden of Obstructive Lung Disease (BOLD) initiative was designed to develop standardized, objective methods for estimating COPD prevalence worldwide that would be practical for use in countries with different economic development profiles, and also to then estimate the economic burden of COPD (Buist et al., 2005). This showed that the prevalence of COPD increased with age and pack-years of smoking (multiples of 20 cigarettes per day for 1 year, which is one pack-year), although other risk factors, such as use for biofuels for internal home heating and/or cooking, occupational and environmental dust/fume/gas exposures and airway infections, could also contribute significantly to COPD in a location-specific way (Buist et al., 2007). Halbert et al., (2006) conducted a systematic review with random effects of meta-analysis to quantify the global prevalence of COPD. The overall prevalence of physiologically-defined COPD by GOLD criteria in adult's ≥40 year of age was again approximately 9–10%. The authors also reported the prevalence of chronic bronchitis, i.e., a chronic cough and sputum production alone as 6.4% from 38 studies and for emphysema alone as 1.8% from eight studies.

From all these studies, COPD prevalence has been found to range from 0.2% in Japan to 37% in the USA, but varied widely across the populations reported, with variations dependent on diagnostic methods and groups reported. The burden of COPD was more commonly reported in older populations, socially with median population age greater than 75 years. Although, the prevalence of COPD has increased over time, this rate of increase has declined in recent years, particularly among male populations. Globally, COPD affects

approximately 329 million people, which accounted for nearly 5 percent of the total world population at the time assessed (Vos et al. 2010)

Respiratory diseases, which includes airway diseases such as asthma and COPD, are the third most lethal non-communicable diseases (NCDs) causing 4.2 million deaths globally in 2008 and are expected to become even more so in the quite near future (WHO 2014). COPD alone resulted in 2.9 million deaths in 2013, which increased from 2.4 million deaths in 1990, in data published as an international collaborative global burden of disease project ('Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013' 2015). Also, these figures do not take into account the death burden from lung cancer, which is the most lethal malignancy now in both men and women (5-year survival only about 15%), and increasingly understood as closely associated with COPD rather than just smoking as such.

1.3.2 National burden of COPD (Australia)

Interestingly in Australia, the death rate due to COPD has approximately halved between 1979 and 2011 among males, although it was increasing among females before now starting to decline (Figure 1.4). Furthermore, an increase in death rates, almost 2.5 folds higher are observed among the indigenous community with COPD compared to that of the non-indigenous population according to AIHW 2012, while this is more prevalent in the rural areas where poor socio-economic factors could be the reason.

Although there is a declining trend in COPD (mainly due to ongoing governmental and non-governmental initiative using various other social awareness programmes), up until 2013, it is still one of the leading cause of death and disease burden after heart disease, stroke, and cancer. Again, remembering that much of cancer mortality is related to COPD, though not currently considered in the national statistics. Lung cancer and chronic obstructive pulmonary disease (COPD) coexist in smokers, and the prevalence of COPD increases the risk of developing lung cancer by 4-5 folds, even after

smoking history is controlled (Eapen et al., 2016). The major risk factor for COPD in Australia is cigarette smoking, as for like other developed and now increasingly for under-developed countries, COPD as of 2014 is the fifth leading cause of mortality and caused 7,025 in this year alone which was 4.9 percent of the total deaths in Australia (AIHW, 2014). An estimated 750,000 Australians have COPD, which has progressed to a stage, such as breathlessness may already be, affecting their daily lives (Toelle et al., 2011). While half of these people do not have a doctor's diagnosis of COPD and are therefore not taking the essential steps to slow down the progression of the disease.

The median age of death from COPD was reported to be 81 years in 2007–2011, which suggests that perhaps there is not too much to worry about. However, more recently convincing data from the Sax Institute's 45 and up study found that two-thirds of the deaths in existing smokers could be directly attributed to smoking, which is higher than the previous international estimates of 50%, and in addition, current smokers are estimated to die an average of 10 years earlier than non-smokers (Banks et al., 2015). The age of 45 seems to be something of a watershed, such that if a smoker stops smoking before then, they come out mostly unscathed, but beyond that, the real trouble starts. This study also emphasized the substantial morbidity in the 20-40-year group from smoking-related anxiety and depression.

The Australian limb of the BOLD study estimated that approximately 14.5% (one in seven) of Australians >40 years have airflow limitation/obstruction, which increased to 29.2% in the >75 years. Moreover, around 7.5% of Australians >40 years have subjective COPD-type symptoms, and it was estimated that about half of COPD was not diagnosed (Toelle et al., 2013).

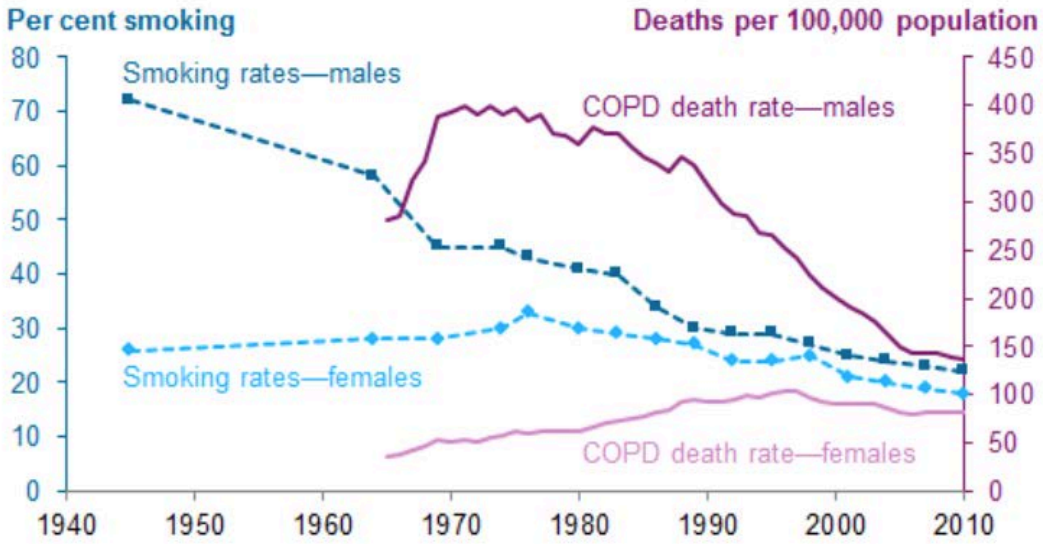


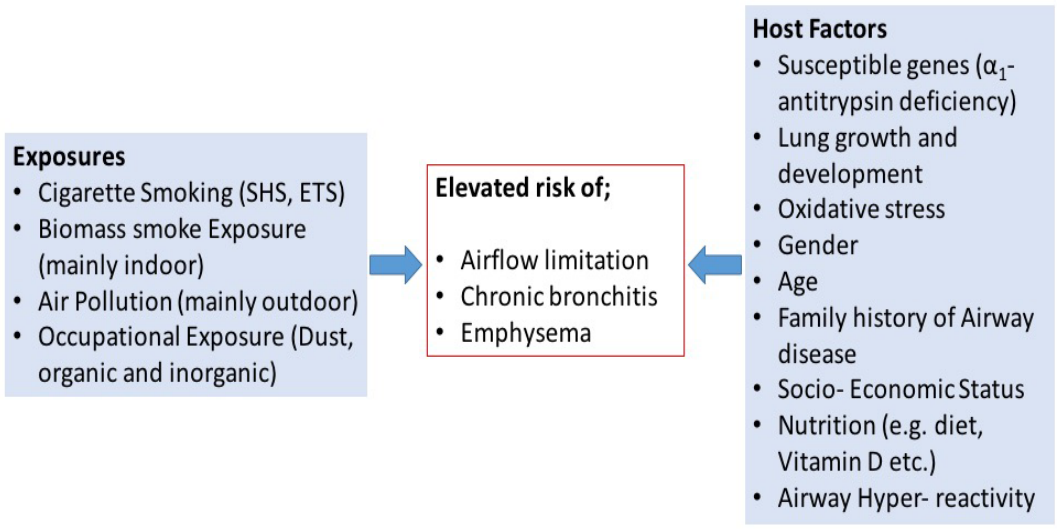
Figure 1.4 COPD death rates in Australia related to smoking rates. The data is presented on 3-year moving averages. Smoking rates and death rates are obviously closely linked, albeit with about a 20-year lag. *Source: (AIHW 2012).*

COPD is more expensive per case than cardiovascular disease (CVD) and exerts substantial economic burden on Australia. In fact, COPD ranks second in the most number of inpatients being hospitalized (Glover J, 2007). However, the major financial costs in COPD which are estimated to $8.8 billion, is due to the loss of productivity due to COPD lower employment, absenteeism and the workplace impact of the premature death of Australians with COPD. The direct cost to the Australian health care system is estimated to be $900 million. In addition to the above costs on the public and private sector purse, there are the costs that are harder to quantify – those of lost wellbeing because of COPD.

1.4 Risk Factors associated with COPD

There are several risk factors associated with COPD patients which can thus be broadly classified as external exposures and host-related (Figure 1.5). Exposures include those from cigarette smoking, biomass exposure, air pollution and occupational hazards among others. However, host-related such as (genetic) factors predisposing to lung pathology and other factors mentioned in the figure below are extremely difficult to control. These factors are briefly summarized here, with a significant focus on cigarette smoke exposure.

Figure 1.5 Known risk factors for COPD.



The pulmonary function (FEV_1), which that increases during childhood and adolescence up to the age of 18–22 years, then plateau before declining during a normal ageing process (Eisner et al., 2010). Various factors that affect the average lung growth phase lead to decreased maximal lung function and this is an area under active current investigation, e.g., by the Tasmanian Longitudinal (Respiratory) Health Study (TAHS) which is closely aligned with our group. Of importance, both in the TAHS and elsewhere it has become increasingly recognized that reduced lung function in children leads to poorer maximal lung function and is a significant contributor to diminishing lung

function in adults (Bui et al., 2017). In this section, I will focus more on factors that cause more rapid subsequent decline from whatever the maximal attained.

1.4.1 Cigarette Smoking

Currently, World Health Organisation (WHO) has estimated that on an average there are about six million annual deaths due to tobacco use (both smoking and smokeless). According to the projection by Bilano et al., globally there are approximately 1.1 billion daily smokers, which are estimated to increase to 1.6 billion daily smokers by 2025 (Bilano et al., 2015). The authors further observed that there were huge disparities among the countries in tobacco consumption rates and many were not on track to achieve tobacco control targets and further in low-income and middle-income countries, there is an increased risk of worsening tobacco epidemics. Similar projections by earlier by Mathers and Loncar, (2006) -also attributed tobacco-related deaths per year to increase at an alarming rate from 5.4 million in 2005 to 6.4 million in 2015, which could further rise to 8.3 million by 2030. However, as mentioned, the picture among countries remains mixed, with tobacco-related deaths in more developed nations projected to decline by estimated 9% between in 2030 , while at the same time will double from 3.4 million to 6.8 million in low- and middle-income countries (Mathers and Loncar, 2006). The difference can be attributed to the lack of education, poor awareness of the risk of smoking and an ineffective government tobacco control policy in place in these nations. Direct or indirect exposure to cigarette smoking is by far the main casual factor for COPDs; Forey et al., (2011) in a systematic meta-analysis review of 218 studies found that smoking history had a direct impact on all three symptomatic outcomes COPD, chronic bronchitis and emphysema. Interestingly, the decline in lung function and mortality rate did not mitigate even after COPD subjects quit smoking,

The indirect effects of smoking are significant, especially for children. A recent study evaluating participants from the Tasmanian Longitudinal Health Study cohort (TAHS) concluded that children exposed to extensive maternal smoking (smoking history greater than twenty pack per year) during their childhood increased the prevalence of post-bronchodilator (BD) fixed airflow obstruction

in their middle-age. (Perret et al., 2016). The effects of parental smoking on children are multiple: these children themselves are more likely to become long-term adult smokers; they have lower childhood and so lower maximal early adult lung function, and on both counts they are likely to have accelerated loss of lung function in middle age (Perret 2016, plus more recent unpublished TAHS data)

The death rate among current smokers was found to be two to three times higher than that of non-smokers but taking into account other deaths caused by disease associated with current smokers involving heart, kidney, liver, intestine, infection and various cancers, the increase is further substantial. However, among former smokers, the relative risk for mortality had reduced as the number of years since quitting increased (Carter et al., 2015). Moreover, compared to the smokers and ex-smokers the decline in lung function (FEV_1) in current smokers remained persistent over time as demonstrated a (figure 1.5). This thus emphasizes the need for early smoking cessation for patients with chronic conditions such as COPD (Vestbo et al., 1996).

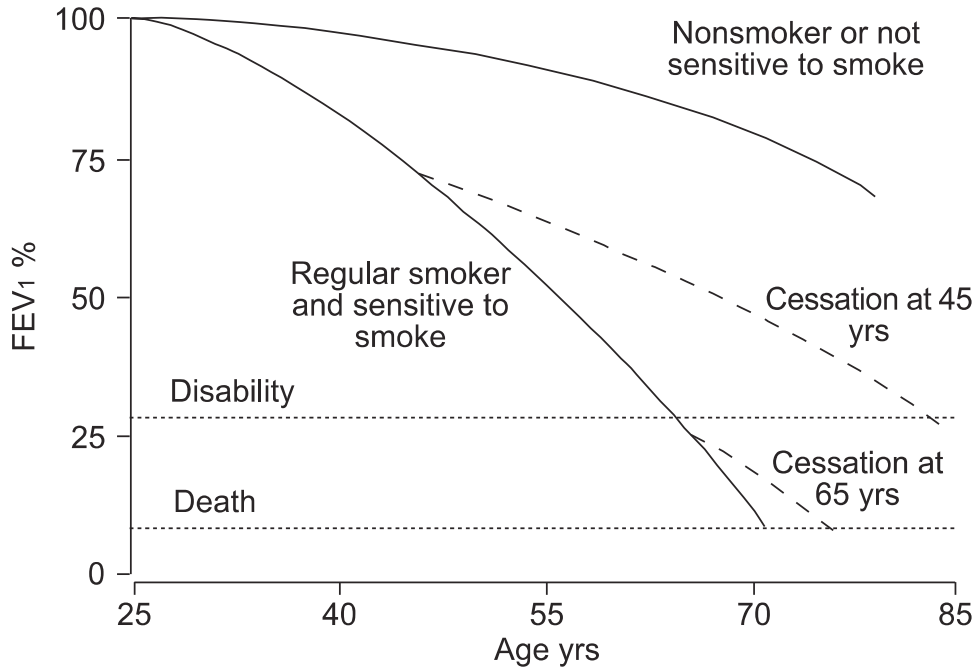


Figure 1.6 Decrease in forced expiratory volume in 1 second, according to smoking status (*Reproduced from Fletcher & Peto (1977).*

The strategies for smoking cessation at a personal and community level are beyond the scope of this account, but a combination of limiting access to tobacco (e.g., price), public media campaigns, quit facilities including reasonable access to nicotine replacement therapy and other pharmacological aids, and potentially limiting supply (e.g., Tobacco Free Generation legislation in Tasmania) are all critical (Walters et al., 2015).

1.4.2 Environmental exposure to particulate matters (air pollution)

Particulate matter (PM) can be characterized by the size of the particulates. The PM mass suspended in the atmosphere which is on average 10 microns or less in diameter (PM10) generated from plants, tree pollens, road dust, agricultural outputs, and industrial processes that generate smoke and dust. Smaller particles that are on average 2.5 microns in size are generated by vehicular pollution, industrial pollution, natural burning of biomass fuels and forest fires; these particles especially remain suspended in the atmosphere for a long period and can be carried by the winds for long distances (Hogg et al., 2017).

The PM deposition into the surface of the lung is also mainly dependent on particle size. Thus, larger particles of PM10 tend to settle more towards the central areas of the lungs while particulate matter with an average diameter of 2.5 penetrate deeper into the airways and can initiate deleterious effects on the small airways, including increased oxidative stress and inducing pro-inflammatory cytokines. They may also impair the phagocytic capacity of macrophages (Donaldson et al., 2002).

Further, it has been observed that in COPD patients when exposed long-term to the airborne particulate matter had an increased risk of premature deaths and acute care hospitalization (Zanobetti, Bind & Schwartz 2008; Sunyer 2001). The mechanisms of airway obstruction due to air pollution are likely to be the similar to those due to cigarette smoking, but there is limited mechanistic evidence for this.

1.4.3 Occupational exposure

Occupational exposures are most thought about regarding interstitial lung disease (the Pneumoconioses) or asthma development. However, several longitudinal studies show an association between occupationally generated dust exposures and COPD. For example, a Swedish cohort of construction worker exposed to dust had increased mortality rates than those unexposed; cigarette smoking and aging were found to further enhance these effects (Toren and Jarvholm, 2014). A population-based study found positive associations between several occupational exposure measures (mineral dust, metal dust and fumes, organic dust, irritant gases or vapors, sensitizers, organic solvents, diesel exhaust, and environmental tobacco smoke) with COPD, among both ever-smokers and never-smokers (Weinmann et al., 2008).

1.4.4 **Biomass smoke inhalation**

Various form of biomass fuel that is used as energy source in third world countries. This includes traditional combustible material such as wood, coal, dried animal dung, and crop residues. One-third of the world rely on biomass fuel for cooking, heating, and lighting. The combustion of these materials is associated with high levels of indoor air pollution and increases the incidence of respiratory infections, including pneumonia, tuberculosis and chronic obstructive pulmonary disease, both in adults and children. (Fullerton et al., 2008). Depending on the fuel type, the combustion of biomass fuel generates significant amount of smoke and adds significantly to indoor and local air pollution (Naeher et al. 2007). The indoor biomass pollution has also been observed to affect women more than men, mainly due to their extended exposure to cooking related activities. Further, the exposure to biomass fuel combustion in these women may trigger their development of COPD and show similar clinical characteristics and, increased mortality rate to those of tobacco smokers (Ramirez-Venegas et al., 2006). Further, repeated and sustained exposures to acrid to low-efficiency biomass smoke while cooking or heating with poorly or non-ventilated houses has again been related to increased mortality worldwide (de Koning et al., 1985). Given the conditions of use, women and children are especially exposed. When compared to the third world nation, the majority of industrialized countries, biomass fuel use is below <5% (Rehfuess et al., 2006).

Chapter 2

Current published evidence of cellular and inflammatory changes in the airways of COPD patients

Note: This chapter is published with 'Expert Review of Respiratory Medicine' and is explained in with the overall textual context.

Mathew Suji Eapen, Eugene Haydn Walters, Sukhwinder Singh Sohal. Airway Inflammation in Chronic Obstructive Pulmonary Disease (COPD) - A True Paradox. Expert Review in Respiratory Medicine Volume 11 Issue 10: pages 827-839. DOI:10.1080/17476348.2017.1360769.

2.1 Tissue Composition and Cellularity

The lungs can be compartmentalized into airways and lung (alveolar) parenchyma, and air-containing lumen and actual tissue in both areas. The airway walls (tissue) consists of epithelium, sitting on its basement membrane and reticular basement membrane (RBM), and then the underlying layers of lamina propria), smooth muscle and the outer sub-mucosal adventitia, respectively. The adventitia abuts and anchors the alveolae. Much of the airway wall is composed of a complex glycoprotein extracellular matrix (ECM), and changes in composition and quantity of this are intimately related to wall thickening.

An increase in luminal cellularity could be attributed to cellular infiltration via systemic migration of cells at the site of infection and damage/injury. In COPD, the literature, especially in the sampling of cells from induced sputum and in the BAL, suggests an increase in the total cell counts, especially of macrophages and to lesser extent neutrophils, compared to healthy non-smokers; it occurs early and overlaps with a smoking-only effect. (Lacoste et al., 1993a, Traves et al., 2002, Leckie et al., 2003, Profita et al., 2010). However, no difference in sputum total cell counts was observed between exacerbating and non-exacerbating stable COPD patients (Bhowmik et al., 2000). Sputum, which mainly consists of cells from the more proximal end of the airways were found to be relatively more neutrophilic, while BAL fluid, which

more represents the cells from the distal end of the respiratory tract, had a more macrophage profile (Traves et al., 2002).

The cellularity in the airway wall, including the proximal large airways and distal small airways, is more heterogeneous, with variable cellular composition. The epithelial layer is classified as “pseudostratified columnar”, with a basal “stem” cell population and then more mature and differentiated cells, but all component cell directly attached to the basement membrane. Differentiated epithelial cell types include the majority of columnar ciliated cells, non-ciliated secretory goblet cells, and in the small airway club cells which secrete surfactant-like molecules (Mercer et al., 2006). Unlike the epithelium, the lamina propria is highly heterogeneous, with varied cell types, mainly stromal, vessel, smooth muscle and inflammatory cells of various sorts. Until recently there have been few attempts to describe the cellularity of the airway wall in any comprehensive detail. Sohal et al., (2013) were the first to report a decrease in total lamina propria cellularity in COPD patients in the large airways and also observed that these patients had a prominent cleft formation in the RBM with cells within these clefts, features notably absent in normal non-smokers.

2.2 Inflammatory changes in COPD

2.2.1 Neutrophils

Neutrophils are the first innate immune cell to migrate rapidly from residing lung tissue to a site of infection or injury with further recruitment from the blood. They are also implicated in mucous metaplasia in chronic bronchitis and play a vital role in peripheral lung tissue damage in emphysema. Neutrophil infiltration is a consequence of the release of chemoattractants such as IL8 from activated epithelial cells and alveolar macrophages.

Neutrophils phagocytose bacteria, inactivate pathogens directly and indirectly, and cause local tissue damage through secretion of neutrophil elastase, metalloproteases, and oxidative reaction species (Shaykhiev and Crystal, 2013). In COPD, neutrophils are observed to be increased in the central and peripheral airway lumen of COPD patients as found in sputum and BAL fluid (Pesci et al., 1998, Brusselle et al., 2011, Di Stefano et al., 1998, Stanescu et

al., 1996, O'Donnell et al., 2004). The increase in neutrophils correlates with the severity of disease (Di Stefano et al., 1998b, Keatings et al., 1996).

However, in airway wall tissue, there have been conflicting results. . Lacoste et al., (1993) showed a decrease in neutrophils in the large airway wall in patients with both chronic bronchitis and COPD, while others found no change in their numbers (Saetta et al., 1993, O'Shaughnessy et al., 1997). In contrast, DiStefano et al., (1998) showed a significant increase in the large airways neutrophils of COPD patients when compared to smokers without airflow obstruction but lacked normal controls for comparisons. In the small airways Hogg et al., (2004) provided evidence of an increase in percent neutrophils in severe COPD phenotypes (GOLD stage III and IV), However, interestingly they were lower in the mild COPD when compared with healthy smokers. Further, Lams et al., (1998) found no change in the number of neutrophils in the small airways of smoker and non-smokers which significantly decreased in life-long smokers. In contrast, studies by Isajevs et al., (2011) showed an overall increase in neutrophils in both large and small airways resected tissue in the airway mucosa, while specific area remained undefined. The inconsistencies in findings in the airway wall of COPD may be partly due to the variability in the measurement strategy employed by different groups, though a shorter half-life of neutrophils in the tissue or dysfunctionality in neutrophils could be a significant factor.

Neutrophil counts in peripheral blood of stable COPD patients are slightly but significantly, higher than those of healthy controls, and during acute exacerbation of the disease, there is an even more significant peripheral blood neutrophilia (Gunay et al., 2014). This may be a response to infection or even treatment (e.g., corticosteroids). In moderate to severe COPD patients (GOLD Stage II-IV), but not in milder COPD, peripheral blood neutrophils showed reduced levels of spontaneous migration and chemotaxis to the chemotactic peptide fMLP and also IL-8. This suggests that long-term smoking or more severe disease (perhaps mediated by chronic infection) may induce post-stimulation exhaustion in the cells (Yoshikawa et al., 2007). Further, studies by Sapey et al., (2011) demonstrated that systemic neutrophils from COPD patients showed aberrant migratory patterns in the presence of chemoattractant such as GROα, IL-8 which strongly resembled neutrophils

from patients with alpha1 trypsin deficiency. Further, neutrophils from COPD patients were also shown to be deficient of pseudopodia formation, a vital feature for phagocytosis and bacterial clearance

In summary, there is a considerable literature that suggests an increase in blood, BAL, and sputum (i.e., luminal) neutrophils, but the overall picture in the airway wall is not yet definite.

2.2.2 **Macrophages**

Two distinct populations of macrophages have been identified in the lung, one that resides in the airway sub-epithelial layer of the mucosa, and the other being the mature alveolar macrophages, which reside within the alveoli. Both cell types are strategically located to play a significant role in maintaining tissue homeostasis (Nicod, 1999). Activated alveolar and tissue macrophages belong to the innate immune system (Ambarus et al., 2012), but whether the presence of such a limited set of cells should be called “inflammation” in the broad sense, may be debatable.

An increase in the total numbers of macrophages in the airway lumen has previously been observed in smokers and is accepted as the firmest inflammatory cellular change in the airways and lung parenchyma in COPD patients (Kaku et al., 2014, Kuschner et al., 1996). In the sub-epithelium, the literature suggests variable outcomes with macrophages. Rutgers et al. showed an increase in the number of macrophages in the large airway lamina propria of COPD patients with severe chronic bronchitis (Rutgers et al., 2000). . Saetta et al., (1993) showed an increase in macrophages in a mixed population of chronic bronchitis patients which included smokers with and without COPD, but this lack of rigorous phenotyping challenges the validity of these findings. Notably, Di Stephano et al., (1998) found significantly increased macrophage numbers in severe but not in mild-moderate COPD subjects, suggesting it was not a fundamental pathogenic issue. Lams et al., (2000) found no change in the numbers of macrophages in the large airway lamina propria in COPD in comparison to smoker and non-smoker healthy controls.

There are fewer reports on macrophage numbers in the small airways. While one study suggested an increase in macrophage numbers (Utokaparch et al., 2014) in the COPD group, another reported the opposite (Lams et al., 1998). Our group recently found that macrophage numbers are unchanged in both smoking and COPD groups in both the large and small airways, though even in healthy non-smoking individuals, a higher number of macrophages reside in the small airway wall than in the large airways (Sohal et al., 2015).

Macrophages with their increased activation of Nuclear Factor-kappaB (NF-kB) have been suggested to have a substantially longer survival time than neutrophils, and this may be true in the alveolar macrophages in lungs of smokers and patients with COPD, (Caramori et al., 2003, Park et al., 2005). Elastolytic enzymes such as matrix metallopeptidase-9 (MMP-9), neutrophil elastase and cathepsins are secreted by alveolar macrophages in COPD (Russell et al., 2002), and these may be significant players in the progression to emphysema.

Efferocytosis, the removal of cellular debris by active macrophage phagocytosis, has been shown to be decreased in BAL macrophages of COPD patients, and this could lead to increase in neutrophilic inflammations, but the reason and consequences remain unclear (Mukaro and Hodge, 2011). Since there is an increase in total macrophages in BAL in smokers/COPD, the overall efferocytosis function should not be decreased but indeed be markedly improved. However, understanding the complexities of macrophage phenotypes, and phenotype switching that may occur, and consequent alterations in functions have not been done for COPD as it has in other disease processes, and would offer new insight into the role of these innate immune cells in COPD. Until I have such a full picture, it is hard to judge the current empiric observation about any alterations in airway macrophage function.

The concept of differentiation of macrophages into the M1/M2 classification (Mantovani et al., 2004) follows on from the similarly structured Th1/Th2 paradigm for lymphocytes (Mosmann et al., 1986). M1 macrophages are defined as classically activated macrophages with pro-inflammatory properties.

They are thought to be involved in the eradication of intracellular pathogens and to secrete pro-inflammatory cytokines. They have a robust antigen-presenting capacity and promote Th1 immunity (Martinez and Gordon, 2014). M2 macrophages are defined as “alternatively activated” macrophages with anti-inflammatory and more pro-fibrotic properties, linked to tissue repair and secretion of anti-inflammatory mediators; they display poor antigen-presenting capacity and promote T cell (Treg) development (Martinez and Gordon, 2014). Alternative pathways of L-arginine substrate catabolism have been identified as functional markers for M1 and M2 macrophages. iNOS (inducible nitric oxide synthase)-produced NO (nitric oxide) expresses M1 cytotoxic properties and Arg1 (arginase)-produced L-ornithine promotes M2-mediated collagen synthesis, cell division, and growth (Benson et al., 2011).

M2 alveolar macrophages (over-expressing CD163, CD204, and CD206) have been shown to be upregulated in the alveolar lumen in severe COPD (Kaku et al., 2014). Further, CD204 overexpression induced by the presence of MSR1 gene SNP is associated with high susceptibility to COPD (Ohar et al., 2010). Further, transcriptional profiling of BAL alveolar macrophages in smokers and COPD showed significant deactivation of M1-related genes and induction of genes related to the polarization of macrophages towards the M2 phenotype. (Shaykhiev et al., 2009) However, in contradiction to this, higher levels of iNOS have been described in macrophages within induced sputum of COPD patients than in normal controls (Ichinose et al., 2000). Tissue macrophages in the airway walls are yet to be investigated and classified into M1 and M2 phenotypes. However, further M1/M2 macrophage identification may offer a significant step in better understanding the pathology in airway and lung compartments of COPD and the relative involvement of macrophages to inflammation versus fibrosis in the disease.

2.2.3 **Eosinophils**

The presence of eosinophilic inflammation has been associated traditionally with allergic asthma rather than COPD. Thus, previous studies by Lacoste et al., (1993) compared eosinophil numbers in bronchial biopsies and BAL

samples of asthma and COPD subjects and found that while asthmatics had eosinophilic inflammation, in COPD and chronic bronchitis (CB) patients, eosinophil numbers were similar to normal non-smokers. However, in contrast, Rutgers et al., (2000) suggested a significant increase in eosinophils in COPD subjects in the large airway lamina propria and induced sputum in COPD over healthy non-smoker controls. The increase in eosinophils was further corroborated by the presence of an increase in eosinophil cationic protein (ECP) which was also found to be increased in sputum of stable COPD although much less than in asthma patients (Balzano et al., 1999). These paradoxes may be related to recent evidence that eosinophilia is present in some (approx. 30%) of COPD subjects in induced sputum and blood, but not in the majority of COPD cases, and defines their corticosteroid responsiveness (Singh et al., 2014, Prajakta et al., 2015, Bafadhel et al., 2011).

2.2.4 **Mast cells**

Mast cells play a critical innate immune function role in mounting responses after recognizing possible pathogens. However, mast cells are also “accidentally” stimulated by allergens reacting with cell surface IgE, causing the cells to degranulate and release mediators such as histamine and bradykinin which cause smooth muscle constriction and vasodilation.

In COPD, there have also been reports of an increase in the number of mast cells in both large and small airways. Grashoff et al., (1997) showed a significant rise in these cells in the small airway epithelium, though no change was noticed in the rest of the small airway wall. In the large airway wall, our group showed a significant increase of mast cells both in the epithelium and lamina propria of asymptomatic smokers (Ekberg-Jansson et al., 2005) and the lamina propria of COPD current and ex-smokers (Soltani et al., 2012). In contrast are the observations of Andersson et al., (2010) who reported a significant decrease in mast cell numbers in the sub-epithelial region of the small airway mucosa of COPD patients across GOLD stage I-IV subjects, with the decrease correlating with the severity of the disease. A similar reduction in tissue mast cells was also found by Gosman et al., (2008) in COPD subjects when compared to control. It is hard to reconcile these difference between

findings of different groups, although where exactly in the airways was this being assessed in the airway is essential as Soltani et al. (2012) demonstrated; they found that most of the increase in mast cells in COPD was specifically around blood vessels (Soltani et al., 2012). Further, Gosman et al., (2008) also showed that the increase in mast cells they found was far more in the peripheral small airways than in the central airway in both COPD and control groups. Interestingly, this group, though only using morphological analysis based on cellular conformation, observed no change in the percent of degranulated mast cells in COPD compared to their control group, which suggests that the mast cells were recruited to but not activated in the airway wall, perhaps in response to local chemo-attractants or bacterial infection rather than to allergens, as in the classic allergic model of inflammation (Gosman et al., 2008). This finding, however, actually requires further validation using a more specific degranulation marker such as lysosome-associated membrane protein-1/2 (LAMP-1/2); a decrease in this marker was recently shown to be related to cellular dysfunction in CD8 cellular degranulation in COPD when ex-vivo exposed to influenza virus (McKendry et al., 2016).

2.2.5 **Dendritic cells**

Dendritic cells (DCs) are part of the adaptive immune system, including in the airway. They are monocyte-derived and play a crucial role in recognition, uptake, and presentation of antigen to lymphocytes, and are on par with macrophages as an antigen presenting cells. However, although fewer in numbers in comparison to macrophages and neutrophils, the airway DCs are present in the airway epithelium and subepithelium, the lung interstitium and the pleura, at least in immature forms. In the airways, mature and active DCs are especially closely associated with epithelial cells, and their extended cytoplasmic protrusions are thought to sample luminal antigens and environmental signals (Shaykhiev and Crystal, 2013).

However, studies of DC specifically in COPD in human tissues have been few. A study by Tsoumakidou et al., (2009) demonstrated a decrease in mature CD83+ DC in the small airways and alveoli of COPD subjects in comparison to

normal controls. They also showed that DC numbers did not change between COPD current and ex-smokers or between steroid-treated and steroid-naïve patients. A similar decrease in sputum mature CD83+ DCs was observed in COPD current smokers when compared to non-smokers (Tsoumakidou et al., 2009). Again, there are contrasting papers, with Vassallo et al., (2010) finding an increase in mature CD83+ DC and CD1a Langerhans cell mRNA in lung tissue in COPD compared to normal. Specifically, the increase in Langheran-207+ cells was found to be pronounced in the small airway epithelium while remaining unchanged in the subepithelium (Demedts et al., 2007, Van Pottelberge et al., 2010). Paradoxically, though, other dendritic cell markers such as DC-SIGN and BDCA-1 showed no change in the small airway wall of COPD patients (Van Pottelberge et al., 2010). Sohal et al., (2011) reported the presence of CD11c dendritic cells closely associated with basal epithelial cells and in the RBM of the large airway of COPD patients, but they were few. Utokaparch et al., (2014) recently reported no change in CD1a dendritic cells in the small airway wall of COPD subjects. The variability in dendritic cell types and the lack of more specific marker have contributed to inconsistent reports in COPD research and will warrant further investigation, especially since in an animal model DCs were suggested to be central to COPD development (Hashimoto et al., 2015)*.*

2.2.6 **CD8 and CD4 T cells**

The role of CD8+ and CD4+ T cells as powerful components of cell-mediated adaptive immune response, in general, is well established. Both CD8s and CD4s are also thought to play a crucial role in the pathology of COPD especially in progression to or in the presence of severe disease. However, once again the true picture in earlier disease especially is quite unclear. O'Shaughnessy et al., (1997) observed both CD4+ and CD8+ T cell numbers to be increased in the large airways of COPD subjects with chronic bronchitis. In contrast, Di Stefano et al., (2001) found a decrease in large airway lamina propria CD8+ T cells, and progressively so with increasing severity of the disease. Our recent reassessment of the airway wall concluded that there is a decline in both CD4+ and CD8+ T cell numbers in the large airway lamina propria area of mild to moderate COPD in comparison to normal controls (Sohal et al., (2015).

However, they did find a small increase in CD8+ T cells in the lamina propria of the small airway, though this is in contrast to findings of Utokaparch, S., et al., (2014) who showed no significant change in this population of small airway cells between their COPD subject and controls.

The increased dominance of CD8+ lymphocytes may be due to the susceptibility in COPD to viral infection and especially with influenza A (Utokaparch et al., 2014) or a consequence of chemotaxis associated with smoking-induced tissue damage. CD8+ T cells are known for their potent cytotoxic activity, and so may be involved in tissue damage in the lamina propria by their secretion perforins and granzyme B in particular (Mikko et al., 2013). A decrease in CD4+ T cell in COPD may well be attributable to the presence of suppressor CD8+ T cells coexpressing CD103, which are known to reduce the proliferation of CD4+ T cells (71) actively, but this has not yet been studied in the airways. The heterogeneity of findings in this T-lymphocyte area is summarized in Table 2.

Table 2 Comprehensive summary of lymphocyte changes in COPD patients-

Reference	Cell type	Tissue type/ Technique used	Controls		COPD		Findings
			NC	NLFS	CS	ES	
(Freeman et al., 2014b)	CD8, CD4, NK cells	Resected tissue large and small Flow Cytometry	UA	16	Mild 14 Severe 15	UA	Found decrease in CD8+/CD4+ cells co-expressing CD56 in mild and severe COPD patients. Also, no change in NK cells was observed
(Freeman et al., 2014a)	CD4 T cells	Resected tissue Flow cytometry and gene expression studies	UA	16	37	UA	Reduced expression of CD4+ cells was found in COPD patients over smokers with normal lung function
(Forsslund et al., 2014)	CD4 and CD8 T cells	BAL Flow cytometry	40	45	27 (Stage 1 and 2)	11	Increased in CD8+/NKT cells like cells and a decrease in CD4 cells were found in BAL of COPD patients over healthy controls
(Urboniene et al., 2013)	CD8, CD4, and gamma delta T cells subsets	Used BAL, IS and PB Flow cytometry	14	UA	20	UA	Found an increase in CD4+ cells in BAL and IS but decrease in PB. CD4 and gamma delta T cells were found to reduction in all fluids

(Chang et al., 2011)	CD4 and CD8 T cells	Epithelium and LP IHC in large airways	15	UA	16	UA	Co-expression of IL17A and IL17F was carried out along with the expression of CD8+ T cells. Increased expression of CD4 and CD8 cells expressing IL17 cytokines was observed
(Mikko et al., 2013)	CD4 and CD8 cells expressing CD103	BAL and PB Flow cytometry	40	40	38	UA	Increase in CD8 and decrease in CD4 co-expressing CD103 in BAL while there is no change in both cell type over normal in PB
(Mathai and Bhat, 2013)	CD4 and CD8 in peripheral blood	PB Flow cytometry	20	19	21	UA	Decrease in CD8 and CD4 in peripheral blood
(Grundy et al., 2013)	CD8+ cells and TCR zeta CD247 co-expression	BAL and PB Flow cytometry	UA	6	6	UA	Found decreased expression of genes associated with active CD8 cells in smokers over COPD in pulmonary region when compared to the peripheral blood of the same patient
(Freeman et al., 2013)	CD8+ and CD4+ co-expressing TLR receptor	lung resected tissue Flow cytometry	UA	14	20	UA	Found increase in CD8+ and CD4+ cells expressing TLR receptor in COPD over NLFS
(Hodge et al., 2012)	CD8 and CD4 cells	Epithelial brushings of trachea left and right bronchi Flow cytometry	11	8	16 mild to moderate and Ten moderate to severe	UA	No change in CD4 or CD8 expressing cells

(Isajevs et al., 2011)	CD8 macrophages and neutrophils	Lung resected tissue in small and large airway IHC	19	20	20	UA	Significant increase in CD8, macrophage, and neutrophil in large and small airway of the lung
(Hodge et al., 2011)	CD8 cells	PB Flow cytometry	34	15	30	18	No significant change in CD8 cells observed in percent CD8 of CD3 cells
(Roos-Engstrand et al., 2010)	CD8 cells	Large airway epithelium IHC	21	16	16	19	A significant increase in CD8 cells found in normal controls and smokers. Decrease in CD8 cells in ex-smokers
(Olloquequi et al., 2010)	CD4 and CD8 T cells and B cells	Lung resected tissue-IHC	9	8	16 mild and 16 severe COPD		Significant change ($p<0.05$) observed in COPD over normal control in epithelium but no significance in connective tissue area of small airway. Also high increase in B cells found in small airway of severe patients
(Freeman et al., 2010)	CD8 cell that co-express CD69 cells	Resected Lung Tissues Flow cytometry	3 and 6	cohorts 11/8/25	Various cohorts GOLD Stage I II III and IV	UA	Increase in CD8 co-expressing CD69 cells
(Lofdahl et al., 2008)	CD8	Bronchial biopsies Large airway IHC	15	14	22		Significant increase in both CD4 and CD8 cells in COPD over normal controls

(Glader et al., 2006)	CD4 and CD8	PB Flow cytometry	6	8	17	No significant difference found in CD8 and CD4 cells found in PB. An increase in CD4 cells expressing CD69 was found

UA- Unavailable, IHC- Immunohistochemistry, PB- Peripheral Blood, NC- Normal Control, NLFS- Normal Lung Function Smokers, CS- Current Smokers, ES- Ex-Smokers

2.2.7 B cells

B cells are essential adaptive immune cells whose interactions with antigen presenting cells (macrophages and dendritic cells) and T cells are of paramount importance in evoking short-term and long-term (memory) responses with the generation of antibodies primarily targeting microbial infections and 'self' antigens in autoimmune diseases.

In large airways mucosa of COPD (GOLD stage II and III), Gosman et al., (2006) showed an increase in CD20+ B cells in COPD current smoker compared to smoker controls while ex-smoker tended to have more of them suggesting that persistent B cell accumulation occurred even after smoking cessation. In contrast, O'Shaugnessy et al., (1997) observed no change in B cell numbers in the airways of mild-moderate COPD compared to normal controls, though notably, this study had fewer subjects in their analysis. In small airways, Hogg et al., (2004) observed an increase in the number of both B cells and lymphoid follicles (LF) in more severe COPD patients compared to healthy smokers and mild COPD patients. Furthermore, the changes in B cell and LF numbers directly correlated with a decrease in lung function in this COPD group. More recent studies by Polverino et al., (2015) reported a similar increase in LF in both number and size in more severe COPD patients (GOLD stage IV) compared to mild COPDs and smoker controls. The authors importantly describe the presence of two types of LF, the first was smaller in size and were observed mainly in mild-moderate COPD patients. The B cells

in this group of COPDs LFs were highly apoptotic and expressed less of the B cell-activating factor of tumor necrosis factor (BAFF), an essential mediator in B cell maturation, while, in more severe COPD there were larger LFs that contained highly active B cells with higher expression of BAFF. These are significant observation, which suggests that smoking actively suppress immune function in early disease, leading to more susceptibility to infection in more severe COPD group. Thus, the increase in B cells and LF in the latter group points to a more conservative response to acute infections and perhaps related to exacerbation in severe patients. In a separate study, the transcriptomic analysis further confirmed upregulation in gene expression of BAFF and its receptor B cells maturation antigen (BCMF) in airways of COPD patients with emphysema while were absent in patients with bronchiolitis (Faner et al., 2016, Lee et al., 2016). Again, the distinct signature observed in COPD corroborates the heterogeneity in the disease phenotype and warrants selective therapeutic interventions.

2.2.8 TH17 and T regulatory cells

Both Th17 and Tregs are subsets of T cell population are known to have considerable influence in modulating various inflammatory disease conditions. Both cell types play a critical role in autoimmune disease (Cazzola and Matera, 2012). While TH17 cells are considered as pro-inflammatory, with the secretion of IL-17, IL-22, and IL-21, the Tregs produce anti-inflammatory mediators such as IL-10 and TGFβ. Tregs essentially plays an essential role in modulating IL-2, an essential cytokine for T cell expansion.

In both COPD patients (mild-moderate and severe) and smokers, Di Stefano et al. (Di Stefano et al., 2009) demonstrated significant changes in TH17 cellular mediators such as IL-17A, IL-21, and IL-22, with increased expression observed mainly in the bronchial mucosa. A similar increase in these mediators was also reported in the serum and sputum of COPD patients and correlated with decrease in lung function and increase in a number of neutrophils and C-reactive proteins (Zhang et al., 2013). Further, TH17 cells also seemed to be the dominant T cell phenotype peripheral blood of mild-moderate COPD patients, and this increase was attributed to increased Tregs suppressive

activity on CD4+ and CD8+ T cells while being ineffective against TH17 cells (Vargas-Rojas et al., 2011). In contrast, observations that are more recent showed an imbalance in Th17 and Treg cells and associated mediators, both sputum and serum of moderate to severe COPD patients. Furthermore, Th17 to Tregs ratio also showed negative physiological consequences in moderate COPD patients (Wang et al., 2015, Li et al., 2015). Role of Tregs in naïve T cells suppression are well known, though, literature evidence for their presence and activity in human airways are few. Barceló et al., (2008) reported an increase in Tregs in BALF of smokers had more of them than in mild-moderate COPD and normal controls, though unlike previous observation mentioned here, they found no change in peripheral blood population between the groups, suggesting their suppressive activity occurs more in the airway milieu (Roos-Engstrand et al., 2011). Further, a recent study also demonstrated an imbalance among the BALF Treg subtypes (defined by levels of CD25 expression), classified as regulatory (rTreg), activated (aTreg) and pro-inflammatory (FrIII) Tregs. The FrIII subtype secretes IL-17/IFNγ. Interestingly, the ratio of rTreg and aTreg combined to FrIII was reduced in more severe COPDs compared to both smokers and normal controls, suggesting the dominance of pro-inflammatory FrIII cells (Hou et al., 2013)."

Overall, the current literature provides contrasting evidence in the presence of Tregs and TH17 cells in COPD and their interplay, though their regulation remains a vital factor in understanding disease progression.

2.3 Role of Reactive oxygen species in inflammation in COPD

Cigarette smoke contains roughly 4000 different chemicals, with most of them being potential oxidants. Indeed, it is estimated that tobacco smoking generates up to 10^{15} highly reactive free radicals per puff which can cause irreversible damage to lung tissue. Reactive oxygen species (ROS) along with reactive nitrogen species (RNS) possess the ability to interact with vital cellular organelles such as mitochondria and endoplasmic reticulum to cause devastating imbalances in cellular metabolism.

In COPD, substantial evidence points to an increase in cigarette smoke-induced ROS activity and their functional ability to cause various disorders in

the cellular architecture as well as DNA damage. Their effect on inflammatory cells can be profound with the ability to cause dysfunctionality reducing their efficiency in combating bacterial infections (Zuo et al., 2014). Morlá et al., (2006) observed that circulating systemic lymphocytes in COPD patients have shorter telomere length compared to healthy subjects, thus a shorter lifespan. This is attributed to the action of ROS, which is known to accelerate the process of aging and aging-related pathologies. Similar studies by Ceylan et al., (2006) showed that systemic leukocytes had severely damaged DNA in circulating leukocytes with a considerable increase in levels of oxidative stress markers such as plasma malondialdehyde (MDA) and TBA-reactive substances (TBARS). ROS induce cellular senescence via DNA damage arrests cellular growth and function. Immune cells that have senesced activates protein complex called senescence-associated secretory phenotype (SASP) which produce phlogogenic substances such as IL-1, IL-6, and IL-8 (Aoshiba et al., 2013). The cytokine produced are potent attractors and activators of innate immune cells, which cause tissue damage by producing oxidizing molecules released mainly to destroy pathogens (Freund et al., 2010). However, the sustained release of these potential harmful oxidants is directly linked to exhaustion of immune cells and their ability to mount an effective immune response. For example, recent ex-vivo human studies by McKendry et al., (2015) showed a significant decrease in capacity of CD8+ T cells isolated from COPD patient to effective ward off viral infections, and this was due to the reduced degranulation capacity of dysfunctional CD8s. Our recent assessment of the small airway wall showed overall a decrease in degranulating cells in COPD patients, pointing towards a possible dysfunctional cellular phenotype in the tissue (Mathew et al., 2017). ROS also impair neutrophil and alveolar macrophage phagocytosis capacity and cause an imbalance in protease/anti-protease activity causing bacterial colonization and excessive tissue damage (Zuo et al., 2014).

The overall contribution of cigarette smoke derived ROS on dysregulated immune response together with increased oxidant production by the immune cells lead to development and progression of COPD pathology.

2.4 Role of the Inflammasome in COPD

The “inflammasome” is defined as a multimeric protein complex expressed mainly by innate cells such as macrophages, neutrophil, dendritic cells and also by adaptive immune cells in response to pattern recognition signals such as bacteria-derived so-called pathogen-associated molecular patterns (PAMPs) and host-derived danger-associated molecular patterns (DAMPs) (Schroder and Tschopp, 2010). These signals are recognized by an array of primitive germ-line-encoded pattern recognition receptors (PRR) such as Toll-like receptors (TLRs) that scan both the extracellular milieu as well as the endosomal compartments within the cells. The inflammasome complex, in innate cells, is now known to help maturation of cytokines such as IL-1β and IL-18 through the activation of proinflammatory caspase-1 (Schroder and Tschopp, 2010). There are numbers of PRRs associated with inflammation, with the most studied and best characterized being the NACHT, LRR and PYD domains-containing protein-3 (NLRP3) (Kim et al., 2015).

There has been much interest in the role of the inflammasome in COPD since the disease pathology is strongly associated with both infection and tissue destruction. However, studies by Di Stefano et al., (2014) found no correlation between expression of NLRP3, caspase-1, IL-1β and severity of the disease, in either the epithelial or subepithelial layer of large airway tissue in COPD. In contrast, the expression of inflammasome inhibitory molecules NALP7 and IL-37 were shown to be significantly increased in COPD in comparison to healthy smoker controls. Also, no change in IL-1β, IL-18 or their receptors in BAL samples were found in this study, although other investigators have suggested contrasting finding from indirect observation (Kim et al., 2015a). Further, Franklin et al., (2014) showed an increase in inflammasome formation in the cellular compartment of COPD lungs via increased interaction of NLRP3 with adaptor protein Apoptosis-associated speck-like (protein) containing a caspase recruitment domain (ASC). The ASC can bind to pro-caspase-1 and convert it to active caspase-1. The authors also observed an increased level of ASC specks accumulated in the extracellular matrix of COPD subjects and mouse models. These excreted ASC specks were found to be stable and retained their ability to transform pro-IL-1β to IL-1β. The author further showed an increase

in ASC specks in BAL samples from COPD and a cigarette smoke-induced mouse model of experimental COPD indicative of potential activity in luminal fluids (Franklin et al., 2014). With little-published literature, this field warrants further mechanistic studies to characterize their potential role as pro-inflammatory factors in COPD.

2.5 Inflammation and airway remodelling in COPD

Unlike “airway (wall) inflammation,” which is an etiological concept that is now a dogma in COPD though not truly evidence-based, well confirmed structural changes have been observed in all compartments in the airway wall in COPD patients, including the epithelium, RBM and lamina propria in both the large and small airways.

2.5.1 Epithelial Remodelling

Epithelium forms the “skin” that is the frontline of the airway in dealing with exposure to the outside world. It is the primary barrier in the innate defense mechanisms against microorganisms and toxic chemical entities such as smoke, through mechanical mucus production and ciliary clearance; biochemical antibacterial, antioxidant, and anti-protease functions; and intercellular epithelial tight junctions to prevent penetration of agent s into deeper tissues (Puchelle et al., 2006). Changes are seen in the normal architecture of the epithelium in smokers with COPD, with squamous metaplasia of the normal pseudostratified columnar tissue organization, mucus hyperplasia with an increase in goblet cells and mucous glands, and basal cell hyperplasia and decreased integrity in tight epithelial junctions (Shaykhiev and Crystal, 2014, Shaykhiev et al., 2011, Peters et al., 1993).

It is believed that the fundamental alteration leading to such well-established epithelial changes is a crucial process of basal epithelial (stem) cell reprogramming (Crystal, 2014). Further, there is no evidence that this also may underlie airway wall remodeling through induction of epithelial-mesenchymal transition (EMT) which, as previously observed in smoking-related idiopathic pulmonary fibrosis (IPF), is now considered a core pathophysiological factor in

COPD development (Milara et al., 2013, Sohal et al., 2010, Sohal, Mahmood et al., 2015). The increased expression of mesenchymal markers such as S100A4 and vimentin especially in the basal cells has been observed both in the large and small airway in COPD along with characteristic EMT-related Rbm fragmentation and also increased epithelial growth factor receptor (EGFR) activity (Mahmood et al., 2015, Sohal et al., 2010). There is a strong relationship between markers of EMT activity such as S100A4 and vimentin with decreased lung function and airway obstruction (Mahmood et al., 2015). EMT in COPD potentially driven by the canonical pathways via TGFβ that induces nuclear transcription factor such as pSMAD2/3 while reducing the inhibitory SMAD7 (Mahmood et al., 2017). Further, this increased expression of pSMAD2/3 showed a high correlation to decrease lung function parameters and airway obstruction. I have also reported that has the potential to ameliorate EMT in COPD patients (Sohal et al., 2014). Further, epidemiological studies reveal that the use of ICS is associated with a decrease in lung cancer risk in COPD; I suggest that this might be through anti-EMT effects of ICS. There have been few direct reports that evaluate the influence of luminal cytokines, chemokines and growth factors on EMT-related changes. However, Milara et al., (2013) showed that cigarette smoke extract (CSE) induced EMT activity in normal small airway primary bronchial epithelial cells (pHBECs) via ROS production, increased TGFβ1 expression and decreased cAMP levels. Further, they demonstrated that the epithelial transition could be abrogated by elevating cAMP levels using a PDE4 inhibitor (Milara et al., 2014). Further, inhibiting TGF-β1, via targeted monoclonal antibodies, during in vitro differentiation of pHBECs prevented mesenchymal vimentin expression and fibronectin release (Gohy et al., 2015). Similar findings of increased expression of mesenchymal markers such as N-cadherin and α-smooth muscle actin (αSMA) with a concomitant decrease in E-cadherin and α-catenin was observed by Wang et al. in small airway epithelial cells when stimulated in-vitro with CSE (Wang et al., 2013). They further suggested a role for urokinase plasminogen activation receptor (uPAR) in the modulation of EMT and exhibited a positive correlation between uPAR and vimentin expression in human small airways. Recent in-vitro epithelial-fibroblast co-culture studies also pointed to the probable role of IL-1α in promoting EMT. The group reported an increase in IL-1α expression in

pHBECs derived from COPD patients when stimulated by CSE (Froidure et al., 2016, Osei et al., 2016). These studies cited are preliminary as yet and will thus require further mechanistic validation to identify the role played by these mediators in promoting EMT; -if anything I am now beginning to get an expanded understanding of the potential mechanisms on airway EMT, but it does seem to be a real phenomenon in the airway pathology in COPD which warrants urgent attention.

2.5.2 Fibroblasts, Myofibroblasts and the extracellular matrix (ECM) in COPD

The primary source of airflow limitation in COPD is the result of airway wall especially in the small airways, tissue remodelling and scarring, i.e., reorganization of the extracellular matrix (ECM). These changes in the ECM have profound effects, the most important being the gradual obliteration of the small airway lumen. In contrast, the other major pathophysiological process that occurs in COPD subjects i.e. breakdown of the alveolar wall, is a destructive process leading to emphysema, that results in decrease air-blood gas exchange and decreased elastic recoil of the lung, with worsening airflow, is a later complication in only about 50% of COPD subjects.

The cell type that is primarily involved in ECM production is the myofibroblast. These cells are of mesenchymal origin, have spindle-shaped morphology, and are highly contractile. The contractile ability of the myofibroblasts directly relates to the increased expression of alpha-smooth muscle actin (αSMA) myofilaments which form in non-muscle cells (stress fibers). Interestingly, isolated rat lung fibroblasts produced more αSMA and showed increased contractibility when compared to subcutaneous fibroblasts (Hallgren et al., 2012, Hinz et al., 2001).

Studies in COPD based on αSMA as a protein marker for myofibroblasts in human bronchi and bronchiolar tissue have been variable. Lofdahl et. al. in their histological staining of large and small airway tissue from operative resections, showed an increased expression in αSMA positive cells in the lamina propria (LP) of the large airway in COPD patients when compared to non-smoker controls, although similar changes in the expression level was not observed in

the crucial small airway (Lofdahl et al., 2011). In contrast, findings from in-vitro studies with fibroblasts isolated from the distill end of the airway from COPD patients showed increased contractile properties associated with increased myofibroblasts (Hallgren et al., 2012). These findings suggest myofibroblasts may be important in both the small and the large airways, but the situation needs to be clarified.

Figure 2.1

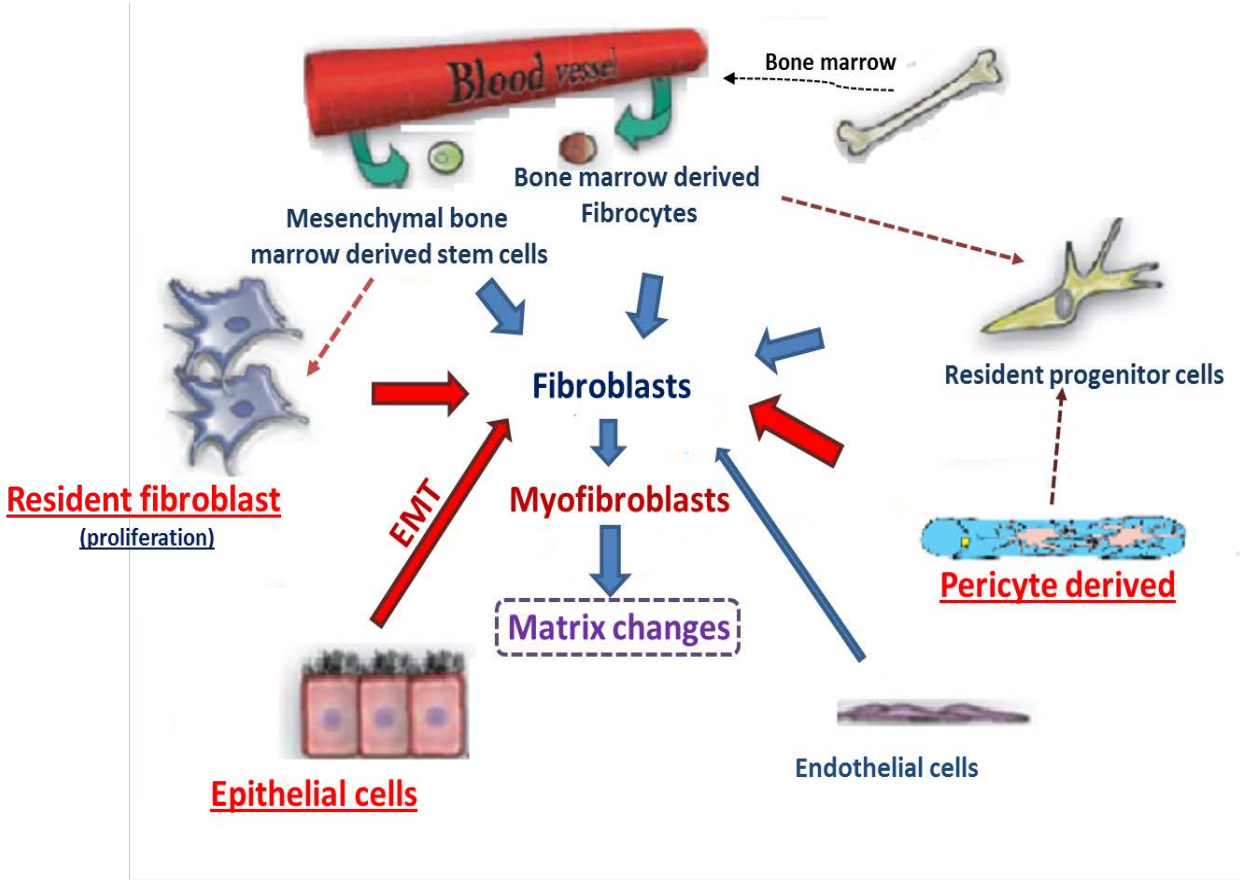


Figure 2.1 Illustrates the various possible origins of fibroblasts and their transformation to myofibroblast in the lung tissue.

Myofibroblast is known to secrete a broad array of ECM proteins which includes fibrous proteins such as collagen and elastin, as well as glycoproteins such as

fibronectin, tenascin C, and proteoglycans. Fibrillar collagens type I, II, III, V, and XI are the most abundant matrix proteins and constitute approximately 15-20% of the dry weight of the tissue (Pierce and Hocott, 1960). In patients with COPD, variability in collagen subtype deposition in both the large and small airways has been related to disease stage (GOLD classification). In large airway biopsies Harju et al., (2010) observed an increase in expression of both collagen I and III in stage I and II COPD in the lamina propria region, while stage IV COPD patients showed a decrease in expression of collagen I and an increase only in expression of collagen III, when compared to normal lung function smokers and non-smoker controls. Small airway tissues showed an overall increase in both collagen subtypes in early stage COPD (stage I and II) but subsequently, the decline in Stage IV. Another interesting observation was the co-localization of αSMA positive fibroblastic cells along with collagen III expressions in the small airway which was not apparently present in large airways (Harju et al., 2010). In contrast, again, Annoni et al., (2012) showed a decrease in collagen type I and no change in type III and IV in mild to moderate COPD patients over that of non-smokers in resected tissue in either large or small airway. The studies so far in this critical area have been small and contradictory, and again more work is required, given the importance of these issues.

Recent evidence has shown that ECM glycoproteins such as tenascin C and fibronectin may have an immune modulation as well as tissue remodelling in COPD. Myofibroblasts secrete these molecules and play a critical role in the structural stabilization of the tissue by interacting and cross-linking with collagen and elastin fibers in the airway. The changes to these glycoproteins were investigated in COPD patients by Karvonen et al., (2013) who showed an increased expression of tenascin C in mild to moderate COPD patients and correlation with myofibroblast numbers in the lamina propria area of large airway biopsies. A similar increase in tenascin C was reported by Annoni et al. (2012) again mainly in the LP region of both large and small airways in resected tissue sections in mild to moderate COPD patients. For fibronectin, however, neither group found any change in expression in COPD patients over normal non-smokers either in the small or large airway and they showed no correlation of fibronectin to the myofibroblast population. Similarly, in-vitro studies for

evaluation of secretory fibronectin from fibroblasts isolated from non-smokers and COPD patients also showed no differences (Togo et al., 2008). These findings are surprising as both glycoproteins (tenascin C v/s fibronectin) are known to be secreted by myofibroblasts, and the apparent differential expression level could be specific spatial and temporal changes that occur in the ECM under disease conditions.

Proteoglycans consist of a protein core covalently attached to one or more glycosaminoglycan (GAG) chain. Based on the structure, function, and localization in the tissue, the proteoglycans are further subdivided into three subtypes: basement membrane proteoglycans (e.g., perlecan), small leucine-rich proteoglycans SLRPs (e.g., decorin, biglycan, lumican) and hyalectans (versican, aggrecan) (Lozzo, 1998). The proteoglycans have an essential role in maintaining tissue homeostasis; for example, versican has a crucial role in determining the water content, and the viscoelasticity of the tissue, perlecan in wound healing, cartilage formation and angiogenesis, decorins and biglycan are closely involved in regulating fibrosis and tissue stiffness.

Annoni et al. (2012) recently observed no changes in versican, decorin, biglycan or lumican expression in the resected large or small airway or in lung parenchyma among COPD patients in comparison to the non-obstructive smoker and non-smokers controls. In contrast, Postma et al. had earlier observed a decreased expression of decorin and biglycan in the peribronchiolar area of emphysematous lung tissues in complex COPD patients and associated it to decreased elastic recoil and subsequent increases in bronchiolar obstruction (van Straaten et al., 1999). Further, Hallgren et al. (2010) described variable changes in proteoglycan expression in in-vitro culture studies using fibroblasts isolated from both central and distal airways. Distal airway fibroblasts from COPD patients showed enhanced production of versican, which correlated with emphysema – related decreased elastic recoil. Lower perlecan production was observed from centrally derived cells in COPD. Although there is substantial report on ECM changes in other lung diseases such as lung parenchymal interstitial disease (ILD), including idiopathic pulmonary disorder (IPF), and also on the airway wall in asthma, investigations into changes in the ECM in COPD patients have so far been quite limited. The lack of differential markers to distinguish myofibroblasts from other fibroblasts

and other mesenchymal stromal cells have impeded this research. New markers such as CD44 and CD90 (Thy1), have emerged as plausible specific tools that could improve sensitivity (Karvonen et al., 2013, Hagood et al., 2005). There has also been considerable interest in a general sense over recent results in the roles played by other mesenchymal cells such as pericytes and endothelial cells and their potential transition to myofibroblasts and also the role of macrophages subtypes in maintaining and disrupting the ECM homeostasis. (Figure 1.6) Such insights and questions also need to be detected in airway and lung tissue of COPD patients.

2.6 Concluding remarks for literature review, aims, and hypothesis for the present thesis

There has arisen over the past 20 years an assumption in respiratory research circles that the airway wall pathology in COPD is primarily one of "inflammation." This is true in the lumen, but in the airway wall, there is more contradictory evidence that requires further verifications. Most of the assumptions come from animal studies, which have not matched perfectly with the human situation and does not take into account the published data on decreased total airway wall cellularity in COPD, and, hence could be misleading. This hypo-cellularity needs verification in mild-moderate COPD, while the picture even in more severe GOLD-III and IV stages are yet to be confirmed. In this literature review, I have assessed the whole literature on this and have concluded that this prevailing inflammatory dogma may at the very least be over-simplistic. There is undoubtedly a great deal of fibrotic remodeling and obliteration especially in the small airways in COPD, which is indeed is the primary pathology, but what is driving and meditating this is still poorly understood. Our group believes that EMT is a significant contributing factor and I wish to take the previous studies further in relating EMT to the fibrotic process more directly. My provisional understanding is that EMT is causally related to reprogramming of the basal stem cell layer of the epithelium, which as well as causing dramatic changes in the structure of the epithelium, also leads to an active EMT in these basal cells. The EMT process directly induces changes in

the extracellular matrix in the underlying airway wall. Importantly, small airway destruction occurs early in the disease even before the diagnosis of COPD.

Thus, the **aims** of my thesis were developed to reassess in phenotypically well-characterized population in smokers and well-characterized COPD patients, and are summarized below;

1. To undertake a detailed and systematic analysis of critical inflammatory cell profiles in both the large and small airway tissue from well characterized normal lung function smokers (NLFS), COPD current and ex-smokers compared to non-smoker controls, which has been done to date. Our group has data to suggest that the cellular picture within the airway wall in smokers is not one of classic inflammatory and I wish to confirm or refute this.
2. Further, I would estimate the overall dysfunctionality in inflammatory cell components in COPD patients via their current degranulating activity.
3. There has been much work done in disease process on distinct M1 and M2 population, but this has not been done in the airways. I wish to assess macrophage phenotype switching in COPD in both luminal and wall compartments.
4. There are very few data available on myofibroblast populations in the small airways which are the site of active fibrosis and obliteration in COPD, and I wish to explore this population to a greater extent than previously done and relate it to EMT activity.
5. To evaluate the presence and changes in crucial ECM markers such as collagen - I and, fibronectin in smokers and COPD patients and correlate them to myofibroblasts, EMT activity as well as airflow obstruction and small airway wall thickness.

Overall, I hypothesize that the airway in COPD are not inflammatory at least in the airway wall, but are mainly primed to undergo an alternate “journey” of fibrotic remodelling; that this is related to epithelial basal cell reprogramming and in particular EMT development and activity.

Chapter 3

Materials and Methods

3.1 Subject recruitment

3.1.1 Ethics statements

The Tasmania Health & Medical Human Research Ethics Committee approved all studies (EC00337, H6532, H0013051, and H0012921). All subjects gave written, informed consent to use their tissue, either before volunteer bronchoscopy and biopsy or before lung surgery.

3.1.2 Endo-bronchial biopsies (large airways)

Seventy-two subjects were recruited through advertisement. Bronchial biopsies (BB) from 20 smokers with normal lung function (NLFS), 13 current smoking COPD (COPD-CS) and 14 ex-smokers with COPD (COPD-ES) were compared with up to 25 healthy non-smokers. COPD was diagnosed according to GOLD criteria. Subjects with other respiratory diseases, a history of recent acute exacerbation of COPD and those on systemic or inhaled corticosteroids were excluded from the study. The diagnosis of COPD was made according to GOLD guidelines based on FEV1 /FVC ratio and categorized into two groups based on current versus ex-smoking history. For normal lung function current smokers the inclusion criteria were; a minimum 10 pack-year history of cigarette smoking with spirometry within normal limits, i.e., FEV1 >70% and no scalloping out of the expiratory descending limb of the flow-volume curve to suggest small airway dysfunction. Normal healthy volunteers had no history of respiratory illness or smoking and had normal lung function.

3.1.3 Bonchoscopy

Bronchoscopy was performed using standard techniques. Briefly, subjects were pre-medicated with nebulized salbutamol (5 mg) 15–30 min before the procedure. Sedation was achieved with intravenous midazolam (3–10 mg) and fentanyl (25–100 µg). Lignocaine (4%) was used for topical anaesthesia above the vocal cords and 2% lignocaine was used to anaesthetize the airways below the cords, in 2 ml aliquots as required, up to a maximum of 6 ml. Subjects were

monitored by pulse oximetry throughout the procedure and oxygen was administered routinely. Eight biopsies from secondary carinae of segmental and sub-segmental bronchi in the right lower lobe were obtained. There were no complications from the procedures. Bronchial biopsies were fixed in 4% neutral buffered formalin for 2 hours and subsequently processed into paraffin through graded alcohol and xylene using a Leica ASP 200 tissue processor

3.1.4 Resected lung tissue (small airways)

Forty patients consented for inclusion in this study. Subjects all had primary non-small cell lung cancer, with an approximately equal distribution of squamous and adenocarcinoma, and consented for their surgical tissue to be used for research at Royal Hobart Hospital. Twenty patients had demonstrated mild-moderate, Global Initiative for Obstructive Lung Disease (GOLD) stage I and II COPD of which nine were COPD-CS and eleven COPD-ES (>1year smoking cessation). Eleven individuals NLFS. Ten non-smoking tissues were obtained from the James Hogg Lung Registry, the University of British Columbia with approval from the Providence Health Care Research Ethics Board H00-50110, and were included as a control group (NC) for comparison. Subjects with other respiratory diseases, a history of recent acute exacerbation of COPD and those on systemic or inhaled corticosteroids were excluded from the study. The surgically resected material was taken well away from the primary tumour and contained non-cancer affected small airways).Emeritus Late Professor HK Muller, a professional pathologist, inspected and confirmed that these tissues were normal, and without the hallmarks of smoking or asthma.

Table 3. Demographic and lung function data for participants in the cross-sectional study for endo-bronchial large airways and bronchoalveolar lavage fluid (BALF).

Groups (numbers)	NC (n=25)	NLFS (n=20)	COPD-CS (n=13)	COPD-ES (n=14)
GOLD I/GOLD II‡	N/A	N/A	6/7	6/7
Age (years)	44 (20-68)	50 (30-66) (p=0.313)	61 (46-78) (p=0.001) *****	62 (53-69) (p=0.001) *****
Smoking (pack years)	0	32 (10-57)	45 (18-78)	51 (18-150)
FEV_1% predicted (Post BD) †	113 (86-140)	99 (78-125) (p=0.01) *****	83 (66-102) (p<0.001) *****	83 (54-104) (p<0.001) *****
FEV_1/FVC % (Post BD) †	82 (71-88)	77 (70-96) (p=0.218)	59 (46-68) (p<0.001) *****	57 (38-68) p<0.001) *****

Data expressed as median and range.
NC- Normal control; NLFS-Normal lung function smoker; COPD-CS (current smoker); COPD-ES (ex-smoker)
*****Significance difference from NC
† Post BD values after 400µg of salbutamol
‡ Diagnosis of COPD as per GOLD guidelines (GOLD 2013).

Table 4. Demographic and lung function data for participants in the cross-sectional study for small airway resected tissue.

Study groups	NC	NLFS	COPD-CS	COPD-ES
n	10	11	9	10
Age (years)	68 (63-75)	70 (52-79)	64 (59-78)	68 (56-85)
Smoking history (Pack-years)	N/A	33 (0.3-60)	28.5 (2-50)	33 (18-36)
FEV_1/FVC (%)*	N/A	76 (70-90)	66.0 (59.9-70)	63.9 (54.9-69)
$FEF_{25-75\%}$ L/sec Post BD %pred.	N/A	81.5 (70-116)	37.0 (28-47)	40.5 (20-55)

Data expressed as median and range.
NC, Normal control; NLFS, Normal lung function smoker; COPD-CS, COPD current smoker; COPD-ES, COPD ex-smoker;
N/A, Not any.
*Post BD values after 400µg of salbutamol

3.1.5 BALF collection and processing

BALF were obtained from the same cohort of patients as mentioned in section 3.1.2 and processed as described previously (Ward et al., 2002). Briefly, participants were pre-medicated with nebulized salbutamol (200µg), intravenous atropine (0.6 mg), and sedation was achieved with intravenous administration of midazolam (3–10 mg) and fentanyl (25–100µg). Lignocaine (4%) was applied topically to the nose, pharynx, and larynx, and 2% lignocaine was administered below the cords. Participants were monitored by pulse oximetry and administered oxygen throughout the procedure. The BAL fluids was immediately transported to the laboratory at 4°C for processing and analysis

BAL was performed with three aliquots, 60 mL each; of phosphate buffered saline (PBS) at 37° introduced by steady hand pressure into the right middle lobe and recovered by suction at less than -80mm Hg pressure. Total cell counts were determined on unfiltered BALF using a Neubauer haemocytometer and 1 ml of the BAL was cyto-centrifuged using CytoSpin 4 Cytocentrifuge (ThermoScientific) at 850 rpm for 5min producing two cytospots onto each glass slide. The cytospins were frozen at -80°C until use.

Further, BAL fluids was filtered through a 200µm stain-less mesh and cells pelleted at 100Xg max for 15min at 4°C. Aliquots of the BAL supernatants were stored in at -80°C, for subsequent used in cytokine profiling.

3.1.5.1 *Exclusion criteria*

1. Subjects with asthma, which included symptoms in childhood, related atopic disorders, significant a history of wheeze, eczema or hay fever, rather than progressive breathlessness and any who had previously used ICS (oral or inhaled) were excluded.
2. Further, uncontrolled comorbidities such as angina or cardiac failure, diabetes and other associated respiratory illnesses including pulmonary fibrosis and bronchiectasis.

3. Subjects with inability to provide written informed consent were also excluded.

3.1.5.2 *Inclusion criteria*

1. COPD Current-smokers aged at 40 years and above with smoking history equal more than 15 pack-years and subsequently obtained BAL fluid had to be free of bacterial colonisation; FEV_1 40% to 80% predicted, with FER (FEV_1/FVC)≤70% post-bronchodilator with definite scalloping out of the descending limb of flow-volume loop on spirometry. COPD ex-smokers with at least six months of smoking cessation were included.
2. Normal healthy never smoking controls and current smokers with normal lung function recruited also underwent bronchoscopy examination and physiological lung function test. They were more tha eighteen year old and had a FEV_1/FVC ratio of greater than 70% or higher and FEV_1% predicted of 80% or higher.
3. All never-smoking controls individuals were devoid of any respiratory illness or smoking history at the time of extraction. For normal lung function current smokers the inclusion criteria were; a minimum 10 pack-year history of cigarette smoking with spirometry within normal limits (FEV1 (forced expiratory volume) >80% of predicted, and FEV1/FVC (forced vital capacity) >70%) and no scalloping out of the expiratory descending limb of the flow-volume curve, suggesting small airway dysfunction.

3.2 Processing of biopsies and resected tissue

Both biopsies and resected sections were cut up to 3.5 microns thick from individual paraffin blocks and stained with hematoxylin & eosin to assess morphology and damages.

3.2.1 Hematoxylin and eosin (H&E) stain

1. Slides with tissue sections were placed in Mayer's hematoxylin for 5 minutes to elaborate nuclei, followed by rinsing in running water.
2. Tissue sections were placed in approximately in 400ml of water with eight drops of ammonia 30 seconds and rinsed well in running water.
3. Slides were then placed in eosin solution for 2 minutes and rinsed quickly in running water to remove excess eosin and then placed into 95% ethanol for 30 seconds with agitation.
4. Further dehydrated with three changes of 100% ethanol was carried out (1 minute each).
5. Clearing of sections was done using two changes of fresh xylene (2 minutes each).

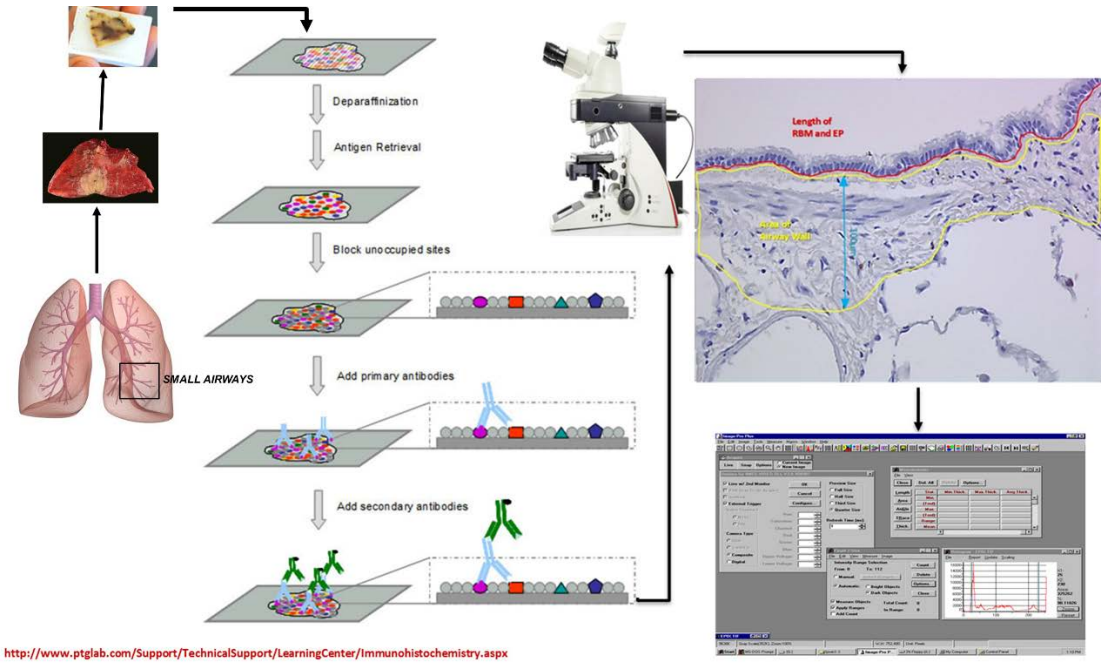
Sections were mounted in Depex using Dako Coverslipper (Dako, Denmark A/S) and dried on hotplate overnight.

3.2.2 General procedure (Tissue slide preparations for immunohistochemical analysis)

1. Paraffin blocks (both biopsies and resected tissues) were sectioned at 3.5 microns after cooling in –20 freezers or on ice blocks 5-10 minutes.
2. For biopsies, two sections (approximately 3µm thick) separated by 40-50 (approximately ten sections) were cut and placed on Dako FLEX IHC microscopic slides (Code K8020, Dako Denmark A/S). For resected tissue, only one section of tissue was used due to a greater size.
3. Slides were either left to dry on a hotplate at 56°C for one hour or left overnight in a 37°C incubator.
4. Slides were barcoded for easy identification and further marked on the edges with a wax pen to avoid spillage of reagents.
5. Dewaxing of the tissue sections was carried out with lab grade Xylene twice for 5 minutes each in a fume hood.
6. Hydration to water was carried out using 100% ethanol followed by 95% then 70% (3 minutes each) followed by rinsing the sections in running tap water for approximately 2 minutes.

Note- Unless otherwise stated, all immunohistochemical staining was done using Dako auto-stainer which provided a more reliable and consistent output.PT link (Dako), a regulated heating device, was used for antigen retrieval with either high pH Target Retrieval Solution, High pH (*50x Tris/EDTA buffer, pH 9*) *or low pH (50x buffer, pH 6) pre-diluted antigen retrieval solution available from DAKO*

Figure 3.1 Pictorial representation of staining strategy.



3.2.3 Immunostaining for neutrophils (Neutrophil Elastase)

1. For neutrophil elastase staining, sections were placed prior in 3% H_2O_2 in distilled water for 20 minutes to block endogenous peroxidase activity. *Note*: *No antigen retrieval was required as determined by prior optimization.*
2. Tissue sections were washed thrice with Tris HCl pH 7.5 (wash buffer) with two minutes intervals.
3. Primary antibodies neutrophil elastase (Dako, clone NP57, catalog # M0752), was applied to the tissue at 1/500 dilution and incubated for an hour. Matched negative control was used: for neutrophil elastase-isotype-matched immunoglobulin IgG1κ (X0931, Dako, Denmark A/S).

Lung sections from the previous biobank was used as positive controls for confirmation of staining.

4. Sections were further rinsed using wash buffer (thrice, 5 minutes interval) before addition of anti-mouse secondary antibodies- the EnVision+ system-HRP labeled polymer reagent (catalog number K4001; Dako, Denmark A/S) for 30 minutes.
5. After three washes, DAB+ (catalog number K3468; Dako, Denmark A/S) was applied to sections for further 10 minutes and rinsed in wash buffer (twice) followed by once with distilled water.
6. The tissue sections were counter-stained Mayers hematoxylin to elaborate nuclei for approximately 5 minutes, then rinsed thoroughly in running water.
7. Sections were placed in approximately 400 ml of water with 6-8 drops of ammonia for 30 seconds and rinsed again in running water.
8. Dehydration was carried out in 95% ethanol, then two changes of 100% ethanol (2 minutes each). The clearing was done in two changes of xylene (2 minutes each).
9. Sections were then mounted in Depex using Dako Coverslipper (Dako, Denmark A/S) and dried on hotplate overnight.

3.2.4 Immunostaining of total CD68+ cells

1. For CD68+ staining, high pH antigen retrieval was done at 100°C for 15min followed by treatment with 3% H_2O_2 in distilled water for 20 minutes to block endogenous peroxidase activity. Tissue sections were washed thrice with Tris HCl pH 7.5 (wash buffer) with two minutes intervals.
2. Primary antibodies mouse monoclonal CD68+ (Dako, clone KP1, catalog # M0814,) was applied to the tissue at 1/400 dilution and incubated for an hour. Matched negative control was used: for CD68+ isotype-matched immunoglobulin IgG1κ (X0931, Dako, Denmark A/S). Lung sections from the previous biobank was used as positive controls for confirmation of staining.
3. Sections were further rinsed using wash buffer (thrice, 5 minutes interval) before addition of anti-mouse secondary antibodies- the

EnVision+ system-HRP labeled polymer reagent (catalog number K4001; Dako, Denmark A/S) for 30 minutes.

4. After three washes, DAB+ (catalog number K3468; Dako, Denmark A/S) was applied to sections for further 10 minutes and rinsed in wash buffer (twice) followed by once with distilled water.
5. The tissue sections were counter-stained Mayers hematoxylin to elaborate nuclei for approximately 5 minutes, then rinsed thoroughly in running water.
6. Sections were placed in approximately 400 ml of water with 6-8 drops of ammonia for 30 seconds and rinsed again in running water.
7. Dehydration was carried out in 95% ethanol, then two changes of 100% ethanol (2 minutes each). The clearing was done in two changes of xylene (2 minutes each).
8. Sections were then mounted in Depex using Dako Coverslipper (Dako, Denmark A/S) and dried on hotplate overnight.

3.2.5 Immunostaining for CD4+ and CD8+ T cells

1. For CD4+ and CD8+ cell staining, antigen retrieval was done in EDTA buffer (pH 8.0) at boiling temperature for three minutes followed by treatment with 0.3% H_2O_2 in methanol for three minutes. Tissue sections were washed thrice with Tris HCl pH 7.5 (wash buffer) with two minutes intervals.
2. Primary antibodies mouse monoclonal CD4+ (Leica Novo Castra, clone 1F6, catalog # NCL-CD4-4B11) and CD8+ (Leica Novo Castra, clone 4B11, catalog # NCL-CD8-4B11) were applied to the tissue at 1/20 dilution and incubated for an hour. Matched negative control was used: for CD4 isotype-matched immunoglobulin IgG1κ (X0931, Dako, Denmark A/S) and CD8 isotype-matched immunoglobulin IgG2b (X0944, Dako, Denmark A/S) respectively. Lung sections from the previous biobank was used as positive controls for confirmation of staining.
3. Sections were further rinsed using wash buffer (thrice, 5 minutes interval) before addition of anti-mouse secondary antibodies- the

EnVision+ system-HRP labeled polymer reagent (catalog number K4001; Dako, Denmark A/S) for 30 minutes.

4. After three washes, DAB+ (catalog number K3468; Dako, Denmark A/S) was applied to sections for further 10 minutes and rinsed in wash buffer (twice) followed by once with distilled water.
5. The tissue sections were counter-stained Mayers hematoxylin to elaborate nuclei for approximately 5 minutes, then rinsed thoroughly in running water.
6. Sections were placed in approximately 400 ml of water with 6-8 drops of ammonia for 30 seconds and rinsed again in running water.
7. Dehydration was carried out in 95% ethanol, then two changes of 100% ethanol (2 minutes each). The clearing was done in two changes of xylene (2 minutes each).
8. Sections were then mounted in Depex using Dako Coverslipper (Dako, Denmark A/S) and dried on hotplate overnight.

3.2.6 Immunostaining for mast cells, degranulating Mast cells, and degranulating cells

For the enumeration of Degranulated mast cells in the small airway, dual staining was done by using mast cell tryptase AA1 and lysosomal-associated membrane protein-1 (LAMP-1).

1. Antigen retrieval was done for the tissues in high pH buffer at boiling temperature for three minutes followed by treatment with 3% H_2O_2 in water for three minutes. Tissue sections were washed thrice with Tris HCl pH 7.5 (wash buffer) with two minutes intervals.
2. Primary antibodies mouse anti-human mast cell tryptase (AA-1) monoclonal antibody (M7052; Dako; 1/1500 dilution) and a rabbit anti-LAMP-1 antibody (Abcam; ab24170; 1/200 dilution) were sequentially added and further incubated for an hour. Matched negative control was used: isotype-matched immunoglobulin IgG1κ (X0931, Dako, Denmark A/S) and rabbit serum (X0903, Dako, Denmark A/S) at appropriately adjusted concentrations. Lung sections from the previous biobank was used as positive controls for confirmation of staining.

3. Tissue sections were washed thrice with wash buffer with two minutes intervals.
4. Bound Tryptase AA-1 antibodies were elaborated using Dako REAL detection system (catalog number K5005; Dako) and were visualized with BCIP/NBT (5-bromo-4-chloro-3'-indolyphosphate and nitro-blue tetrazolium) in a ready-made substrate system (catalog number K0598; Dako). Endogenous alkaline phosphatase activity was inhibited by the addition of levamisole to the visualization substrate (catalog number X3021; Dako).
5. Sections were further rinsed using wash buffer (thrice, 5 minutes interval) and bound LAMP-1 before addition of anti-mouse secondary antibodies- the EnVision+ system-HRP labeled polymer reagent (catalog number K4001; Dako, Denmark A/S) for 30 minutes.
6. After three washes, DAB+ (catalog number K3468; Dako, Denmark A/S) was applied to sections for further 10 minutes and rinsed in wash buffer (twice) followed by once with distilled water.
7. The tissue sections were counter-stained nuclear fast red to elaborate nuclei for approximately 2 minutes, then rinsed thoroughly in running water to remove the excess stains.
8. Dehydration was carried out in 95% ethanol, then two changes of 100% ethanol (2 minutes each). The clearing was done in two changes of xylene (2 minutes each).
9. Sections were then mounted in Depex using Dako Coverslipper (Dako, Denmark A/S) and dried on hotplate overnight.

3.2.7 Immunostaining for M1 macrophages (CD68+ and iNOS)

1. To identify M1 macrophages, resected small airway tissues and BALF cytospins, were dual stained with CD68+ and iNOS. Antigen retrieval was done with high pH buffer at boiling temperature for three minutes followed by treatment with 3% H_2O_2 in water for three minutes. Tissue sections were washed thrice with Tris HCl pH 7.5 (wash buffer) with two minutes intervals.
2. Primary antibodies mouse anti-CD68 monoclonal antibody (KP1, Dako, M0814, 1/400 dilution) and a rabbit anti-iNOS polyclonal antibody

(Thermo Fisher Australia, PA1-21054, 1/100 dilution) were sequentially added and further incubated for an hour. Matched negative control was used: isotype-matched immunoglobulin IgG1κ (X0931, Dako, Denmark A/S) and rabbit serum (X0903, Dako, Denmark A/S), Dako, Denmark A/S) at appropriately adjusted concentrations. Lung sections from the previous biobank was used as positive controls for confirmation of staining.

3. Tissue sections were washed thrice with wash buffer with two minutes intervals.
4. Bound CD68+ antibodies were elaborated using Dako REAL detection system (catalog number K5005; Dako) and were and visualized with BCIP/NBT (5-Bromo-4-chloro-3'-polyphosphate and nitro-blue tetrazolium) in a ready-made substrate system (catalog number K0598; Dako). Endogenous alkaline phosphatase activity was inhibited by the addition of Levamisole to the visualization substrate (catalog number X3021; Dako).
5. Sections were further rinsed using wash buffer (thrice, 5 minutes interval) and the bound LAMP-1 before addition of anti-mouse secondary antibodies- the EnVision+ system-HRP labeled polymer reagent (catalog number K4001; Dako, Denmark A/S) for 30 minutes.
6. After three washes, DAB+ (catalog number K3468; Dako, Denmark A/S) was applied to sections for further 10 minutes and rinsed in wash buffer (twice) followed by once with distilled water.
7. The tissue sections were counter-stained nuclear fast red to elaborate nuclei for approximately 2 minutes, then rinsed thoroughly in running water to remove the excess stains.
8. Dehydration was carried out in 95% ethanol, then two changes of 100% ethanol (2 minutes each). The clearing was done in two changes of xylene (2 minutes each).
9. Sections were then mounted in Depex using Dako Coverslipper (Dako, Denmark A/S) and dried on hotplate overnight.

3.2.8 Immunostaining for M2 macrophages

1. To identify M2 macrophages both CD163 and Arginase-1 (Arg-1) were used as markers in both small airway wall and BALF cytospins samples. For Arg-1, a high pH antigen retrieval was done in at 97°C for 15min, while for CD163 no antigen retrieval was used, as determined during optimization. This was followed by treatment with 3% H_2O_2 in distilled water for 20 minutes to block endogenous peroxidase activity. Tissue sections were washed thrice with Tris HCl pH 7.5 (wash buffer) with two minutes intervals.
2. Primary antibodies mouse monoclonal CD163+ mouse anti-CD163 (EDHu-1, AbD Serotec, MCA1853, 1/100 dilution) and mouse anti-Arg1 (BD Biosciences, 610708, 1/100 dilution) antibodies was applied to the tissue for 90 minutes. Matched negative control was used: for CD163 and Arg-1 isotype-matched immunoglobulin IgG1κ (X0931, Dako, Denmark A/S). Lung sections from the previous biobank was used as positive controls for confirmation of staining.
3. Sections were further rinsed using wash buffer (thrice, 5 minutes interval) before addition of anti-mouse secondary antibodies- the EnVision+ system-HRP labeled polymer reagent (catalog number K4001; Dako, Denmark A/S) for 30 minutes.
4. After three washes, DAB+ (catalog number K3468; Dako, Denmark A/S) was applied to sections for further 10 minutes and rinsed in wash buffer (twice) followed by once with distilled water.
5. The tissue sections were counter-stained Mayers hematoxylin to elaborate nuclei for approximately 5 minutes, then rinsed thoroughly in running water.
6. Sections were placed in approximately 400 ml of water with 6-8 drops of ammonia for 30 seconds and rinsed again in running water.
7. Dehydration was carried out in 95% ethanol, then two changes of 100% ethanol (2 minutes each). The clearing was done in two changes of xylene (2 minutes each).
8. Sections were then mounted in Depex using Dako Coverslipper (Dako, Denmark A/S) and dried on hotplate overnight.

3.2.9 Immunostaining for αSMA+ Myofibroblast

1. For αSMA+ staining, a high pH antigen retrieval was done in at 97°C for 20min. This was followed by treatment with 3% H_2O_2 in distilled water for 20 minutes to block endogenous peroxidase activity. Tissue sections were washed thrice with Tris HCl pH 7.5 (wash buffer) with two minutes intervals.
2. Primary antibodies mouse monoclonal mouse anti- αSMA (Dako, M0851, 1/400 dilution) antibodies was applied to the tissue for 90 minutes. Matched negative control was used: immunoglobulin IgG1κ (X0931, Dako, Denmark A/S). Lung sections from the previous biobank was used as positive controls for confirmation of staining.
3. Sections were further rinsed using wash buffer (thrice, 5 minutes interval) before addition of anti-mouse secondary antibodies- the EnVision+ system-HRP labeled polymer reagent (catalog number K4001; Dako, Denmark A/S) for 30 minutes.
4. After three washes, DAB+ (catalog number K3468; Dako, Denmark A/S) was applied to sections for further 10 minutes and rinsed in wash buffer (twice) followed by once with distilled water.
5. The tissue sections were counter-stained Mayers hematoxylin to elaborate nuclei for approximately 5 minutes, then rinsed thoroughly in running water.
6. Sections were placed in approximately 400 ml of water with 6-8 drops of ammonia for 30 seconds and rinsed again in running water.
7. Dehydration was carried out in 95% ethanol, then two changes of 100% ethanol (2 minutes each). The clearing was done in two changes of xylene (2 minutes each).
8. Sections were then mounted in Depex using Dako Coverslipper (Dako, Denmark A/S) and dried on hotplate overnight

3.2.10 Immunostaining for ECM proteins

9. For collagen-1, a low pH antigen retrieval was done in at 97°C for 15min while for fibronectin high pH buffer was used. This was followed by treatment with 3% H_2O_2 in distilled water for 20 minutes to block

endogenous peroxidase activity. Tissue sections were washed thrice with Tris HCl pH 7.5 (wash buffer) with two minutes intervals.

10. Primary antibodies mouse polyclonal mouse anti-collagen-1 (Abcam, AB34710, 1/250 dilution) and polyclonal rabbit fibronectin (Dako, A0245, 1/1000 dilution) antibodies was applied to the tissue for 90 minutes. Matched negative control was used: immunoglobulin IgG1κ (X0931, Dako, Denmark A/S). Lung sections from the previous biobank was used as positive controls for confirmation of staining.
11. Sections were further rinsed using wash buffer (thrice, 5 minutes interval) before addition of anti-mouse and anti-rabbit secondary antibodies- the EnVision+ system-HRP labeled polymer reagent (catalog number K4001; Dako, Denmark A/S) for 30 minutes.
12. After three washes, DAB+ (catalog number K3468; Dako, Denmark A/S) was applied to sections for further 10 minutes and rinsed in wash buffer (twice) followed by once with distilled water.
13. The tissue sections were counter-stained Mayers hematoxylin to elaborate nuclei for approximately 5 minutes, then rinsed thoroughly in running water.
14. Sections were placed in approximately 400 ml of water with 6-8 drops of ammonia for 30 seconds and rinsed again in running water.
15. Dehydration was carried out in 95% ethanol, then two changes of 100% ethanol (2 minutes each). The clearing was done in two changes of xylene (2 minutes each).
16. Sections were then mounted in Depex using Dako Coverslipper (Dako, Denmark A/S) and dried on hotplate overnight

3.3 Microscopic and Image analysis

Computer-assisted image analysis was performed with a Leica DM 2500 microscope (Leica Microsystems, Germany), Spot Insight-12 (Spot Imaging Solutions, USA) digital camera and Image Pro Plus 7.0 (Media Cybernetics, USA) software. Firstly, as many images as possible was taken of the tissue from the area of interest (for this study it was mainly epithelium, Rbm, lamina propria), strictly avoiding overlapping of tissue. All image analyses were done

using the above-described image analyzer. Randomly five selected images from the total number of images were used for desired measurements.

Important Note- In all studies, the slides from the pathological and normal subject evaluated here were blinded by an independent observer and was revealed to me only after the data was evaluated.

3.3.1 Immune cells and Cellularity Estimation

Neutrophil Elastase, CD68+ macrophage, CD4 and CD8 stained cells were counted in the LP up to 150 microns deep for large airways and full thickness airway wall (or up to 100 microns deep) in the SA wall; cells per mm^2 area were calculated. Stained cells in the epithelium and Reticular Basement Membrane (Rbm) were counted and presented per mm of Rbm length. For total cell counts, hematoxylin stained nuclei were considered as an individual cell and measured with similar strategies as mentioned above.

3.3.2 Mast cell and Degranulated cell Estimation

Full-thickness SA wall up to 100 microns deep were quantitated as cells per mm^2 area surveyed, excluding the smooth muscle layer. Cells that stained for mast cells (both degranulating and non-degranulating) and degranulating cells in the epithelium and sub-epithelium were counted and are presented per mm of Rbm length for the epithelium and as per mm^2 of small airway sub-epithelium.

3.3.3 M1 M2 macrophage estimation

3.3.3.1 *Small Airway wall*

Five random fields selection of small airways less than two mm in thick (a minimum of two airways per subject) were chosen for comprehensive analysis without ad hoc area selection, although muscle bundles and glands were excluded from the area surveyed. Small airways sub-epithelium up to 100 microns deep were quantitated. Stained M0, M1 and M2 cells in the sub-epithelium and epithelium were separately counted and is presented here as

per mm^2 of the area surveyed and per mm of reticular basement membrane (RBM) length respectively.

For arginase-1 expression, a separate analysis was done for the total sub-epithelium (excluding muscle areas) and epithelium and further, the data here is represented as percent of tissue expressing arginase-1 (Arg-1) expression.

3.3.3.2 *BALF*

BAL cells counts were done using bright field microscope (Olympus BX53) assisted by Visiopharm newCAST™ software. An automated motorized system provided an unbiased uniform random area sampling for 12 fields per cytospot, and stained cells were manually counted for each selected field. Counts were normalized with BAL dilution factor and presented here as cells per ml of the original BAL sample.

3.4 Airway wall thickness Measurements

All airway thickness were measured using thickness measurement analysis programme in the Image ProPlus version 7.0 software. I took random non-overlapping pictures of as many images as possible (with smooth muscle layer captured in each picture), from a minimum of three airways per section and, further, eight picture were randomly selected by using an online randomiser programme. The tissue was divided into three sections lamina propria (the distance between Rbm and top of the muscle layer), smooth muscle layer and adventitia (the distance between the lower layers of the muscle to the airway septum). For calculation of LP, thickness two lines were drawn; one at the base of the epithelium and the other was just above the smooth muscle wall a layer. Similarly, lines were drawn on top and bottom of muscle layer, and for the adventitia thickness, lines were drawn from below the muscle layer, outward into the start septal areas (Figure 3.1).

Further, based on orientation, horizontal, vertical or curvature thickness was measured by the image analytic software, and average distances in microns is calculated.

Figure 3.2

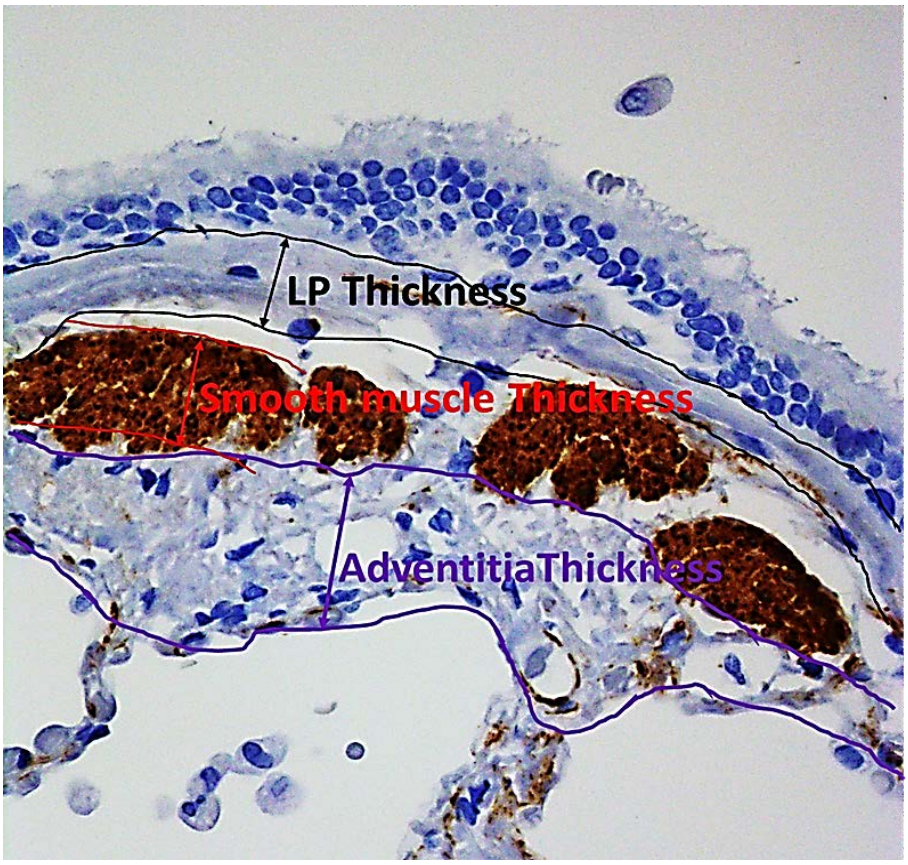


Figure 3.2 Representative image of the zones used for the thickness analysis in the small airways.

3.5 αSmooth muscle actin (αSMA) and ECM estimation

αSMA positive cells were enumerated in Rbm, lamina propria, and the adventitia regions of the small airway wall as illustrated in figure 3.1. αSMA positive cells in the Rbm were enumerated as cells per mm length of the small airway Rbm, while for the lamina propria and adventitia the cells were enumerated as per mm^2 of the respective area. For collagen-1 and fibronectin, per object area of the lamina propria, and the adventitia was analyzed and is represented here as the percentage expression of the surveyed area.

3.6 Cytokine Analysis

For estimating M1/M2 cytokine profile cell-free equal volumes of BALF supernatants were thawed on ice and concentrated ten-folds, using 3kDa cutoff Amicon® Ultra-4 Centrifugal Filter Units (UFC800308 Merck Millipore) and centrifugation (2000g, 30 min, 4°C). Human cytokines for M1/proinflammatory cytokines (IL-12, lFNγ,) and M2 (IL-4, IL-13, CCL22, IL-10, IL-6), and IL-1β TNFα were quantitated using multiplexing (MPHCYTOMAG60; Millipore Multiplex kits), and analysis was done using Luminex™ MagPix Multiplex technology platform according to manufacturer instructions. Chemokine/cytokine quantitation was derived from the standard curve and represented here as picograms per ml of original BAL sample.

3.7 Statistical analysis

For all cross-sectional data, I determined the normality of the data using D'Agostino-Pearson omnibus normality test. Based on the distributions results which were skewed upwards, the analysis presented here are as medians and ranges; non- parametric analyses of variance were performed, Kruskal-Wallis Test, comparing medians across all the groups of interest and specific group differences and correction for the multiple comparisons was done using Dunn's test.

For correlation studies, I performed a regression analysis using Pearson or Spearman's rank test. Linear regression analysis for potential confounders were undertaken, and differences between groups in gender balance, age, and atopy were found to be non-contributory.

Statistical analyses were performed either using SPSS 21.0 for Mac, 2012 (IBM Corporation, Armonk, NY, USA) or GraphPad Prism 7.0 (2012) for Windows, (GraphPad Software Inc., La Jolla, CA, USA), with a two-tailed p-value ≤0.05 being considered statistically

Chapter 4

Profiling cellular and inflammatory changes in the airway wall of mild to moderate COPD

Note: This chapter is published with 'Respirology' and is explained in with the overall textual context.

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4.1 Introduction

COPD is a devastating global disease caused especially by cigarette smoking. The disease is characterized by slowly progressive fixed airway narrowing due to small airway fibrosis and obliteration. Up to fifty percent of COPD patients also go on to varying degree of emphysema (Sohal et al., 2013), and as a group, they are highly vulnerable to lung cancer, suggesting a common pathogenesis (Mahmood et al., 2015).

The literature has reported convincing increases in both innate and adaptive cells in the airway lumen in COPD (Pesci et al., 1998, Rutgers et al., 2000). It has also become accepted that airway wall inflammation also plays a critical role in COPD, but the evidence for this is quite limited Better understanding of COPD pathophysiology requires more detailed information from airway wall tissues (Persson and Uller, 2010).

There are reports of both increases and decreases in inflammatory cell profiles in the airway wall (Di Stefano et al., 1998a, Di Stefano et al., 2001, Saetta et al., 1999). Selection of patients, inadequate approaches to controls, different sources of tissue acquisition, have been important variables. Studies aimed at relatively early disease before secondary complications such as chronic infection and cancer would be valuable for understanding fundamental pathogenesis.

Our group has reported previously that smoking significantly decreased large airway (LA) lamina propria (LP) total cellularity in mild-moderate COPD subjects (Sohal et al., 2013). Surprisingly, few groups have examined this in airway tissue. Our hypo-cellularity finding in large airway biopsies questioned whether COPD could truly be regarded as an “airway wall” inflammatory disease. The limited literature evidence on cell differentials in the airway wall is also variable. O'Shaughnessy et al., (1997) showed that there was an increase in CD8+ T cells in COPD while the same cells were demonstrated to decrease progressively from mild-moderate to severe COPD by Di Stephano et al., and reports on macrophages and neutrophils have similarly varied in both directions (Rutgers et al., 2000, Di Stefano et al., 1998a, Lams et al., 1998, Utokaparch et al., 2014). This inconsistency for cell differentials in the airway wall and the previous finding of overall hypo-cellularity led us to believe that a new thorough, systematic investigation was warranted.

I have also probed in more detail than previously the phenotypic specificity of the commonly used macrophage marker CD68 that has been used rather uncritically by respiratory scientists for many years. Evidence from non-respiratory researchers suggests that this marker is not unique to macrophages alone but are also expressed on fibroblastic cells (Kunisch et al., 2004).

In the current study, I provide total cellularity, “inflammatory” cell differentials for neutrophils, CD68+ and CD8+ cells in both large and small airway tissue from mild-moderate, stable COPD subjects who Ire not on medication, and free from clinical infection.

4.2 Overview of materials and methods

The subjects involved are detailed on pages 65 and 66 in the method section. I employed classical immunohistochemical methods to evaluate neutrophils, macrophages, CD4 and CD8 positive cells in both large and small airway epithelium, Rbm and Sub-epithelium in bronchial biopsies collected from well-phenotyped participants (detailed in chapter 3, page 72-74).

4.3 Results

4.3.1 Total cells

Cell density was greater in SA and LA in normal controls ($p<0.01$), with cells per mm2 of LP compared to diseased. A decrease in overall cell numbers was observed in both LA LP ($p<0.01$) and SA wall $p<0.001$) in COPD when compared to normal non-smokers. In NLFS in LA, TC numbers were intermediate ($p<0.01$) while in ex-smokers the cell numbers were essentially back to NC levels though with wider variation ($p=0.15$). In the SA, TC numbers were significantly fewer compared to normal controls in all smoker/COPD groups with little difference between these three (Figure 4.1 A. and B.).

4.3.2 Neutrophils

Neutrophil numbers were similar between the SA and LA in NC. Neutrophil numbers in the pathological groups in large airways reflected the changes in total cell counts, i.e. were reduced with least density observed in COPD-CS ($p<0.01$). In SA, However, neutrophil density was little different from normal in all groups, even if apparently (non-significantly) slightly reduced in the NLFS (Fig 4.1 C. and D.).

Figure 4.1

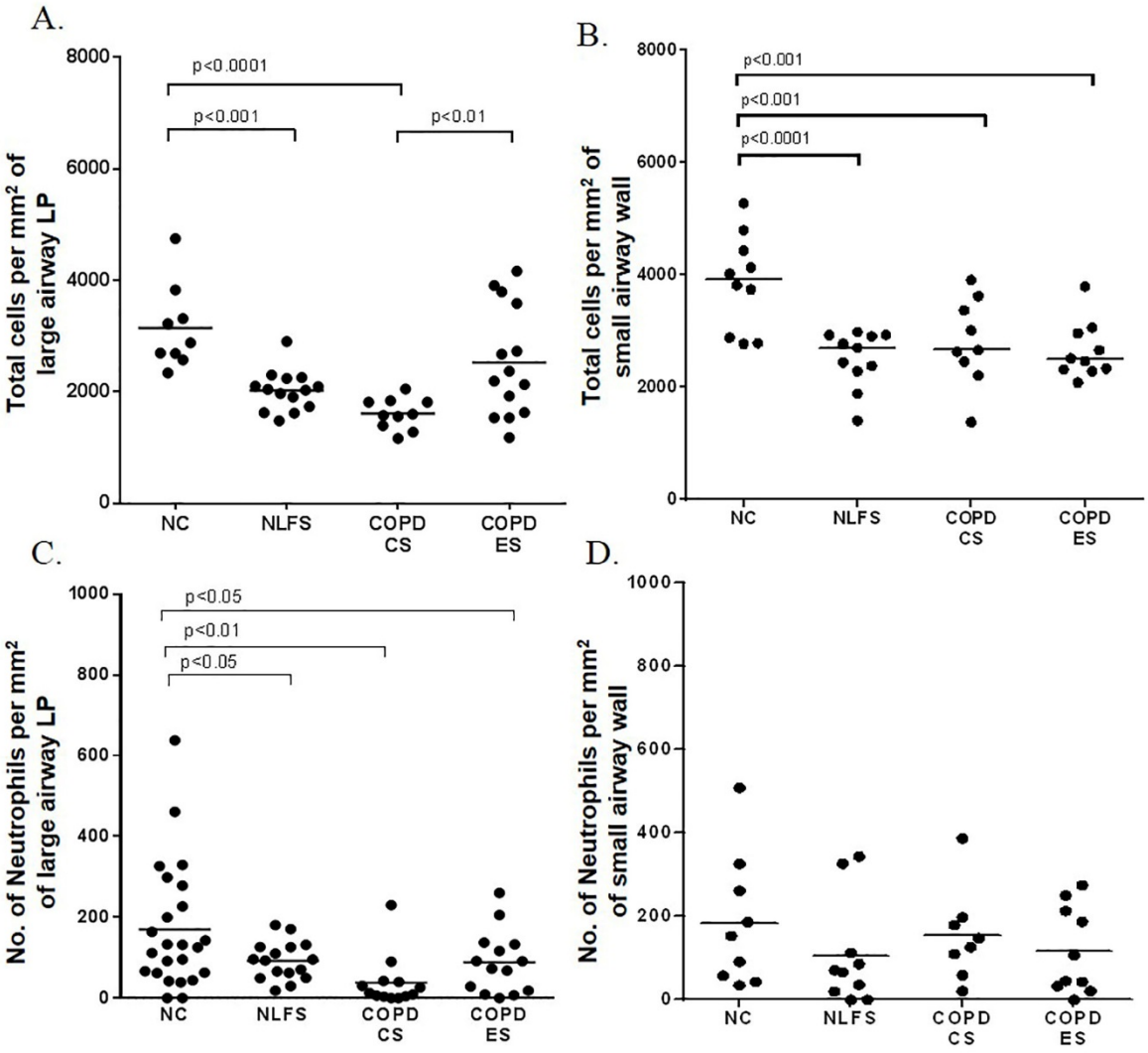

Figure 4.1. Total number of cells per square mm of the airway wall examined: (A). Large Airways; (B), Small Airways. Neutrophils per square mm of the airway wall examined: (C), Large Airways; (D), Small Airways

4.3.3 **CD68+ cells**

I observed two morphologically variable populations of CD68+ cells: round macrophages and spindle-shaped cells, which I will refer to as macrophages and fibroblast-like cells respectively (Fig 4.2 A. and B). In normal, LA these CD68+ fibroblast-like cells were much more numerous than macrophages, but in contrast, macrophages dominated the SA CD68+ profile (Figure 4.3, Figure 4.2 C and D).

In COPD-CS LA, compared to normal controls, I found fewer macrophages ($p<0.05$), while in contrast, macrophages in the NLFS were significantly higher ($p<0.001$) than normal. In COPD-ES subject's macrophages numbers were close to normal levels. In SA, although macrophage numbers were high generally, I found no significant change from normal in their numbers in smoker/COPD groups (Figure 4.3).

Fibroblast-like cells were slightly higher in normal LA than normal SA but not significantly so ($p=0.09$). There was a reduction in all smoker/COPD groups in large airways (significant only for NLFS, $p<0.01$) but fibroblast-like cell densities were unchanged in corresponding small airways (Figure 4.3).

Figure 4.2

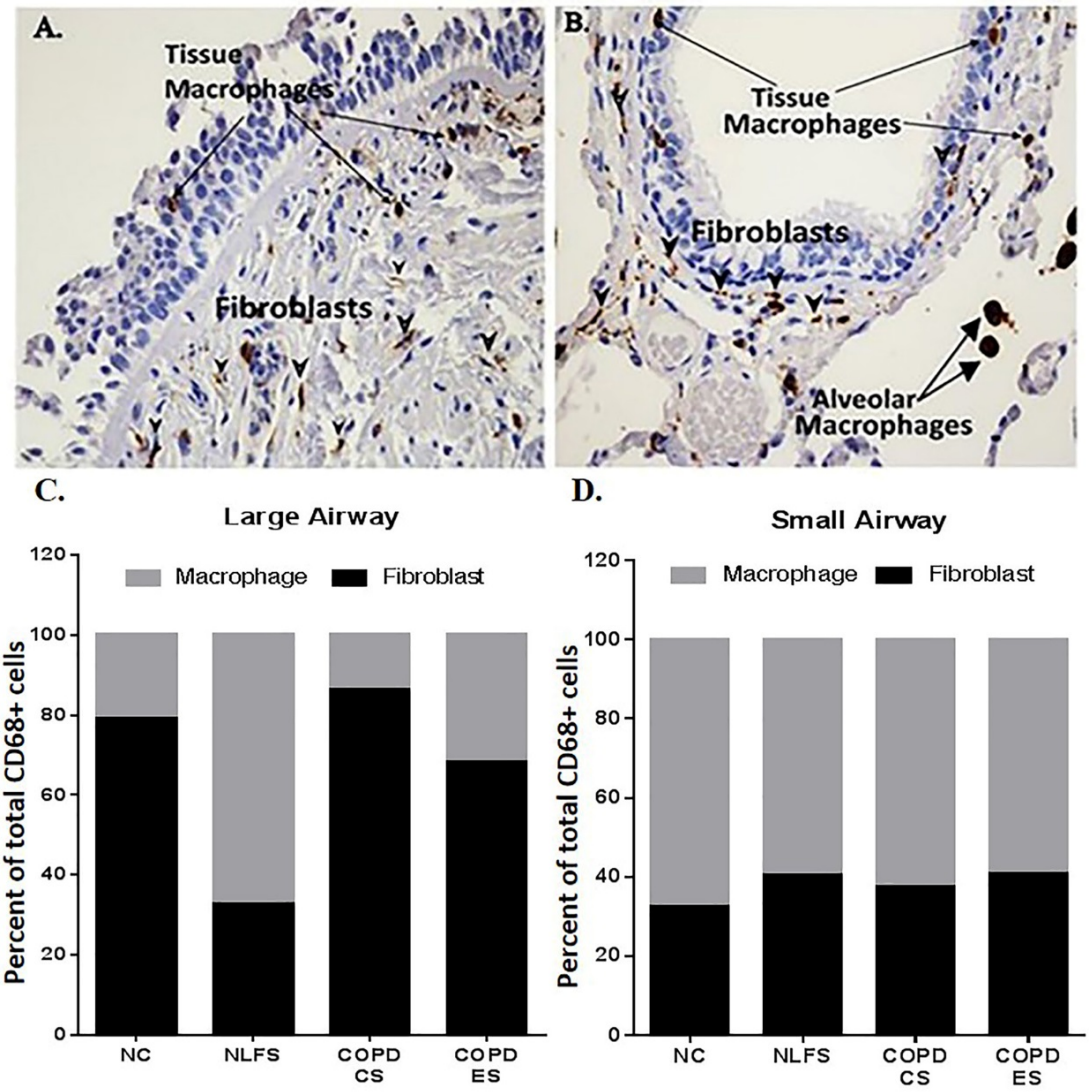

Figure 4.2 Illustrates the presence of two morphologically distinct populations of CD68+ in (A). Large Airways and (B). Small Airways (400X magnification). (C) and (D), represent percentages of the total CD68+ for each morphological phenotype.

Figure 4.3

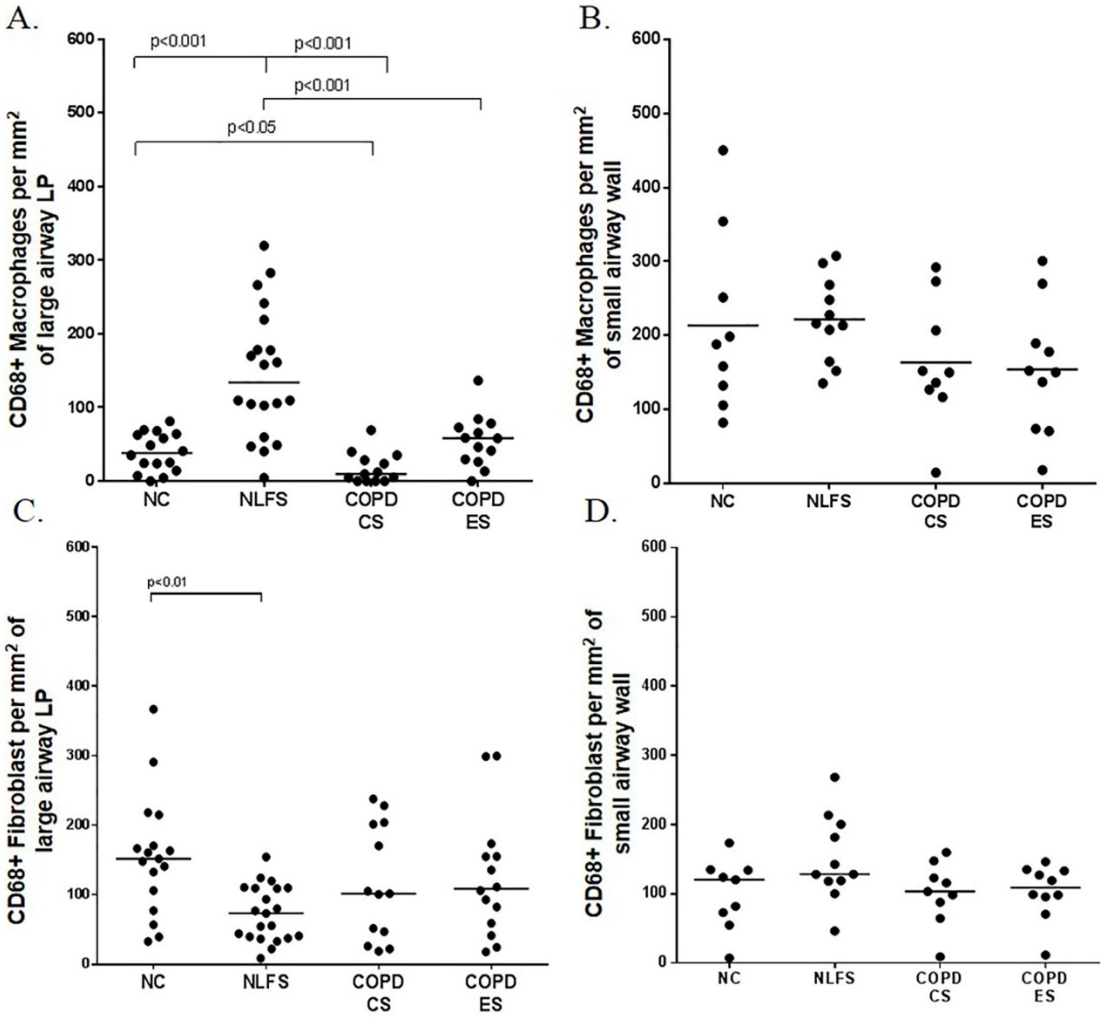

Figure 4.3 A, B, C, and D represents the data for CD68+ve round-shaped macrophages, and spindle-shaped fibroblast-like cell numbers per square mm of airway wall examined.

4.3.4 **Lymphocytes**

In the LA wall, the lymphocyte populations studied were dominated by the CD8+ phenotype. Both CD8+ and CD4+ T cells were found to be lower than normal in the smoker/COPD groups but with some substantial changes in their ratio, especially in the COPD-CS where there was an even more marked relative excess of CD8+ over CD4+ lymphocytes (Figure 4.4D).

There were many fewer CD4+ T cells throughout the airways, and indeed so few in the small airway that I did not formally count them. In contrast in SA, CD8+ cells tended to be higher than normal across all clinical groups, and especially so in COPD-CS ($p<0.01$).

Figure 4.4

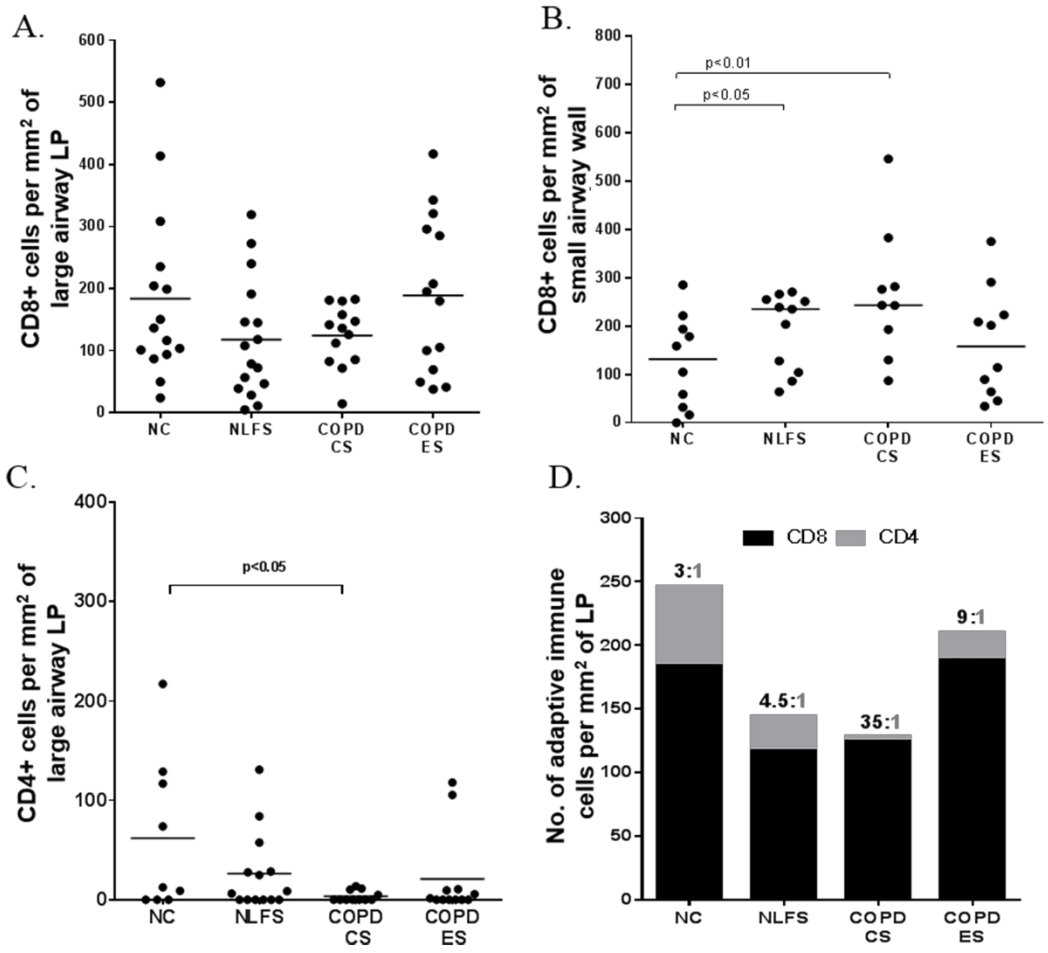

Figure 4.4. Number of CD8+ve T cells per square mm of airway wall examined: (A), Large and (B), Small airways. (C), CD4+ve T cells in LA. (D), Ratio of CD8+ to CD4+ T cells in the LA LP.

4.3.5 **Inflammatory cells in airway epithelium and Rbm**

Overall, the inflammatory cell densities in the airway epithelium and Rbm were far lower in comparison to the LP. Although the absolute number was small, a significant increase above normal was observed for macrophages in the epithelium of NLFS when compared to NC (Table 5).

Table 5. Inflammatory cells in airway epithelium and Rbm in large and small airways

Epithelium					
Large Airways	Neutrophils	Macrophages (CD68+)	Fibroblasts (CD68+)	CD8+	CD4+
NC	0.4 (0-10)	0 (0-2.5)	0 (0-9.3)	3.7 (0-13)	0 (0-1.7)
NLFS	0.5 (0-5.6)	2 **(0-6.5)	1.1 (0-4.9)	7.3(0.5-21.8)	0 (0-4.1)
COPD-CS	0 (0-7.5)	0 #(0-3.9)	0.5 (0-9.3)	5.4 (1-23)	0 (0-0.9)
COPD-ES	0.4 (0-2)	1.9 #(0-1.8)	0.2(0-3.15)	5 (1-25.6)	0 (0-3.2)
Small Airways					
NC	0 (0-0.5)	2.1(0-6.4)	0.5 (0-3.2)	0.5 (0-9.1)	-
NLFS	0 (0-1.4)	3.6 (0.4-9.4)	1.0 (0-6.5)	1.3 (0-12)	-
COPD-CS	0.5 (0-2.4)	3.4(1.2-5.3)	0.5 (0-3.6)	2.6(0-11.3)	-
COPD-ES	0.2 (0-3.7)	2.3 (0.3-6.2)	1.0 (0-3.6)	0.48 (0-2.2)	-
Rbm					
Large Airways	Neutrophils	Macrophages (CD68+)	Fibroblasts (CD68+)	CD8+	CD4+
NC	3.0 (0-13.29)	0 (0-0.36)	0 (0-2.3)	0 (0-4)	0 (0-1.1)
NLFS	2.5 (0.6-9.6)	0 (0-1.8)*	0 (0-3.4)	0 (0-2.7)	0 (0-0.4)
COPD-CS	0.5 (0-10.26)	0 (0-0.33)	0 (0-2.3)	0 (0-2.1)	0
COPD-ES	0.8 (0-7)	0 (0-0.17)	0 (0-2.3)	0(0-1.9)	0
Small Airways					
NC	0.4 (0-4.3)	0.0 (0-1.5)	0.0 (0-1.5)	0 (0-1.2)	-
NLFS	0 (0-0.97)	0.5 (0-2.17)	0.4 (0-2.17)	0 (0-0.4)	-
COPD-CS	0 (0-1.65)	0 (0-1.0)	0.4 (0-1.0)	0 (0-0.4)	-
COPD-ES	0 (0-2.5)	0 (0-0.45)	0.3 (0-0.45)	0 (0-0.6)	-

Data are presented as medians (range).

** and * Represents $P < 0.01$ and $P < 0.05$ compared with NC, respectively; # represents $P < 0.05$ compared with NLFS; — represents not done.

COPD-CS, current smoker with COPD; COPD-ES, ex-smoker with COPD; LA, large airway; NC, normal control; NLFS, normal lung function smoker; Rbm, reticular basement membrane; SA, small airway.

4.4 Discussion

In contrast to the current belief of COPD pathology being primarily an inflammatory airway disease, we have found that the main finding in the COPD "airway wall" is a reduction in total cellularity at least in the mild-moderate disease. This overall decrease was accompanied by quite small fluctuations in specific inflammatory cell types; the most marked change was a general increase in macrophages in the small airway wall compared to the large airways, but this was in both healthy controls and the diseased as well. Changes in neutrophil numbers largely tracked total cell counts. In the COPD large airway wall, CD8+ T cells were the dominant lymphocyte phenotype over CD4+T cells and were the only cell type found to be significantly increased in COPD in the small airway wall.

These data are unique in their comprehensive nature, although the importance of sampling the airway wall has been emphasized as needed to complement more easily obtained luminal cell counts in BAL or sputum (O'Donnell et al., 2004). The activation of an innate neutrophil and macrophage reaction in the airway lumen in COPD is well established. This may be because of smoking itself or secondary to infection, which may have a crucial role (Pesci et al., 1998, Sethi et al., 2006).

An earlier report (Di Stefano et al., 2001) suggested a moderate increase in the number of neutrophils in the sub-epithelial airway wall lamina propria (LP) in large airway biopsies in mild to moderate COPD in comparison to smokers, but typically this finding is difficult to evaluate since there was no normal control group. Our study suggests, in contrast, that in the airway wall the neutrophils are reduced in stable COPD subjects especially in the large airway LP. These data also suggest that the neutrophils trend back towards normal levels in ex-smoker COPD, suggesting mainly a smoking and to a lesser extent a COPD effect.

For the small airways, published neutrophil data have been both limited and contradictory. Studies conducted by Lams et al., (1998) suggested no change in smokers, which was similar to the conclusion of Hogg et al., (2004), in mild COPD subjects (GOLD stage I), while more recent evidence suggested a small increase in neutrophil numbers in mild to moderate COPD patients (Utokaparch

et al., 2014). My findings again showed no significant change in COPD subjects in comparison to either normal lung function smokers or normal controls.

Neutrophils are known to be frontline innate inflammatory defenders against infectious agents; they also play a major role in generating chemotactic factors for recruitment of other cells, especially macrophages. Whether functionally the reductions in neutrophils we described are sufficient to suppress innate immunity within the airway wall is not known, but it is possible. The lack of neutrophilia in the airway wall of smokers and COPD could be hypothesized as being due to active egress of neutrophils into the airway lumen, though there was no obvious gradient of wall neutrophils from deep to superficial (epithelium). Whether such a postulated but unproven trans-epithelial migration would have a beneficial or adverse effect on the airway wall itself needs further investigations (Persson and Uller, 2010, Porter, 2011).

I demonstrated a significant decrease in the number of macrophages in the large airway wall in COPD. Interestingly, macrophages were increased in normal lung function smokers, but it seems doubtful that this reaction is related to COPD development. My macrophage findings are contrary to those of Di Stefano et al., (1998) who suggested a small increase in large airway macrophages in COPD, but they only had smoking controls.

In the current study, I have shown morphological variation in cells expressing the CD68 epitope. Thus, I have observed two phenotypically distinct populations staining with CD68; one is more rounded and darkly staining and considered as typical macrophages phenotypically, while the others more lightly stained, spindly and fibroblast-like. This duality of CD68 staining has been previously shown in other disease tissues and in (Kunisch et al., 2004, Lau et al., 2004, Kunz-Schughart et al., 2003, Gottfried et al., 2008) but so far has received little attention in the respiratory literature.

The small airway wall had far greater number of both “true” tissue macrophages and also the more fibroblast-like CD68+ cells. This would be consistent with small airway fibrosis, but the uniform numbers belied this across groups with no signal here for COPD. The macrophage phenotypes have been established in recent years; macrophages can exist in the pro-inflammatory, tissue-destructive M1 phenotype and also as a phagocytic, anti-inflammatory, anti-bacterial and pro-fibrotic M2 phenotype (Martinez and Gordon, 2014). I

hypothesize that the tissue milieu will determine the fate of macrophages and the variability of the individual population of these cells in the airway wall is actively investigated. Preliminary data (Sukhwinder et al., 2016) indicate that in the airway wall there may be phenotype shift from M2 to M1 macrophages, which could perhaps be the strongest signal found for a true inflammatory signal and has been further confirmed in the next chapter.

The decrease in both CD8+ and CD4+ T cells in the large airway LP in COPD are contradictory to findings from O' Shaughnessy et al., (1997) where both CD4+ and CD8+ T cell numbers were said to be increased, at least in those with "chronic bronchitis". The difference in result may be a consequence of this phenotypic co-morbidity absent in our subjects. However, we agree with the one other report (Di Stefano et al., 2001).

My observations on CD8+ to CD4+ T cell ratios did suggest that the large airway wall in COPD is immunologically skewed toward CD8+ T cells, perhaps a response to the susceptibility of COPD patients to airway viral infection (Utokaparch et al., 2014, Koch et al., 2008). The decrease in CD4+ T cell in COPD could be attributed to the presence of suppressor CD8+ T cells co-expressing CD103, which reduce the proliferation of CD4+ T cells (Koch et al., 2008)), and this should be studied. CD8+ T cells were decreased in large airway LP with both smoking and COPD but CD4+ cells markedly more.

The current report is limited to the more highly cited "classical" inflammatory cells. Also, we have previously observed an increase in mast cells in COPD large airways LP (Soltani et al., 2012), and are studying small airway mast cells. Others have looked at dendritic cells (CD83+) and found them to be few and variable in number and reduced essentially to zero in the airways in COPD (Tsoumakidou et al., 2009). In contrast, Demedts et al. (2007) showed a slight increase in Langerin+ DC in small airways in mild COPD, though absolute numbers were very low. Our assessment of CD11c+ dendritic cells in COPD large airway LP found them to be too few to analyse (Sohal et al., 2011). There are even fewer data available on eosinophils in the airways wall of COPD patients, and these are variable and contradictory (Saetta et al., 1999, Lacoste et al., 1993), but absolute numbers were consistently very low. Thus, even if these cell types were added to the calculation, the total inflammatory cell number in the airway is a small proportion of total cellularity, with over 70%

being “others,” and most likely stromal cells (Fig 6). We are currently trying to define these more systematically.

In conclusion, I have found that there is a marked decrease in total cellularity in both large and small airway walls of smokers and especially in COPD. This change is reflected in a decrease in the number of inflammatory cells in the airway wall in these clinical groups. One is left very hard-put to defend the currently prevalent dogma that COPD is inherently an airway (wall) inflammatory disease; at most the small change in CD8+ to CD4+ T cell balance in the large airways might fit the bill.

Chapter 5

Abnormal M1/M2 macrophage phenotype profiles in the small airway wall and lumen in smokers and chronic obstructive pulmonary disease (COPD).

Note: This chapter is published with 'Scientific reports' and is explained in with the overall textual context.

Eapen, M. S., Hansbro, P. M., Mcalinden, K., Kim, R. Y., Ward, C., Hackett, T.-L., Walters, E. H. & Sohal, S. S. 2017. Abnormal M1/M2 macrophage phenotype profiles in the small airway wall and lumen in smokers and chronic obstructive pulmonary disease (COPD). Scientific Reports, 7, 13392.

5.1 Introduction

With the overall decrease in cellularity in the small airways and further having observed no change in the CD68+ macrophages of COPD patients in their small airways, it was imperative to analyze and provide a more thorough assessment of the role of macrophage subpopulation in driving the inflammatory process.

Macrophages are known to exhibit polarized phenotypes, with, M1 and M2 subpopulations reflecting the paradigm of Th1 and Th2 lymphocytes (Mills, 2012). M1 macrophages have been described as cytotoxic and pro-inflammatory, and are characterized by secretion of the cytokines interferon (IFN)-γ and lL-12, and by promoting Th1-type immunity (Mills, 2015, Beckett et al., 2013). In contrast, M2 macrophages are considered anti-inflammatory and are linked to tissue repair and fibrosis, secreting pro-Th2 cytokines including CCL22, IL-4, IL-13 and IL-10 (Martinez and Gordon, 2014).

Macrophages have the fundamental ability to metabolize L-arginine to nitric oxide (NO) or ornithine, using the mutually substrate-competitive enzymes inducible nitric-oxide synthase (iNOS) or arginase-I (Arg-1), respectively (Benson et al., 2011). Phenotypically M1 macrophages exhibit increased iNOS expression, while M2 macrophages are typified by an increase in Arg-1, which promotes collagen synthesis by making the amino acid proline available to fibroblasts (Benson et al., 2011, Mora et al., 2006). The competition between

iNOS and Arg-1 for L-arginine can drive contrasting pathologies functionally through opposed macrophage phenotypes (El-Gayar et al., 2003).

In the chapter, I have characterized the phenotypic and metabolic regulatory dichotomy of airway wall macrophage populations and their microenvironments in human lung tissue and bronchoalveolar lavage (BAL), and related phenotype is switching to smoking, COPD, and lung function.

5.2 Overview of materials and methods

The subjects in this cross-sectional study involved have been detailed in Table 3 and Table 4 in the method section. I again employed classical immunohistochemical methods including dual staining techniques for the identification of M1 macrophages in the airway wall as well as in the lumen (chapter 3 page 76, 77). The M0, M1 and M2 macrophages were enumerated in the small airway epithelium and sub-epithelium, and airway lumen BAL cells (chapter 3, page 81). Multiplexing strategies were used in estimating the BALF cytokines in all the four groups evaluated (Chapter 3 page 84).

5.3 Results

5.3.1 M1/M2 phenotypes in small airways

In the small airways epithelium, the dominant macrophage type in both in numbers and percent terms was the non-differentiated M0, especially in controls subjects (figure 5.1 a, c), where there were essentially very few M1 (median 0%; range 0-5.2) macrophages. There was a significant increase in M1 population in normal lung function smokers (NLFS) [median 18%; range 0-100; ($p<0.05$)] and COPD current smokers (COPD-CS) [median 21.2% range 0.0-64.1; ($p<0.01$)], which slighty reverted in COPD ex smoker (COPD-ES) [median 10% range 0.0-42; ($p<0.05$)] (figure 5.1 c). Small numbers and percent M2 macrophages were present in the controls (median 6.4%; range 0.0-43.3) but were almost absent in NLFS [median 0% ; range 0.0-9.0 ($p<0.05$)], COPD-CS [median 0% ; range 0.0-0.01 ($p<0.01$)] and COPD-ES [median 0% ; range 0.0-10.1 ($p<0.05$)] (figure 5.1b, c).

In the subepithelium in NC, the M0 population was less dominant than in the epithelium, with fewer percent M1 (median 1.6% range 0.0-6.3) and more M2 macrophages (median 36%; range 0.0-63) (figure 5.1f). A significant rise in the M1 population was observed in the NLFS [median 10.1%; range 0.0-60.2 ($p<0.01$)] and COPD-CS [median 9.6% range 1.6-48 ($p<0.05$)]. There were declines compared to normal in the M2 population in smokers (median 23% range 0.0-64.1) and COPD, again especially significant in COPD-CS [median 8.4%;range 0.0-19.9 ($p<0.01$)] (figure 5.1f). No statistical difference was observed between NLFS and COPD-CS.

There was a positive correlation between smoking pack-years and increase in M1 macrophages in the epithelium (Pearsons $r = 0.55$, $p = 0.006$) (figure 5.3a.) while a negative correlation was observed for sub-epithelial M2 macrophages (Pearsons $r = -0.46$, $p = 0.02$) (figure 5.3b).

Figure 5.1

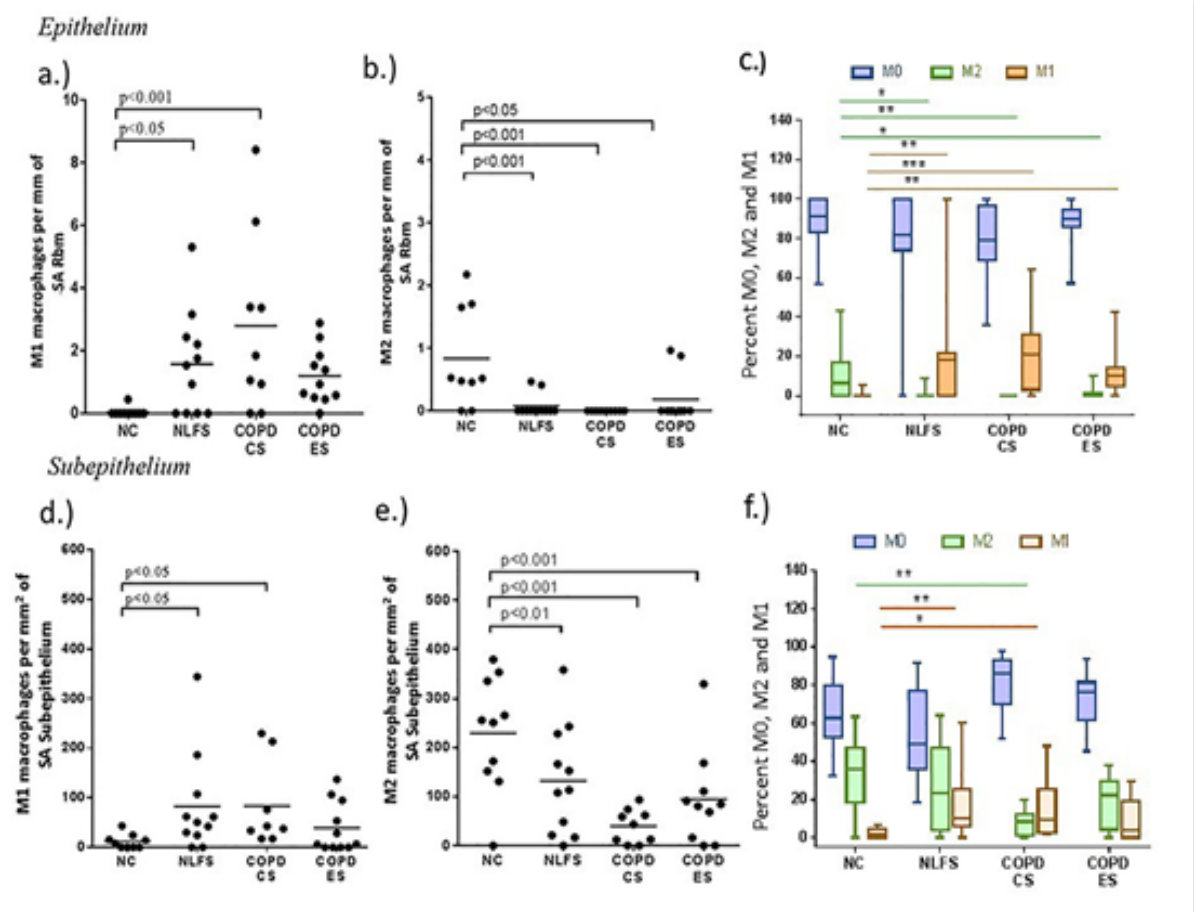

Figure 5.1 Macrophages phenotype numbers in SA tissue. In epithelium: an increase in the numbers of a) M1 and a decrease in b) M2 macrophages observed in smokers and COPDs compared to NC. Similar pattern was also observed in the sub-epithelium d) M1 and e) M2. Percent change in macrophage phenotypes M0, M1 and M2 in c) Epithelium and f) Sub-expressed epithelium (Data in median and range; *p<0.05, **p<0.01).

Figure 5.2

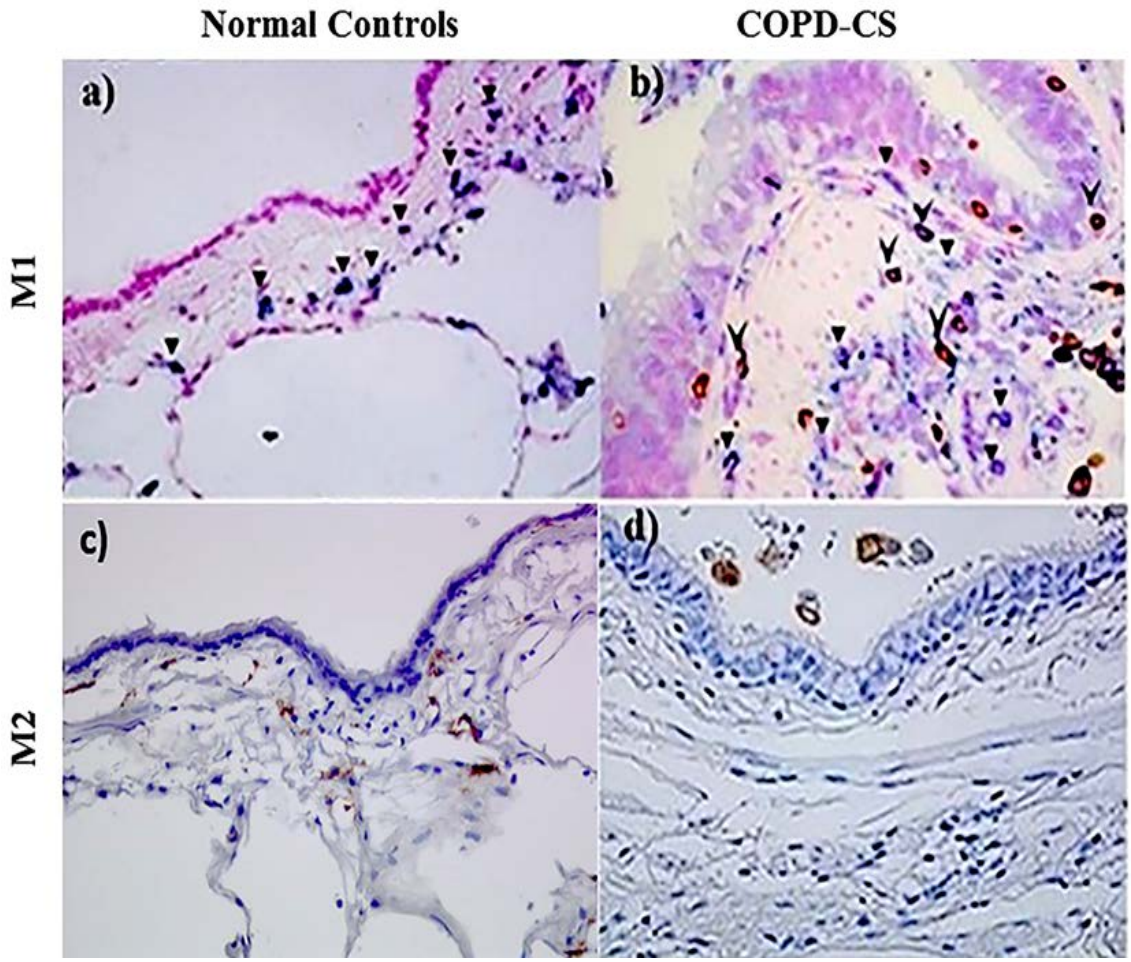

Figure 5.2 Representative micrographs of M1 macrophages dual stained for iNOS (brown) and CD68 (blue). a) Thin epithelium and thin walled normal control, b) thick epithelium and thick walled COPD-CS, counterstained with nuclear fast red (pink). (⮟) Dual stained CD68+iNOS+ cells, (▼) only CD68+ cells. CD163 staining M2 macrophages c) NC d) COPD-CS.

Figure 5.3

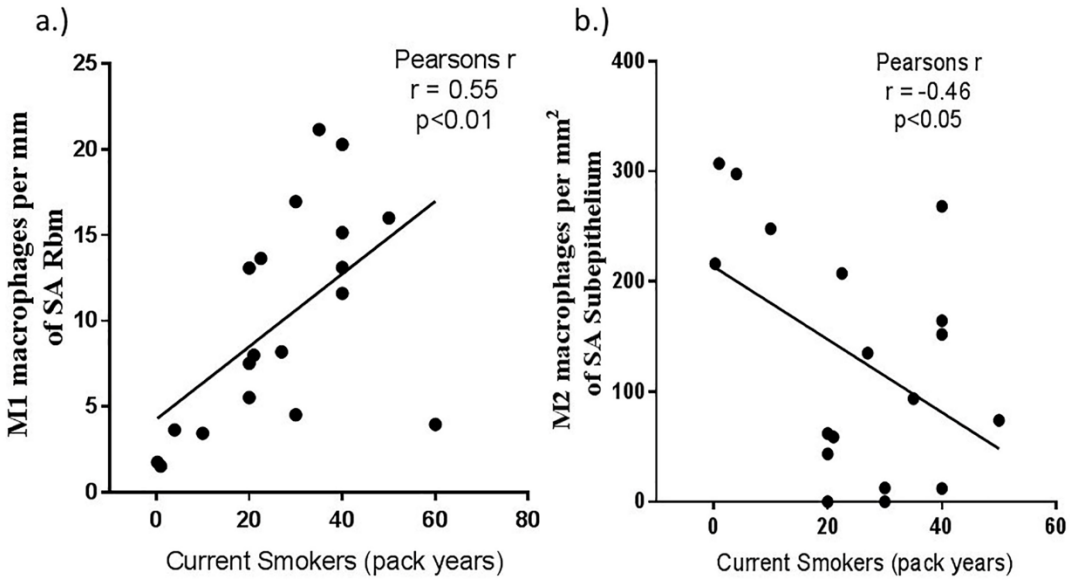

Figure 5.3 Regression analysis for tissue macrophage phenotypes with pack year history for NLFS and COPD-CS for a) epithelial M1 and b) sub-epithelial M2 macrophages.

5.3.2 **Arginase-1 (Arg-1) expression in the SA wall**

The small airways wall tissue of COPD-CS showed a marked overall non-specific increase in tissue expression of Arg-1 in the small airway wall of COPD-CS, both in the epithelium ($p<0.01$) and sub-epithelium ($p<0.001$) in comparison to normal controls (figure 5.4). At this stage, we have not quantified this in non-COPD smokers, but descriptively the staining is present but less abundant.

Figure 5.4

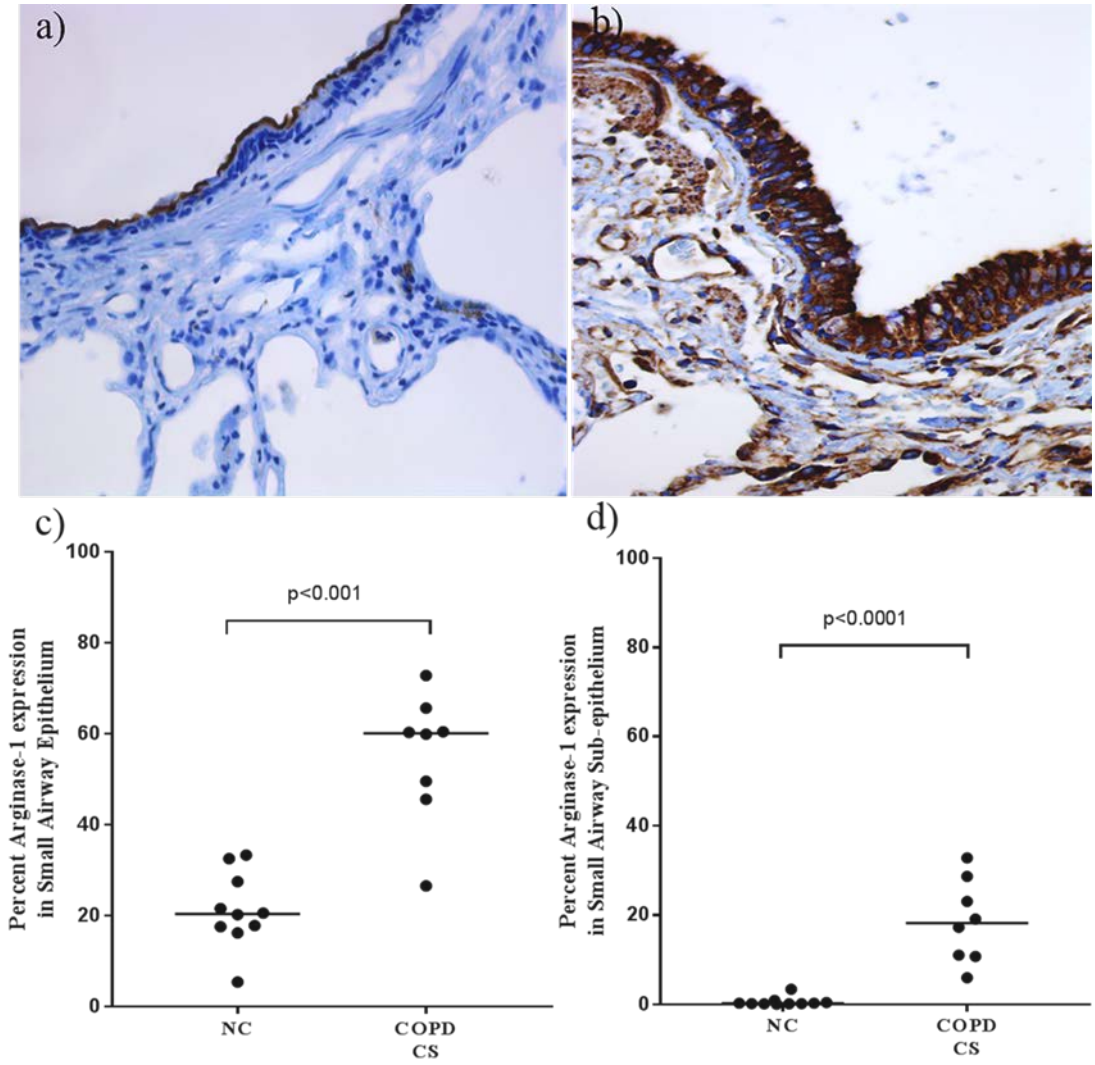

Figure 5.4 Pictorial representation of the Arginase-1 expression in the airway wall of a) NC compared to b) COPD-CS. A significant increase in Arginase -1 expression was observed in both c) epithelium and d) sub-epithelium. (Data presented as median and range; group comparisons with Mann-Whitney two-tailed t-test).

5.3.3 **AM phenotypes in the BAL**

When comparing the alveolar macrophages within the alveolar spaces (figure 5.5 a-c) of resected lung tissue (also containing small airways) of COPD patients, we observed similarity in both morphological and M1/ M2 expression patterns with luminal macrophages derived from BAL lumen (figure 5.6 b, d and f). However, we have provided here only the quantitative results from macrophages from the BAL samples. A two to three-fold increase in total BAL CD68+ AMs was found in NLFS ($p<0.01$) and COPD-CS ($p<0.05$), while in COPD-ES they were similar to normal levels (figure 5.7a). Unlike the tissue macrophage data, there was fewer undifferentiated percent M0 AMs across the groups (figure 7d).

The BAL AMs in NC were predominantly M1 (median percentage 66.3%; range 31.5-91.2) with essentially fewer M2s (median percentage 0% range 0.0-42.7) (figure 5.7d). There was a marked change in phenotype profiles in the clinical groups, with a decrease in the percent of M1 (median percentage 26.3%; range 0.1-53.1) in NLFS, COPD-CS (median percentage 40.4%; range 6.2-82.5) and ES (median percentage 33.6%; range 4.0-80). Further, increases in M2 macrophages was observed in (median percentage 49.5%; range 31.5-91.2) in NLFS, COPD-CS (median percentage 27.15%; range 0.2-71.9) and COPD-ES (median percentage 21.9%; range 1.3-72.7) (figure 5.7d).

Both total CD68+AMs (Spearman's rho (rs) = -0.35, p = <0.05) and M2 AMs (Spearman's rho (rs) =-0.5, p <0.01) (figure 5.8 a, b) correlated negatively with FEV_1/FVC.

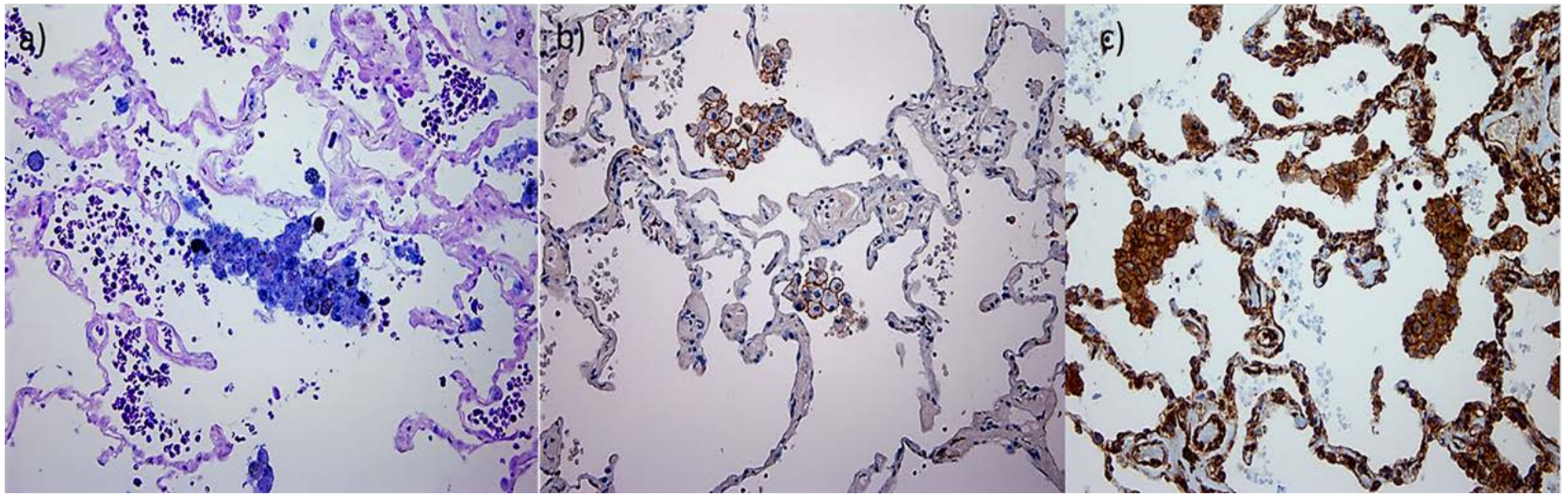

Figure 5.5 Images representing expression patterns of alveolar macrophages (AMs) in the alveolar spaces in COPD patients (200x), a) M1 AMs dual stained for iNOS (brown) and CD68 (blue), counterstained with nuclear fast red (pink), M2 phenotype macrophages stained brown with b) CD163 and c) arginase-1(ARG-1), counterstained with nuclear-stained hematoxylin (blue).

Figure 5.6

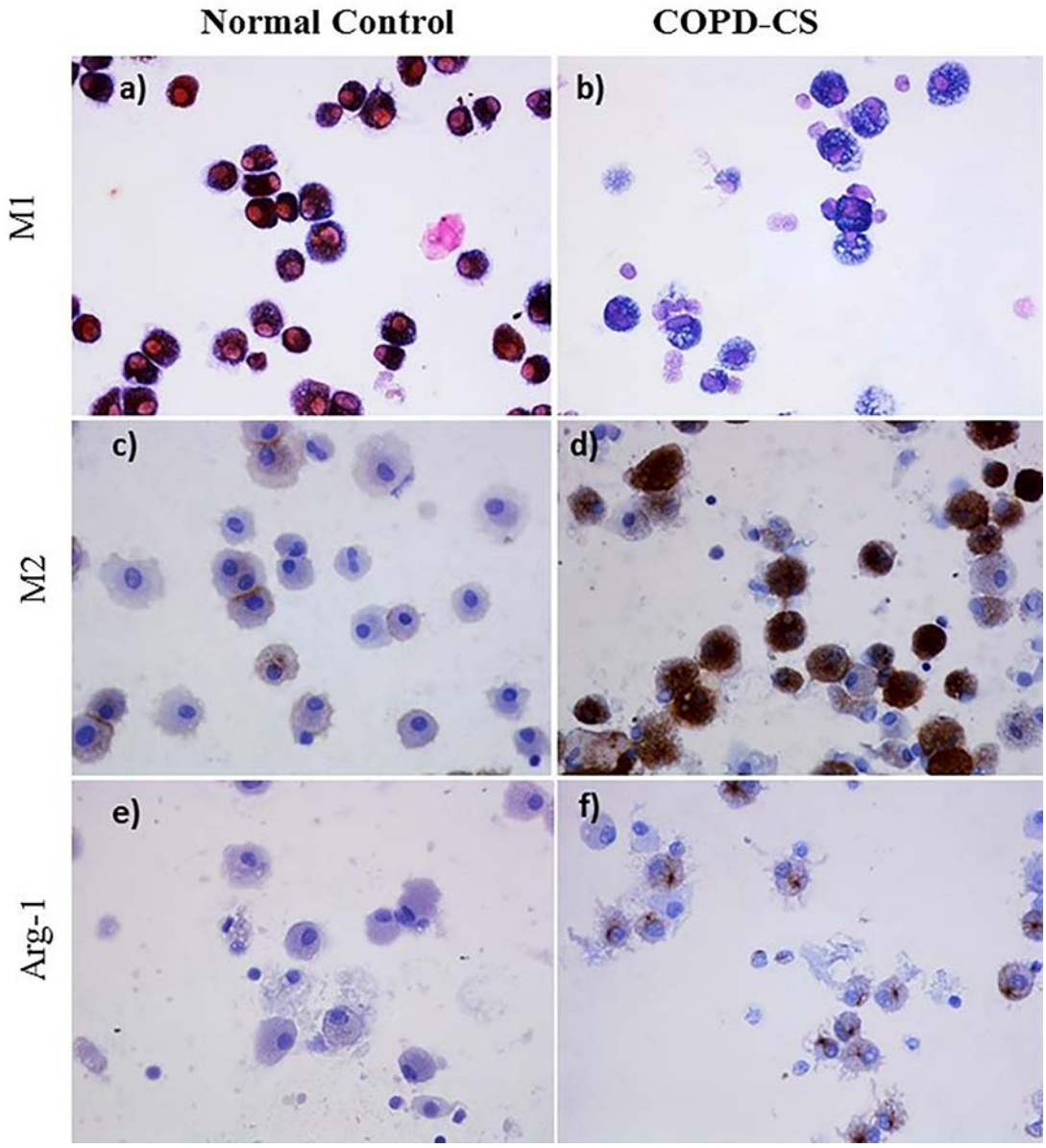

Figure 5.6 Representative pictures (400x) of M1 AMs dual stained for iNOS (brown) and CD68 (blue); a) Normal control, and b) COPD-CS, counterstained with nuclear fast red (pink). M2 phenotype macrophages stained brown with CD163 and arginase-1(ARG-1), (c, e) Normal controls and (d, f) COPD-CS respectively, with nuclear-stained hematoxylin (blue).

Figure 5.7

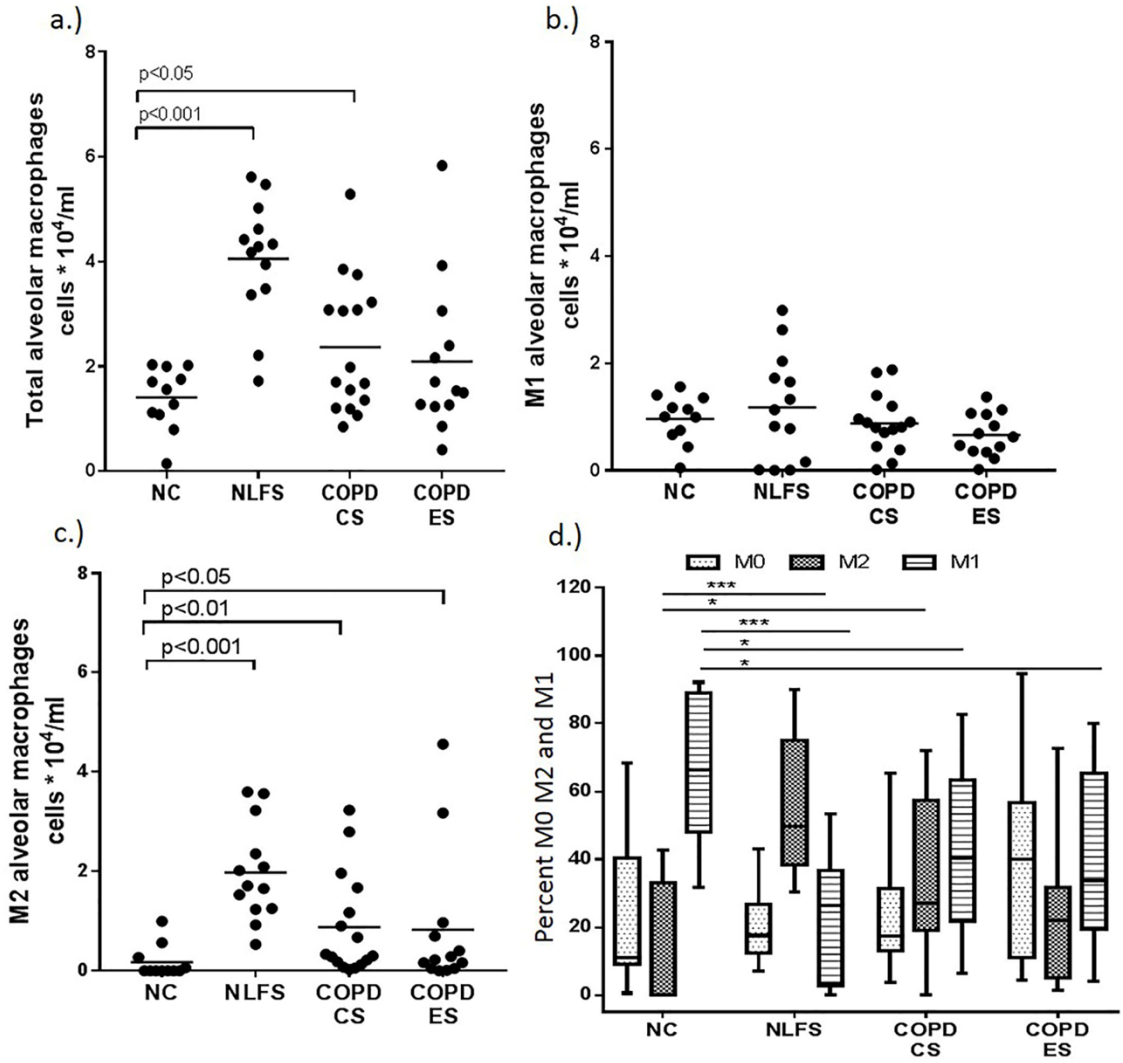

Figure 5.7 AMs numbers in BAL. a) Total AMs, b) M1 and c) M2 macrophages in NC, NLFS COPD-CS, and ES. d) Represent percent of total macrophage for each phenotypic population (Data in median and range; *** p<0.001, *p<0.05).

Figure 5.8

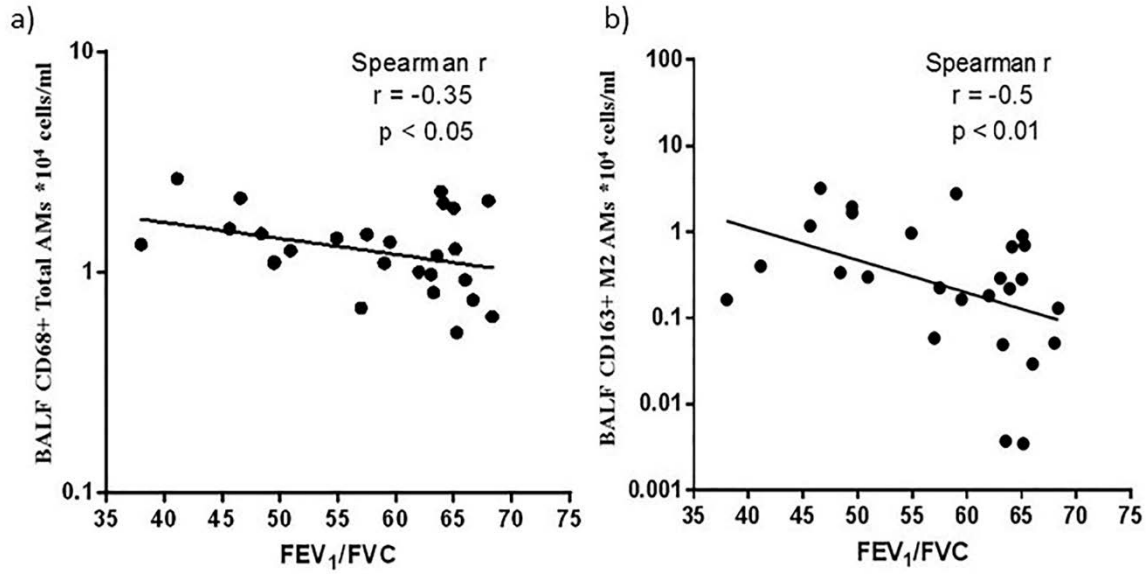

Figure 5.8. Regression analysis for BAL AMs with lung function in COPD groups. a) Total AMs and b) M2 AMs.

5.3.4 **BAL M2-associated cytokines were increased in COPD**

A marked increase in M2-related MDC/CCL22 and IL-4, IL-13, and IL-10 was observed in the BAL supernatants from NLFS and COPD subjects (figure 5.9a), with a decrease in the M1 cytokine IL-12p40 but not IFN-γ (figure 5.9b). We also found a significant increase of the pleiotropic cytokine IL-6 in all pathological (smokers and COPD) group (figure 5.9c). Further, a small but significant increase in proinflammatory IL-1β was observed in smokers but not in COPDs while for TNFα there was an increase in COPD-CS compared to normal controls (figure 5.9c). An increase in the ratio of IL-12p40 to IL-4 (M1/M2 cytokines) confirmed the switch to M2 dominance in BAL COPD-CS (figure 9d). There was a positive correlation in COPD-CS between CCL22 and IL-4 and M2 macrophage numbers (figure 5.10 a, b).

Figure 5.9

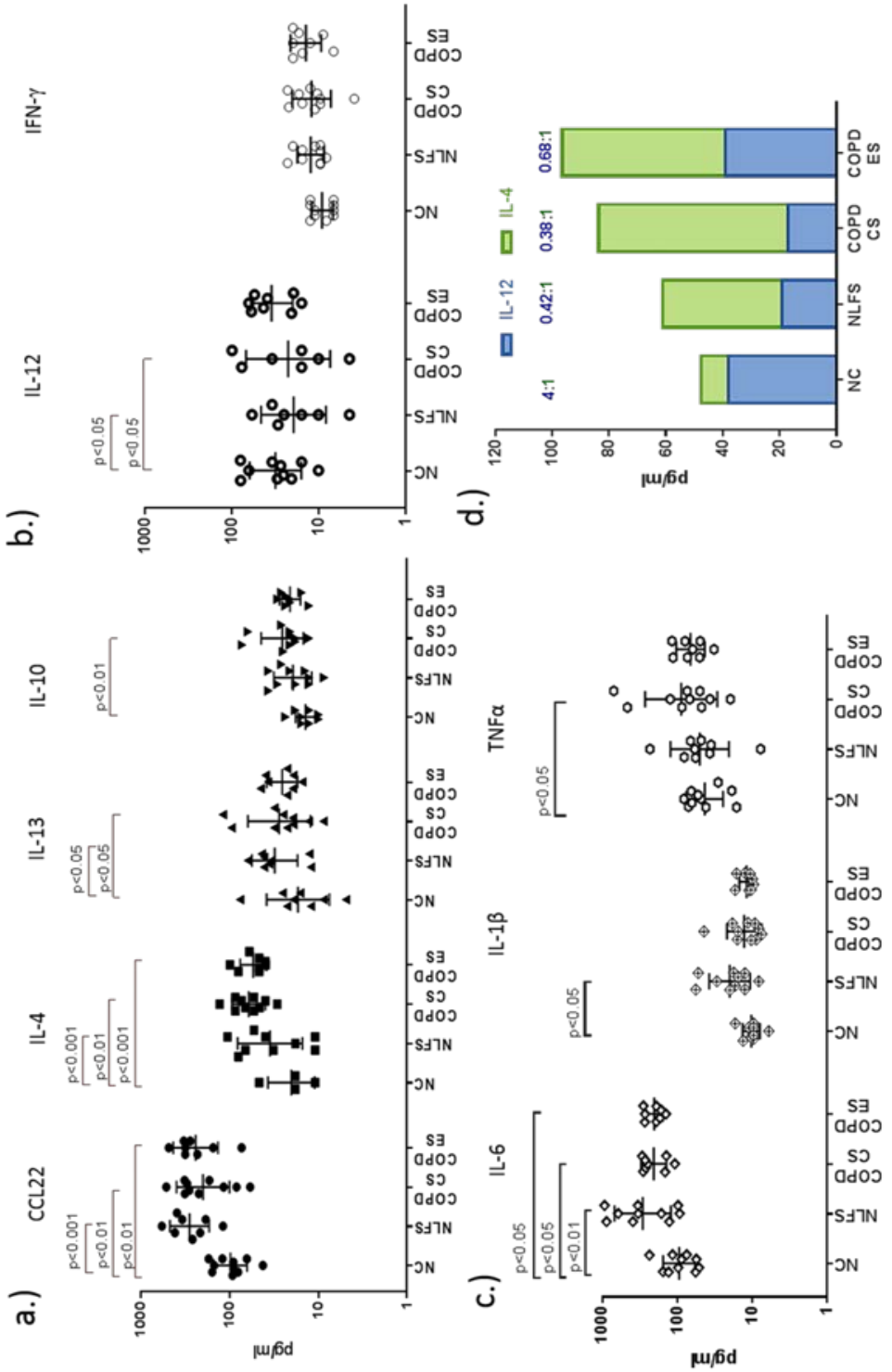

Figure 5.9 Cytokine profile in BAL of NC, NLFS, COPD-CS and ES. a) M1, b) M2, c) inflammatory cytokines (IL-6, IL-1β and TNFα). d) IL-12/IL-4 ratio.

Figure 5.10

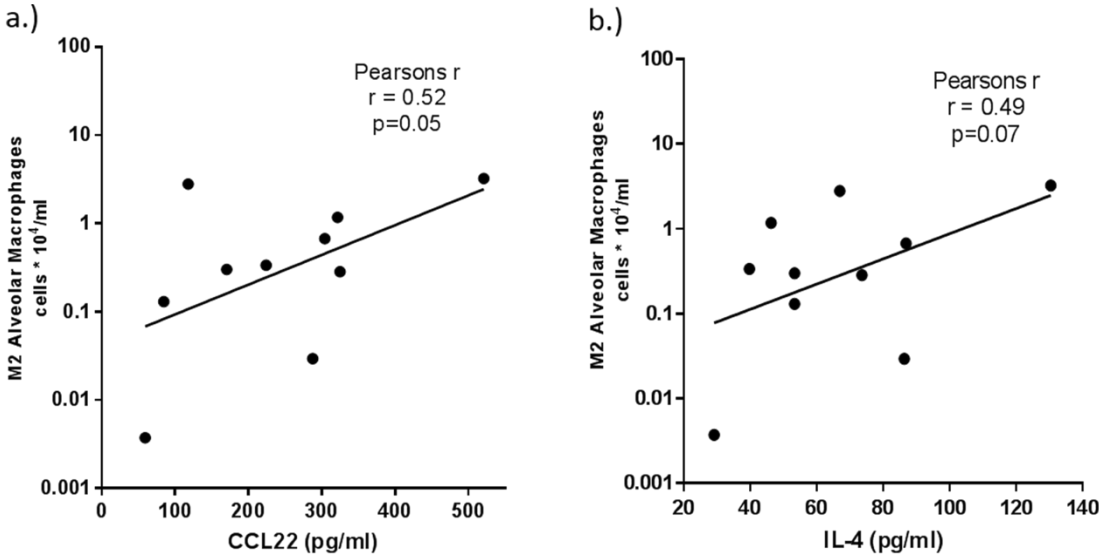

Figure 5.10. Positive correlation observed in M2 macrophage in COPD-CS for d) CCL22 and e) IL-4.

5.4 Discussion

This study is the first to phenotypically differentiate small airway wall and airway lumen macrophage subpopulations based on their M1 and M2 phenotypes in normal, smokers and COPD patients. My observations suggest dynamic differential switching in both the small airways and BAL AMs, but qualitatively these were quite different. In small airways, there was a switch towards predominantly an M1 phenotype in NLFS and COPD-CS, and in BAL AMs there was a switch towards M2 dominance. Further, cytokines in BALF from NLFS and COPD-CS have switched again to M2 characteristics.

M1 and M2 macrophages are considered to be functionally differentiated, with M1 more pro-inflammatory and M2 more pro-fibrotic. For the M1 phenotyping, I chose iNOS+ as a marker (Mills, 2012) with CD68 co-staining to differentiate them from other iNOS producing cells such as dendritic and NK cells. For M2, I used CD163, which is a scavenger receptor that is upregulated in a more TH2 micro-environments (Kaku et al., 2014, Roszer, 2015). Whether the different phenotypic skewing is a compartmental effect or caused by the movement of differentiated cells from wall to lumen needs further investigation. I believe that it is most likely the former as there was no gradient towards the lumen from sub-epithelium to epithelium, and there was no reciprocal decrease in wall cell numbers contributing to the significant increase in luminal macrophages.

The normal predominance of iNOS-expressing M1 macrophages occurs to fight pathogens through luminal production of nitric oxide (NO), an innate immune effector. However, such a response is non-specific, and when uncontrolled can cause considerable damage to host tissues and cells. My observation of reduced percent M1 macrophages in the airway wall in COPD current and ex-smokers compared to normal lung function smokers, therefore, suggests a reduced ability to fight infection (Budden et al., 2017). It may also reflect homeostatic adaptation to avoid excessive tissue damage. Recent evidence suggests that elevated levels of NO inherently suppress the M1 phenotype (Lu et al., 2015).

Given the iNOS changes, I also wished to investigate the functionally reciprocal Arg-1. Interestingly, I found a higher non-specific expression of Arg-1 throughout the airway wall mucosa including epithelium, sub-epithelium and

alveolar septae in COPD-CS compared to normal non-smoker controls. This overexpression of Arg-1 could be the consequence of increased cellular catabolic activity, associated with increased oxidative stress in smokers, catalyzing L-arginine to urea and L-ornithine via urea cycle. L-ornithine is a known precursor to L-proline, a key amino acid in the biosynthesis of collagen, and associated with wound healing (Benson et al., 2011). The excess deposition of collagen, however, leads to airway wall stiffness of the small airways, an important pathophysiological feature in COPD (Hogg et al., 2004). Further, exhaled NO is also well known to be decreased in smokers, but the reason for this has been unclear (Maziak et al., 1998, Hynes et al., 2015).

The finding of an M2 predominance in the airway luminal macrophages in smokers with and without COPD is resonant of two previous studies, although they found an increasing percentage of M2 AMs only in COPD-ex smokers (Kunz et al., 2011, Kaku et al., 2014). One study (Kunz et al., 2011) lacked normal control BAL, and neither assessed the M1 phenotype populations. These findings are in line with the study by Shaykhiev et al., (2009) where gene expression analysis of cytokines and chemokines revealed more M2-polarised AMs in smokers and COPD compared to non-smoking controls. Further, our increase in both total AMs and especially M2 macrophage subtypes correlated to airflow obstruction, suggesting biological plausibility.

The BAL cytokine data reflected the cellular phenotype switching that I observed in that compartment. Invitro studies showed that both IL-4 and IL-13 share a common receptor, IL-4Rα/IL-13Rα1, which signals via the JAK-1/STAT6 pathway, to induce differentially-activated M2 macrophages (Gordon and Taylor, 2005). Again, studies by Rutschman et al., (2001), also showed that the induction of the STAT6 pathway by IL-4 and IL-13 suppressed iNOS expression, and so nitic oxide production, by post-transcriptional modification. Similar studies have also demonstrated that IL-4, IL-13, and IL-10 also synergistically upregulate Arg1 expression in macrophages (Munder et al., 1998). Our current study indirectly corroborates with these findings, but in humans with the clinical disease.

I also observed an increase in CCL22 in BAL in NLFS and COPD. CCL22 is a regulatory chemokine secreted by M2 macrophages in response to TH2 polarized cytokines such as IL-4, IL-5, and IL-13, while it is downregulated

by the TH-1 cytokine lFNγ (Yamashita and Kuroda, 2002, Andrew et al., 1998). Importantly, given the common co-association of COPD and lung cancer, CCL22 has been implicated in tumorigenesis. Its active secretion by M2 tumor-associated macrophages (TAMs), is known to promote malignancy by inhibiting suppressor T cell recruitment. Similar tumorigenic effects have been attributed to IL-6, which I also found to be elevated in smokers and COPD. Further, in vitro studies in macrophages also suggested that IL-6 promotes an M2 phenotype and increased M2-associated markers such as Arg-1 (Fernando et al., 2014).

The differences in the polarization of macrophage subtypes between small airways and lumen are marked. This suggests a difference in the cytokine milieu in each anatomic microenvironment, promoting a shift towards the M1 phenotype in the airway wall but towards M2 in the airway lumen. This may have important implications for the distinct pathologies observed in each site in COPD disease, i.e., infection and ROS-induced innate immune activation in the lumen, but fibrosis and thickening of the airway wall. However, there are limitations to the current study, with tissue originating from two separate patient groups: BAL cells came from volunteer groups designated COPD-CS, COPD-ex-smokers, NLFS and NC, while small airway resections came from cancer patients with physiological categorisation being made on the basis of the lung function tests done for pre-operative work-up. Thus, it is possible that the macrophage phenotypes and cytokine profiles were possibly influenced by the presence of cancer, thought we have quantified the airway wall tissue macrophages well away from cancerous areas. Also, in the current study I have not confirmed M2 data with other potential macrophage sub-type markers such as CD206 and CD163, mainly because of lack of time and because I believe that I was using the best method currently accepted in the literature

5.5 CONCLUSION

The major novel findings in this study are the reduction in M2 and increase in M1 macrophages in the airway wall of SAs in smokers and COPD. M1 are the strongest signal for innate inflammatory up-regulation I have seen in the airway wall to date. The finding of an M2 switch in BAL in smokers with and without COPD was in stark contrast to the macrophage phenotype in the small airway

wall. BALF cytokine profiling revealed promotion of an M2 phenotype. The switching was confirmed in lung tissue from a chronic smoking mouse model. The overall tissue expression of Arg-1 in the SA wall suggests increased catabolic activity, a sign of cellular senescence, but could also have implication for lung fibrosis and airway resistance. These novel findings are potentially important in understanding the pathophysiology of the respiratory tract`s response to smoking and in the aetiology of COPD; they need to be taken into account when considering mostly unexplained cellular functional phenomena associated with COPD, and the specific vulnerability of COPD sufferer to lung cancer.

Chapter 6

Enumeration of Mast cells and degranulating cells in the small airways of COPD patients

6.1 Introduction

Mast cells (MC) play a crucial role in the innate immune response, recognizing possible pathogens and allergens. They are potentially granulating cells originating from hematopoietic stem cells in the bone marrow, which mature into mast cells in the target tissue (Drew et al., 2005). The granules released on activation from MC include mediators that are pro-inflammatory, pro-fibrotic and pro-angiogenic (Soltani et al., 2012). They have been classically associated with allergic reactions whereas they are activated by cross-linking of IgE molecules attached to specific surface receptors by their Fc fragment, but it is now known that there are other ways that degranulation can also be stimulated (Erjefält, 2014, Galli et al., 2011).

In chapter 4, I described the variability in key immune cells in the airway wall, including both innate and adaptive populations. Previously our group reported an increase in the MC population (Soltani et al. 2012) in the large airways of COPD patients in both the reticular basement membrane and lamina propria in endobronchial biopsy samples. In this chapter, I will further discuss the contribution of MCs, and other degranulating cells, in the small airway wall of COPD subjects. Further, I will also be providing information on overall abnormality in the cellular expression of lysosome-associated membrane protein 1 (LAMP-1), which has been used as a marker for active cell degranulation (Hennersdorf et al., 2005, Eskelinen, 2006), although it may also represent a somewhat more complicated phenomenon with links to autophagy (Eskelinen, 2006).

LAMP-1 is a transmembrane lysosomal glycoprotein, which is expressed on activation lysosomes and endosomes and has been used as a marker for degranulation. The lamp-1 expression is known to be increased in MCs when activated with IgE (Grützkau et al., 2004). Further, LAMP-1 has also been demonstrated to be activated in CD8+ lymphocytes; suppression of CD8+ cells

in COPD with inhibition of activation of CD8+ lymphocyte degranulation was associated with a LAMP-1 reduction (Aktas et al., 2009).

Our group has also reported previously that smoking decreased large airway sub-epithelial lamina propria total cellularity in mild to moderate COPD patients (Sohal et al., 2013a), while paradoxically MC was increased in that situation. However, MCs numbers in the small airway were not enumerated in that study. Thus, this chapter will investigate if there is a similar increase in MC numbers in the small airway wall and further estimate their degranulation capacity, which has not previously been attempted in any comprehensive way.

6.2 Overview of materials and methods

The subjects involved are detailed on page 66 in the method section. I employed dual immunohistochemical staining methods to evaluate total MCs as well as degranulated MCs in the small airways of normal controls and the clinical groups (as detailed in Chapter 3 page 75, 76). Further, the enumeration of MCs, degranulated MCs and total degranulated cells were evaluated and is detailed in chapter three, page 81. It is also important to notify here that I have used correlation analysis for mast cells with parameters including collagen-1, fibronectin index, airway wall lamina propria, smooth muscle layer thickness, all of which have been analyzed and presented in Chapter 7.

6.3 Results

6.3.1 Total Mast cells (MCs)

In the small airway sub-epithelium, there were much more MCs than in the corresponding epithelium. There was a significant decrease in the total number of MCs in the small airways of both ex- and current-smoker COPD ($p<0.05$) than in the normal controls with again normal smokers being intermediate. In the epithelium, in contrast, an increase in the number of MC was observed in COPD-CS ($p<0.01$) compared to NLFS and NC, while in ex-smokers their numbers tended back to normal (Figure 6.2).

Figure 6.1

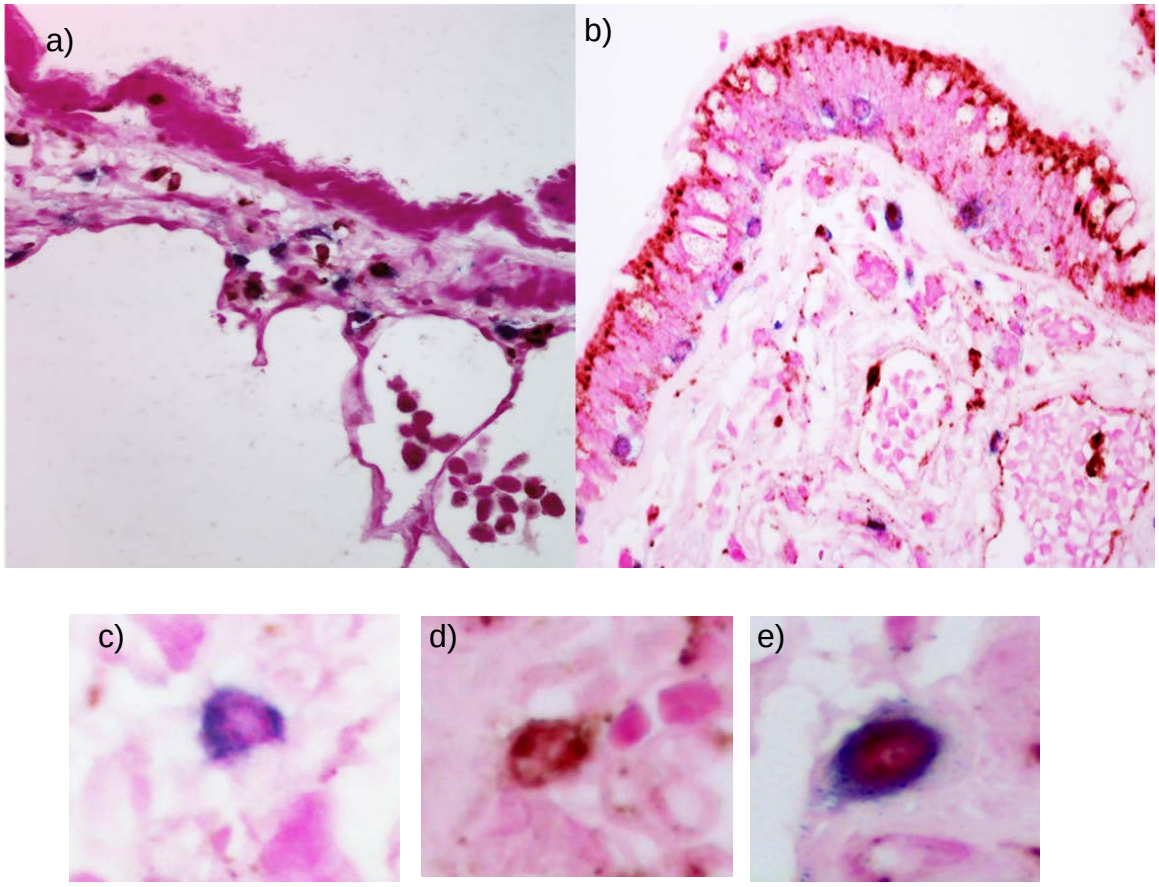

Figure 6.1 Representative images (50x) for dual stained mast cells (mast cell tryptase and LAMP-1+ cells) in the small airway of a) Normal controls and b) COPD-CS. Enlarged images of cells captured from the analysed tissue, c) mast cell tryptase+ cells. d) Only LAMP-1+ (degranulated) cells e) Dual stained mast cell tryptase+LAMP-1+ cells.

Figure 6.2

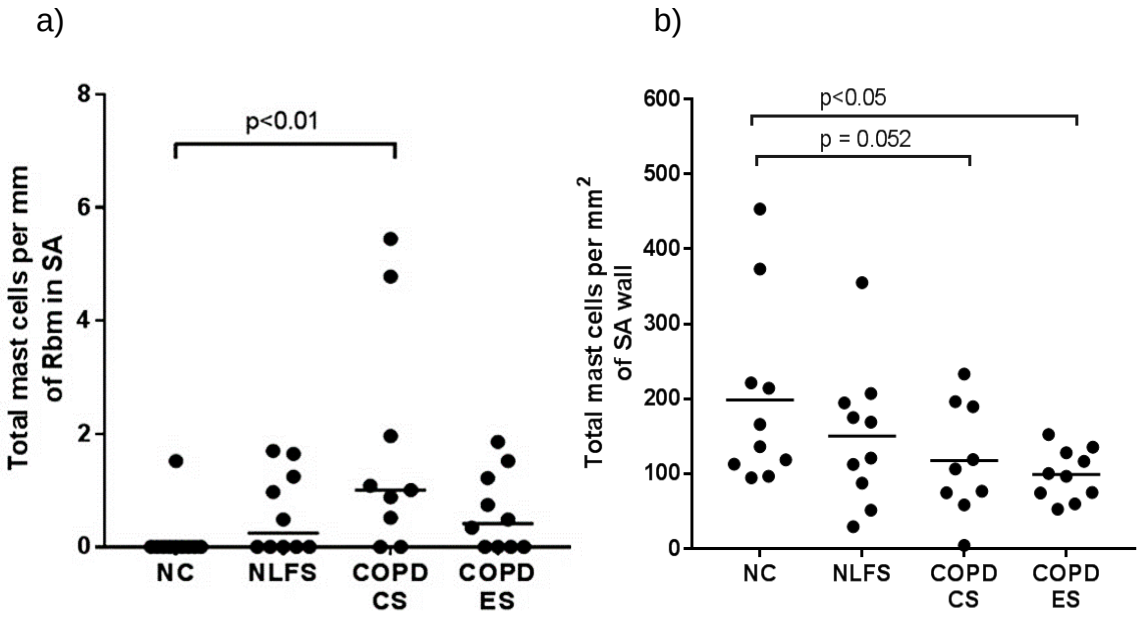

Figure 6.2. Total number of mast cells in the small airway as examined in: a) epithelium; b) sub-epithelium.

6.3.2 **Degranulating Mast cells**

In the epithelium, a slight increase in the absolute number of LAMP-1+ (degranulating) MCs was observed in COPD-CS patients ($p<0.05$) but there was no change from normal in the smoker COPD group in the sub-epithelium in this regard (Figure 6.3 a, b). In percentage terms, there was similarly no change between groups in degranulating MCs in either the epithelium or sub-epithelium (Figure 6.3 c, d).

Figure 6.3

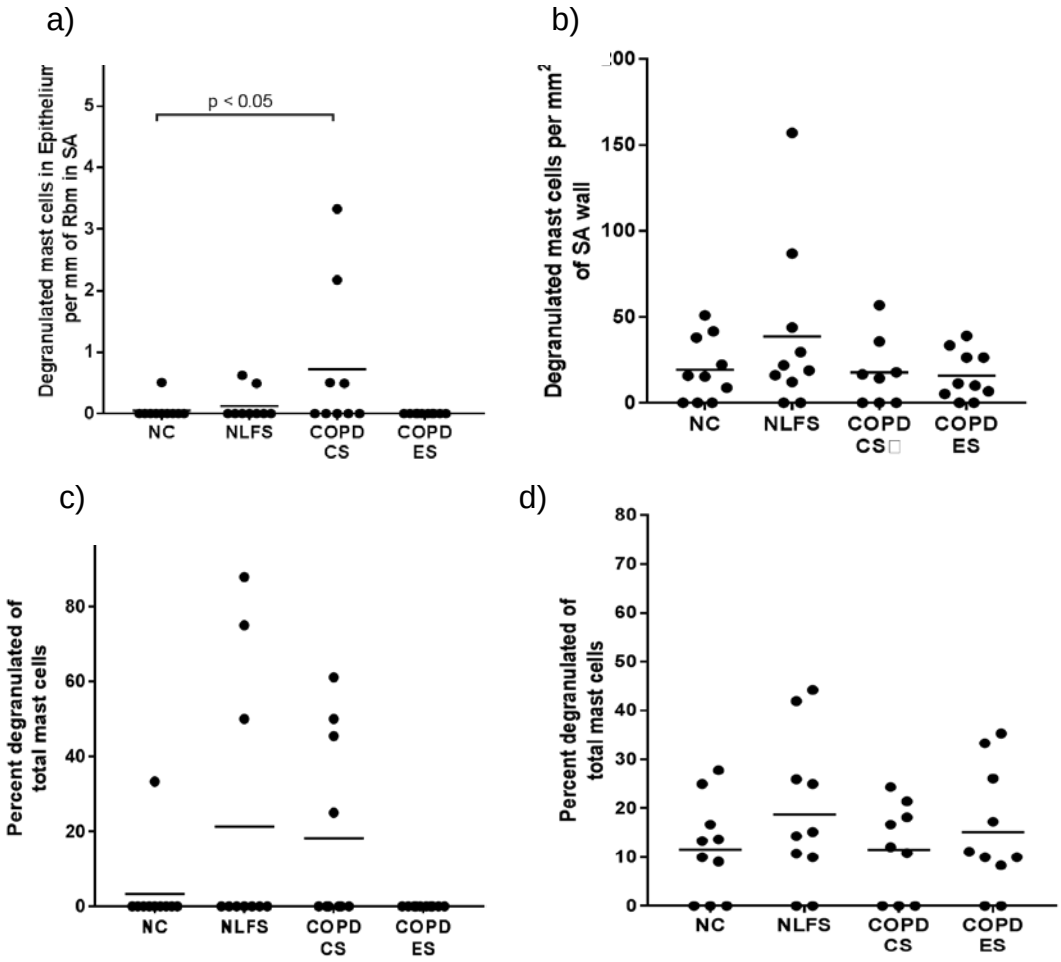

Figure 6.3. Degranulated LAMP-1+ mast cell numbers and their percentage of total mast cells the small airway: a) and c) epithelium; b) and d) sub-epithelium.

6.3.3 **Total Degranulated cells**

The total number of degranulated (LAMP-1 positive) cells in the epithelium was approximately double the number of degranulated MCs, but with no change between the groups. In the sub-epithelium, the density of degranulating cells overall in the normal was about tenfold higher than in the epithelium but was markedly reduced in COPD subjects ($p<0.05$).

Interestingly, and rather paradoxically, degranulating MCs as a percentage of all degranulating cells were increased in both smokers and COPD subjects in both epithelium and sub-epithelium, although of borderline significance, for the most part, suggesting that degranulating NK/CD8 cells are likely to be even more reduced.

Figure 6.4.

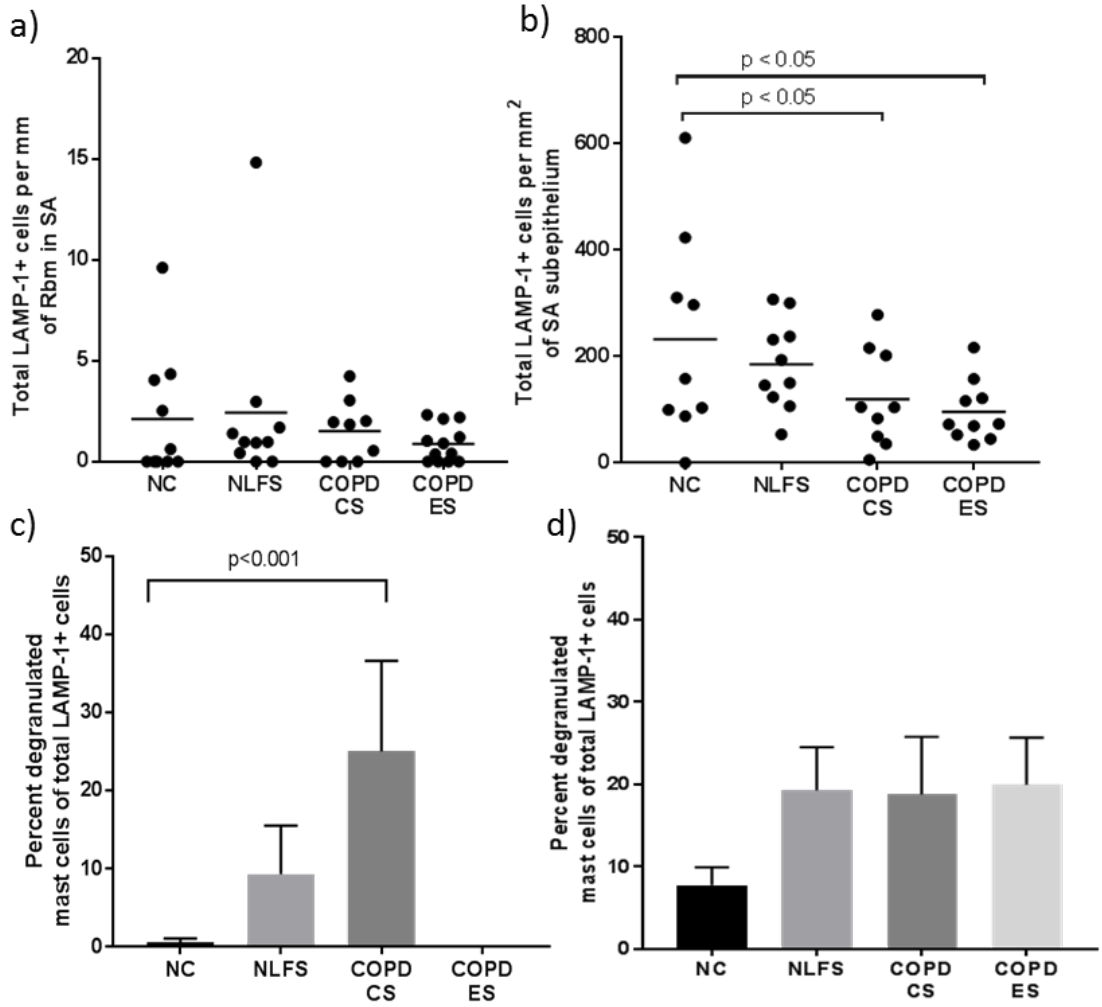

Figure 6.4. Graphical representation of the total LAMP-1+ degranulated cells as examined in the small airway wall and the percent contribution of degranulating mast cells to the total LAMP-1+ cell population a) and c) epithelium, and b, d) sub-epithelium.

6.3.4 **Regression analysis for Mast Cells**

6.3.4.1 ***Correlation to smoking history***

An increase in smoking history (pack-years) correlated directly to the decrease in both total MCs and degranulated MCs in COPD-CS but not in the NLFS subjects (Figure 6.5), suggesting a COPD specific effect.

Figure 6.5

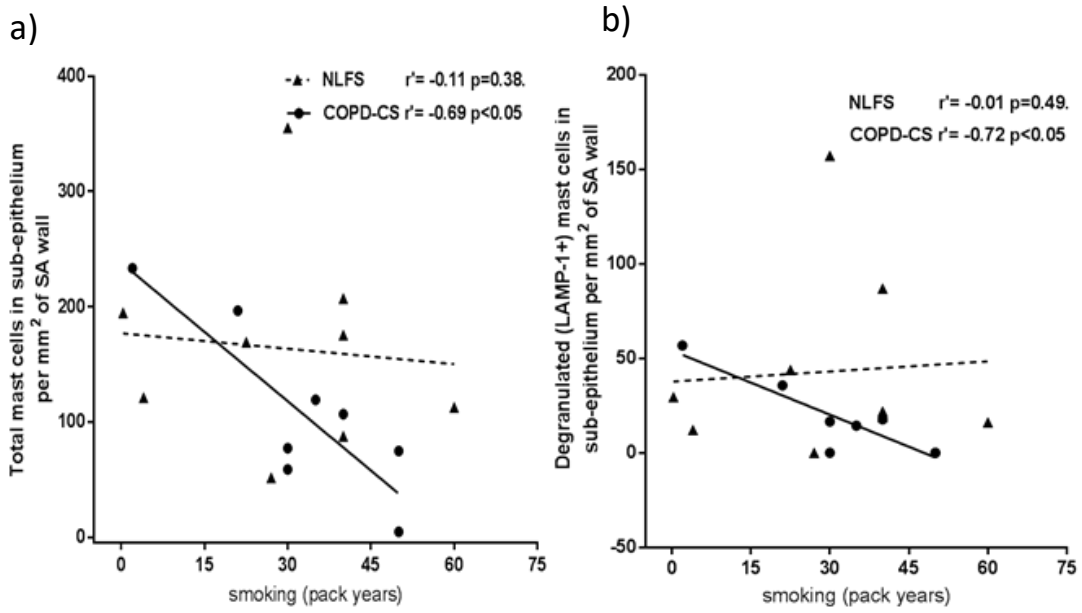

Figure 6.5 Correlation analysis between smoking history (pack-years) and a) Total mast cells b) Degranulated mast cells in NLFS subjects and COPD-CS patients.

6.3.4.2 *Correlation with lung physiology parameters*

A positive correlation was observed between COPD groups MC numbers versus large and small airway obstruction (FER and FEF25-75, respectively) (Fig 6.6, a, b). A less strong and non-significant positive relationship was also observed between total degranulating cells and lung function (Figure 6, c and d).

Figure 6.6

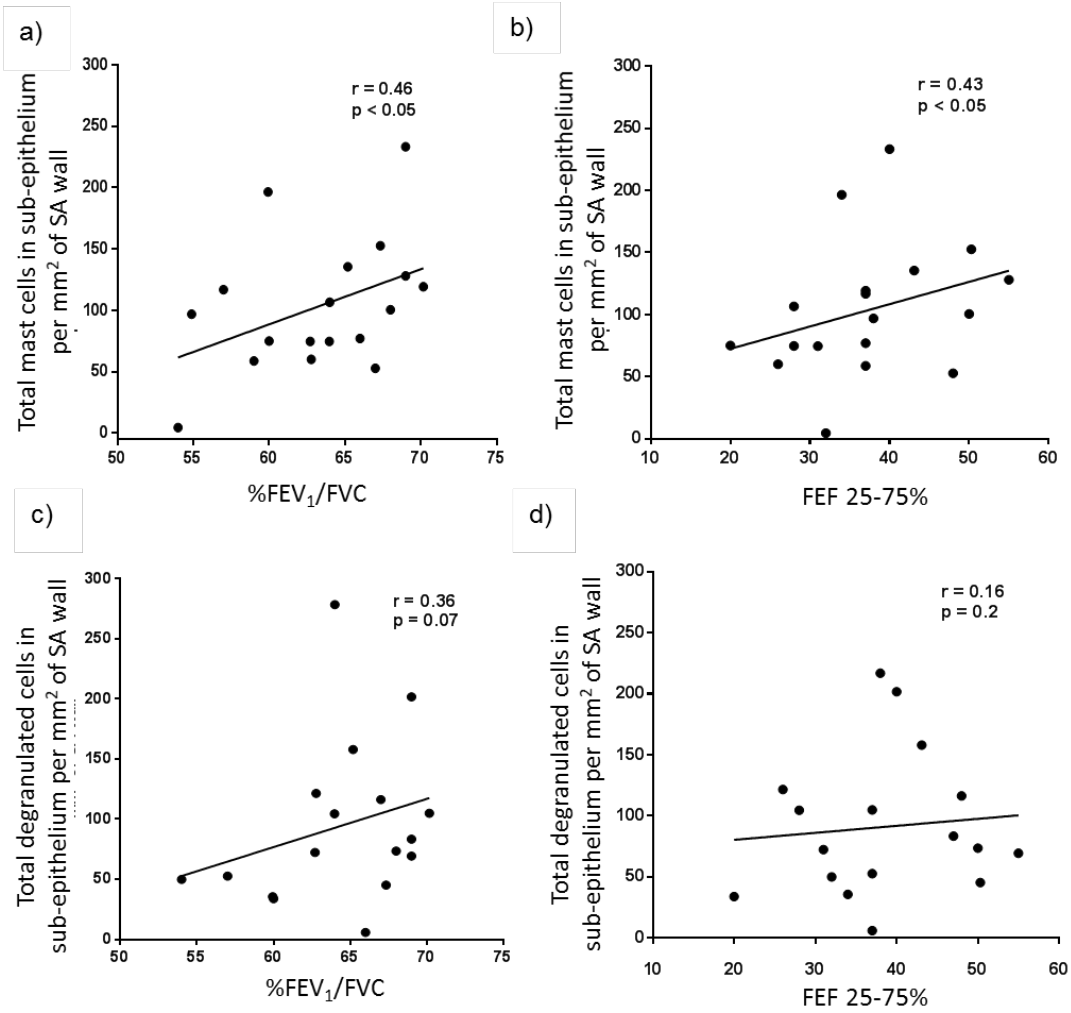

Figure 6.6 Correlation analysis between lung physiology parameters (%FEV_1/FVC and FEF25-75%) and a) Total mast cells b) Degranulated LAMP-1+ cells in COPD groups.

6.3.4.3 *Relation to airway remodelling*

For the COPD groups, there was a strong relationship between decreasing total numbers of sub-epithelial (lamina propria) MCs and indices of small airway remodelling: increased thickening of the small airway wall LP (analyzed as the area between the Rbm and start of smooth muscle layer and deposition of collagen I and fibronectin ECM. In contrast, however, the relationship with the smooth muscle layer was positive, i.e. more MCs, thicker the muscle layer (Figure 6.7).

Note- Features such as airway wall thickness, collagen, and fibronectin index and smooth muscle thickness have been analyzed and presented in the following chapter.

Figure 6.7

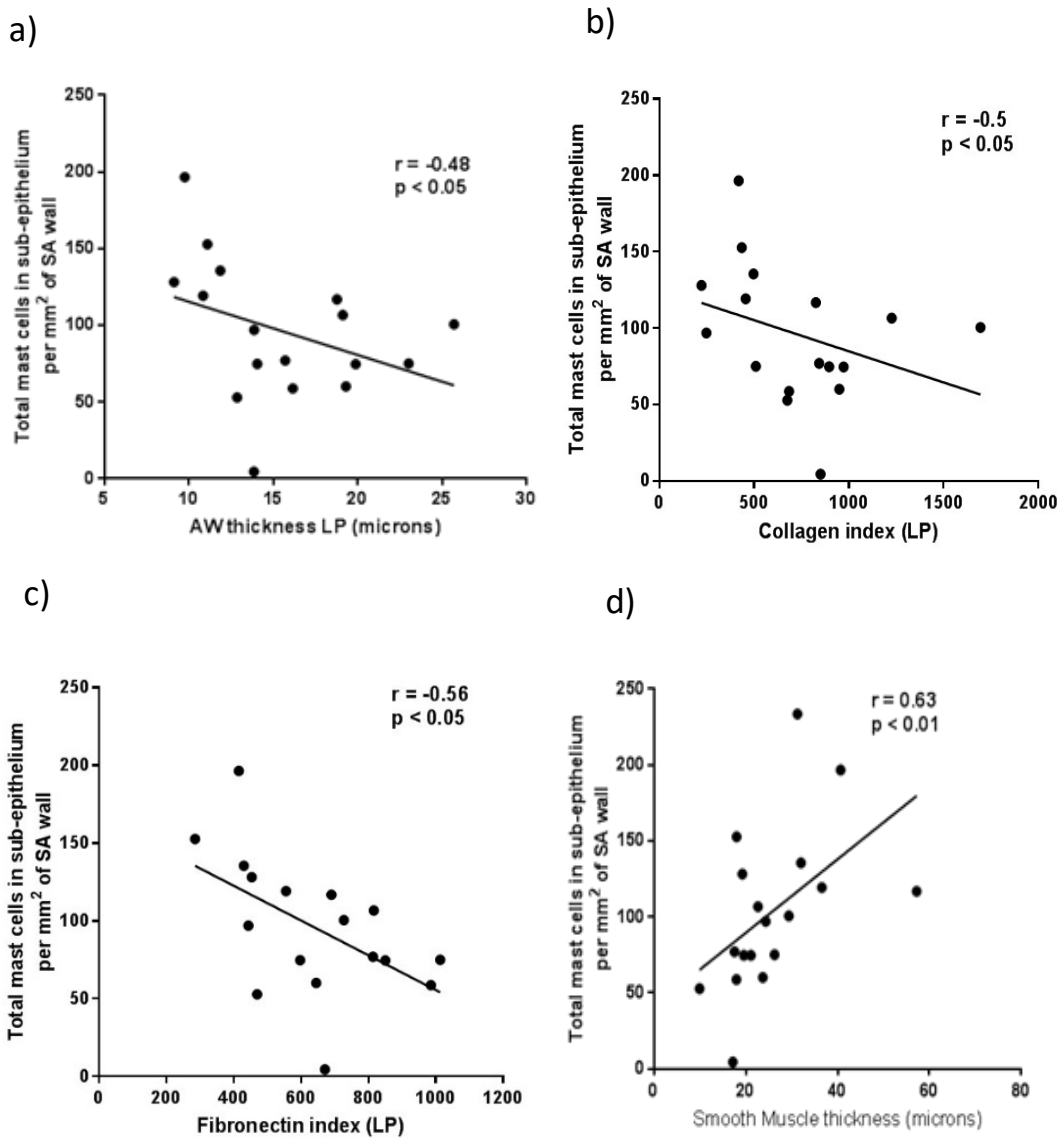

Figure 6.7 Correlation analysis between total mast cell numbers in the small airway wall and a) airway wall thickness b) Collagen-1 index c) Fibronectin index and d) Smooth muscle thickness in the COPD group

6.4 Discussion

This is the first study to evaluate MC numbers, their percentage of total cells, and their functional degranulating status in the epithelium and lamina propria of small airways of COPD patients. The major finding of this study was a decrease in MC numbers in these compartments, while there was no change in their degranulating activity in the sub-epithelium of COPD patients. To complicate the picture, the degranulating activity of other airway wall cells seems even more depressed, so that as a percentage of total actively degranulating cells, those that are marked as MCs are paradoxically increased. Regarding actual mast cells, the current data were in contrast to the previous observation by our group (Soltani et al., 2012) who assessed the large airway wall where there was an increase in the number of in COPD, assessed in bronchial biopsy tissue. Interestingly, MCs in the large airway wall were primarily associated with vascular structures, and in the context of both hypo-cellularity and hypo-vascularity (Eapen et al., 2017). The small airway wall too is hypo-cellular including for MCs, but these are not mainly focused around vessels.

The current observation of reduced MCs in the sub-epithelium LP of COPD patients is comparable to reports by Andersson et al., (2010) who found a similar decrease in MCs in the sub-epithelial region of the small airways of COPD patients across GOLD stage I-IV subjects, which negatively correlated with severity of the disease. My current study provides additional information in COPD ex-smokers where the levels of MCs remained low even after the subjects had quit smoking, suggesting that the effect observed is mainly COPD driven. Moreover, when correlated with smoking history in currently smoking COPD and NLFS group, the suppressions of total MCs and degranulating MCs were more significant in the COPD-CS group, again suggesting that susceptibility to airflow obstruction in addition to smoking influences this pathological MC outcome. Similar to Anderson et al. my finding also showed that the decrease in MCs negatively associated with the lung function, but additionally it was related to decreased small airway calibre as measured by $FEF_{25-75\%}$ in the COPD group. It is difficult and inherently speculative to say

what the meaning of this relationship of induced MCs with airflow obstruction is, but it is unlikely to be causative but more likely that both are related to another more fundamental process.

My observation of an increase in epithelial mast cells numbers in COPD are similar to those of Grashoff et al., (1997) who also observed an increase in MCs in the small airway epithelium of COPD patients; however, in contrast, they found no change within the sub-epithelium LP. They had no data on MC activation. The increase in epithelial numbers could potentially be explained by their active migration from the wall into the airway lumen (Persson and Uller, 2010a). Fate-mapping studies with MCs in an animal model of COPD may well be worth doing to follow this up.

Literature evidence suggests that both tryptase and chymase expression are reliable markers for MCs. In the current study, I used tryptase only as the marker to identify MCs, as these are more specifically located in the airway mucosa when compared to chymase-expressing MCs that are present more in the adventitial connective tissue (Ballarin et al., 2012, Krishnaswamy and Chi, 2006, Galli et al., 2011).

Consistent with my findings, Gosman et al., (2008) found that MCs are concentrated towards the peripheral small airways compared to central airways in both COPD and control groups and suggested that this could be crucial in small airway remodelling in COPD. Interestingly, and perhaps paradoxical to this suggestion, in the current study the reduction in tryptase-positive MCs in the sub-epithelium of COPD patients was quite strongly negatively correlated with airway wall thickening as well as my indices of ECM remodelling changes in collagen-1 and fibronectin. Again, the negative relations would suggest that the MC is not, in fact, driving the remodelling process, in fact quite the opposite, but again that both phenomena are driven by another more fundamental process. However, the current findings may complement earlier observations in other diseases, which have demonstrated the importance of MC tryptase in maintaining tissue homeostasis through the degradation of excessive ECM collagen deposition, indirectly by degrading MMP-9 activity (Wernersson and Pejler, 2014a, Fajardo and Pejler, 2003). Furthermore, MC tryptase is also

known to activate pro-MMP-3 and pro-MMP-13, both of which are crucial for the digestion of ECM proteins such as collagen and fibronectin (Magarinos et al., 2013). Thus, the observed suppression of tryptase-positive MCs in the airway wall of COPDs could perhaps be related to the aberrant accumulation of ECM protein and airway remodelling. It is also interesting that there was a positive relationship between MC numbers in the LP and thickness of the smooth muscle layer, suggesting that there may be some (perhaps mutual) trophic relationship between them.

MCs contains granules (secretory lysosomes) that hold the immense capacity to hold an array of lysosomal proteins such as acid hydrolases (e.g., β-hexosaminidase) (Wernersson and Pejler, 2014b, Schwartz et al., 1979) and biogenic amines such as histamine and serotonin. They also store and secrete extracellularly mature forms of serine and other proteases, which includes tryptase, chymase-1, cathepsin G, granzyme B, and carboxypeptidase among others (Moon et al., 2014). Based on their content MC secretory lysosomes are characterized into three sub-types. Specifically, LAMP-1 marks the more mature or active 'type-1 and type-2' secretory lysosomes that are ready to be exocytosed and containing one or more of these fully active mediators. In COPD research, there are few previously published data on degranulating markers, especially the crucial LAMP-1 (Grutzkau et al., 2004). The previous estimates of mast cell degranulation by Gosman et al., (2008) used morphological and cellular conformational analysis based on the secretion pattern of tryptase by MCs. The limitation of this approach, however, lies in differentiating the pre-formed MC tryptase from its mature form that is stored in secretory lysosomes granules ready for release. In the current study, I provide specific evidence on degranulating activity in MCs by dual staining them for LAMP-1 and mast cell tryptase. I found that the degranulating LAMP-1+ MCs in the LP constituted approximately 15-20% of the total mast cells population, though this was not different overall from normal in spite of some smokers having quite a lot relatively. This was in contrast to the epithelium, where COPD-CS had a significant increase in degranulating MCs though not in percentage terms of total mast cell numbers in the small airway epithelium. Currently, no firm conclusion can be drawn of the role of LAMP-1 or MC

lysosomal exocytosis in COPD, and this is a complex process that may involve multiple types of lysosomes (Moon et al., 2014).

The decrease in LAMP-1 expression that I have described in the COPD airway seemed to be related to an overall cellular dysfunction in potentially degranulating cells and was not limited to just mast cells. McKendry et al., (2016) also recently reported that CD8 cells from COPD patients had a reduced degranulating capacity, also using the LAMP-1 expression, when ex-vivo lung slices were exposed to influenza virus. Indeed, an advantage of using LAMP-1 is that it has been described as a general degranulation marker that can assess the total degranulating cell population in tissue such as the small airway (Hennersdorf et al., 2005).

There is some limitation to my observation. There were fewer subject tissue sample numbers available per clinical group at this stage in my research, though my findings seem statistically robust and consistent, without likely Type-1 or Type-2 errors. Also, as stated above, LAMP-1 alone may not be sufficient to determine the granulation capacity or activity of MCs, and thus more lysosomal markers will be required to be tested as this work goes forward, but I believe that I have shown enough to make this worthwhile (though perhaps more in epithelium than MCs alone). The current study made inferences about non-MCs, and further study needs to do double-staining for LAMP-1 and other specific cell types such as CD8, NK cells, and macrophages that are all granular cells and capable of degranulation on activation. Finally, the current data was mainly obtained using small airways from surgically resected tissue derived from cancer patients; it is conceivable that the presence of cancer in the lungs of these patients affected the MC populations and their activity, although all cell assessments were conducted on airways well away from the cancerous region.

6.5 Conclusion

To summarize, the data provided in this Chapter show that MCs are reduced in number in the lamina propria of the small airway which is congruent with my findings (Chapter 4) of more general hypocellularity. Further, the overall percent change of mast cells to overall cellularity remained unchanged between COPD-CS and normal controls, as did the number and percentage of active MCs, as indicated by LAMP-I positivity indicating degranulation. The data on LAMP-1 staining also indicated a more general decrease in degranulating activity across the range of cell types potentially involved in such processes. These decreases in mast cell numbers in smokers/COPD showed a direct relation to increasing in small airway wall thickening and ECM protein remodelling changes, perhaps pointing to an active role in maintaining normal tissue homeostasis and indirectly, perhaps by default, involvement in airway remodelling.

Chapter 7

Enumeration of Myofibroblasts in the small airways of smokers and COPD patients

7.1 Introduction

In Chapter 4, I highlighted that the airway wall in both large and small airway complements was relatively hypocellular, but in both areas, the 'stromal' cell population may well be the largest cell component. As part of my thesis work, I wished to evaluate this stromal cell population further, but unfortunately, due to time constraints, I have been able to advance this only as far as quantitating the myofibroblast population. However, I felt that this was a priority, as it would help to advance and, in some ways, complete the work of my research group that has been ongoing for a decade. Thus, in addition to straight quantitation, and to placing these cells anatomically within the airway, I wished also to relate them to EMT activity in the corresponding epithelial basal cells, as expression of alpha-smooth muscle actin (αSMA) by transitioning mesenchymal cells has long been described as a late feature in EMT(Kalluri and Neilson, 2003, LeBleu et al., 2013). I also wanted to relate changes in the myofibroblast population to airway wall thickness and deposition of strategic representative ECM proteins. I have focused on small airway tissue for these analyses as the site of functional airflow changes in COPD, and because of this, my analysis has had a strong emphasis on relating all these pathological small airway changes to measures of airflow obstruction.

Myofibroblasts are motile and contractile cells, which are related to the increased expression of alpha-smooth muscle actin (αSMA) myofilaments, which form "stress fibers." Previous studies in COPD based on this protein marker for myofibroblasts using human bronchi and bronchiolar tissue have been once again variable. Lofdahl et al., (2011) in their histological staining of large and small airway tissue from operative resections, showed an increased expression in αSMA positive cells in the lamina propria of the large airway in COPD patients when compared to non-smoker controls, although similar differences in the expression level was not observed in small

airway wall . In contrast, findings from invitro studies with fibroblasts isolated from the distal end of the airway from COPD patients showed increased contractile properties associated with increased myofibroblast numbers (Hallgren et al., 2012). These findings suggest myofibroblast may be important in both airways.

In the current study, I will be using αSMA to identify the myofibroblast population, but with care to dissociate them from smooth muscle bundles. I will descriptively analyze the localization of these cells in the small airway wall tissues. As mentioned, I will also analyze whether the changes in these cell types have likely direct implications for airflow limitation in COPD through airway wall tissue remodelling and scarring, i.e., re-organization of the extracellular matrix (ECM). These changes in the ECM are known to have profound effects, the most important being the gradual obliteration of the small airway. My hypothesis is that all of these potentially inter-related processes are driven by active EMT.

7.2 Overview of materials and methods

The subjects involved in this study are detailed in page 66 in the method section. I employed immunohistochemical methods to stain for αSMA positive cells, collagen-1, and fibronectin in the small airway wall. Details on the staining methodology of the tissue can be referred to in Chapter 3 page 78-80). Further, this chapter evaluates small airway wall thickness, myofibroblast numbers, and ECM deposition, all of which is detailed again chapter 3 pages 82, 83)

7.3 Result

7.3.1 Airway wall thickness in Small Airways

Both the adventitia and the LP were thicker in COPD subjects, with the latter showing at least a tenfold change compared to normal controls, whereas in the former (the adventitia) there was “only” a 2-3-fold increase change observed (figure7.1). Also, the muscle layer was thickened too, but only slightly so. All of these changes in aggregate are reflected in a substantial increase in total small airway thickness in COPD.

It is noteworthy that increased thickness of these compartments also occurred in smokers with apparently normal lung function, and indeed, in the adventitia the change was uniform across smoker/COPD groups. For the LP, the effect was greatest in the COPD subjects. However, there was substantial within-group variability which is likely to reflect real anatomic variability along the airways in the process involved, both within subjects as well as between individuals, given the scrupulous way that we selected airways for quantification (see Methods chapter).

Figure 7.1

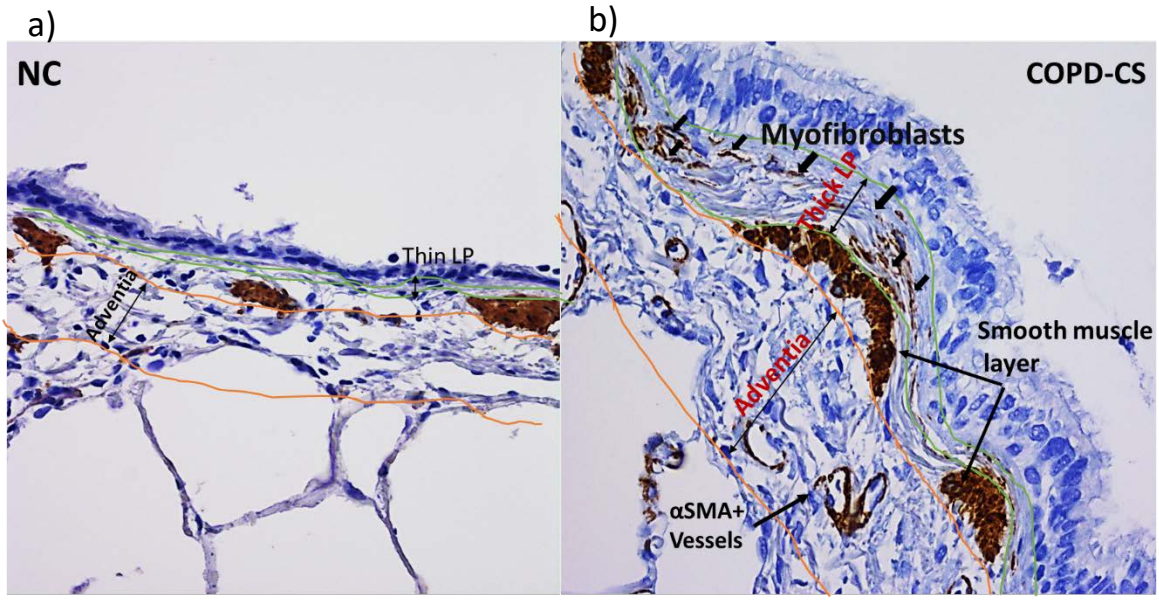

Figure 7.1 (cont…)

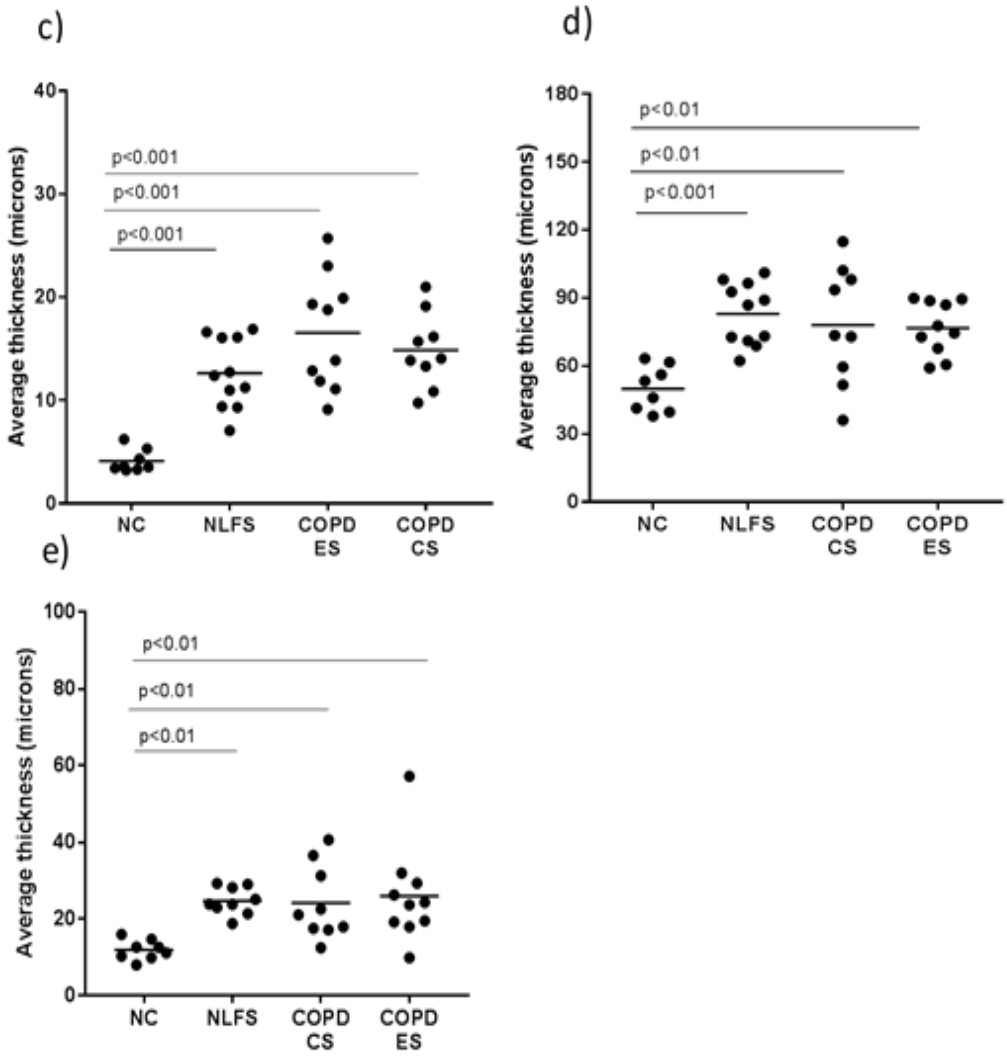

Figure 7.1 Representative images of the airway thickness, myofibroblast population (as marked with arrows within the LP area) in the small airway wall of: a) NC and b) COPD-CS (50x magnification). An increase in thickness was observed in the clinical groups in the c) LP, d) Adventitia and e) smooth muscle layer.

7.3.2 Correlation of airway thickening to physiological function in COPDs

There was a significant correlation between increased small airway LP thickening and decrease in airflow-related lung function in the COPD groups combined.

Figure 7.2

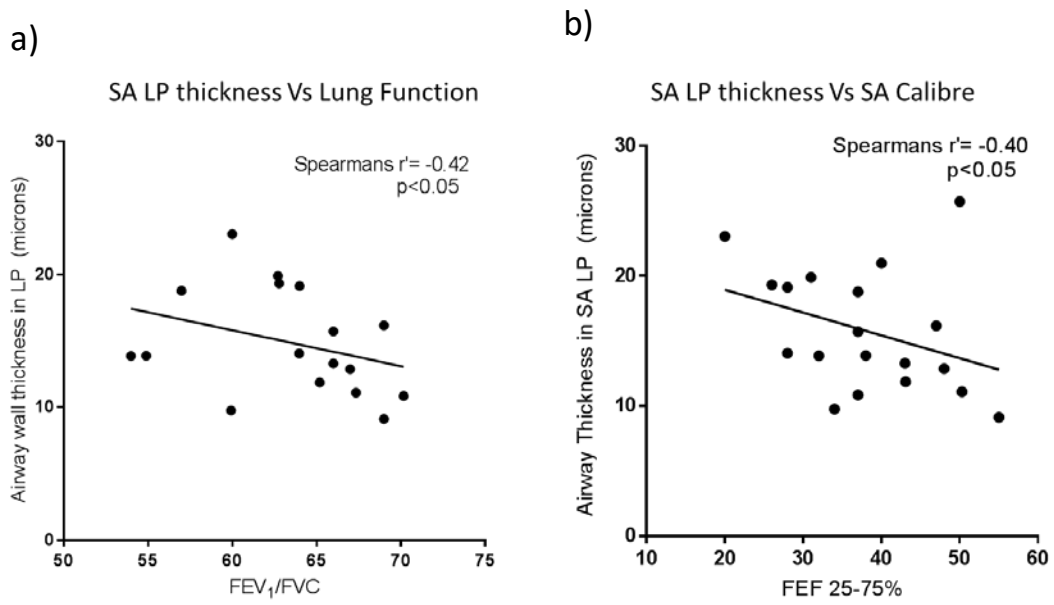

Figure 7.2 Correlation between airway wall thickness and lung function indices a) %FEV1/FVC and b) FEF25-75% (small airway more specifically) in the COPD groups combined.

7.3.3 **αSMA positive myofibroblast in Small Airways.**

Significant increases in small airway αSMA positive myofibroblasts were observed throughout the airway wall in the smokers and COPD groups, but most consistently in the latter. The density and increase in myofibroblasts were especially striking in the LP and in actively smoking COPD. Again, there was a great deal of real within-group variability.

Figure 7.3

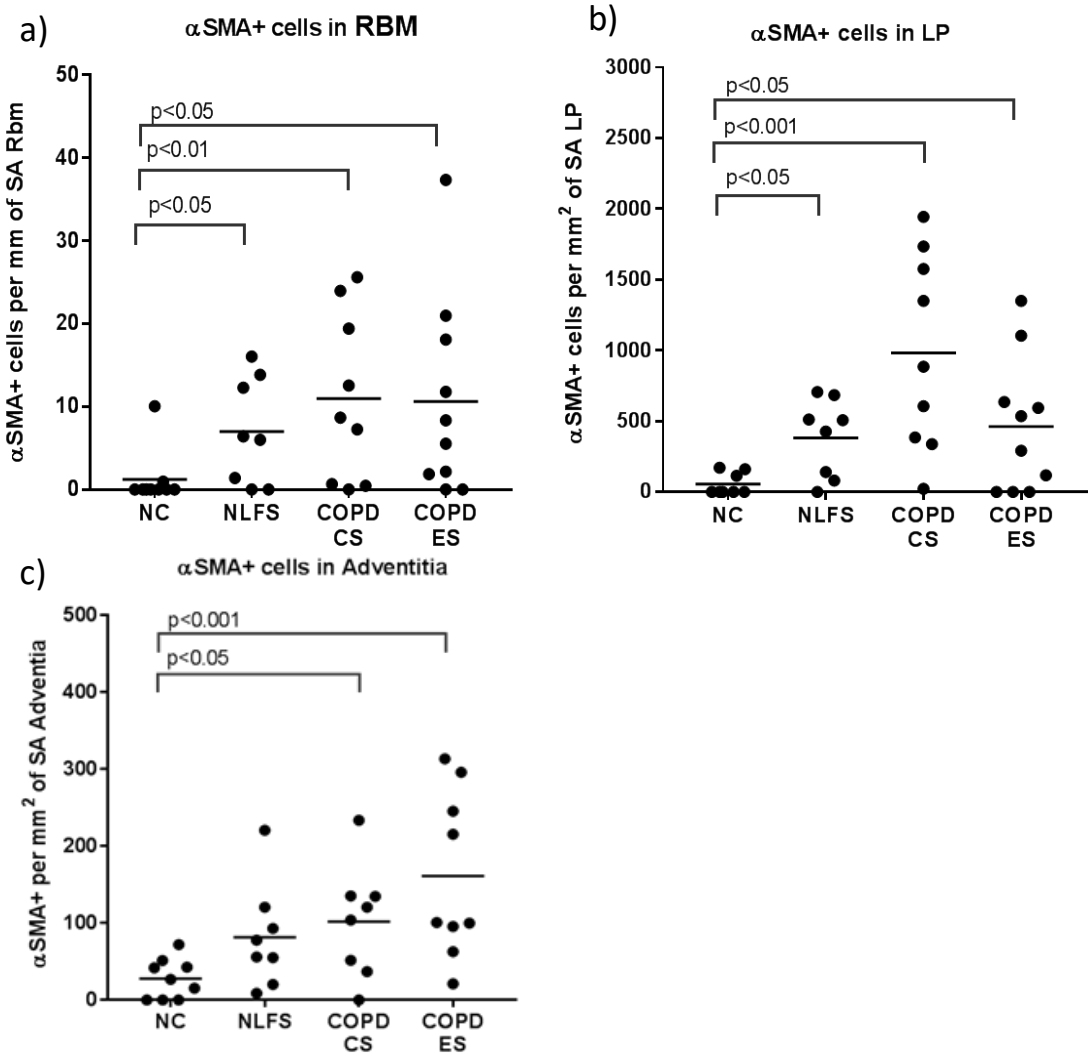

Figure 7.3 Increase in αSMA+ myofibroblast cells were observed in all three compartments of the sub-epithelial areas of the small airway wall, i.e. a) Rbm,

b) LP and c) Adventitia Correlation to αSMA with physiological parameters and small airway wall LP thickening

Figure 7.4 is complex but is an attempt to show together the relationships between myofibroblast numbers in the LP and the Rbm against airflow obstruction in the three clinical groups. Although numbers are rather small, there was a significant or near significant correlation between myo-fibroblasts versus decreases in lung function (airflow, both as %FEV1/FVC and FEF25-75%) in COPD groups but not in the NLFS (Figure 7.4). Relationships were most consistently between airflow obstruction and myofibroblast density in the currently-smoking COPD group.

Figure 7.4

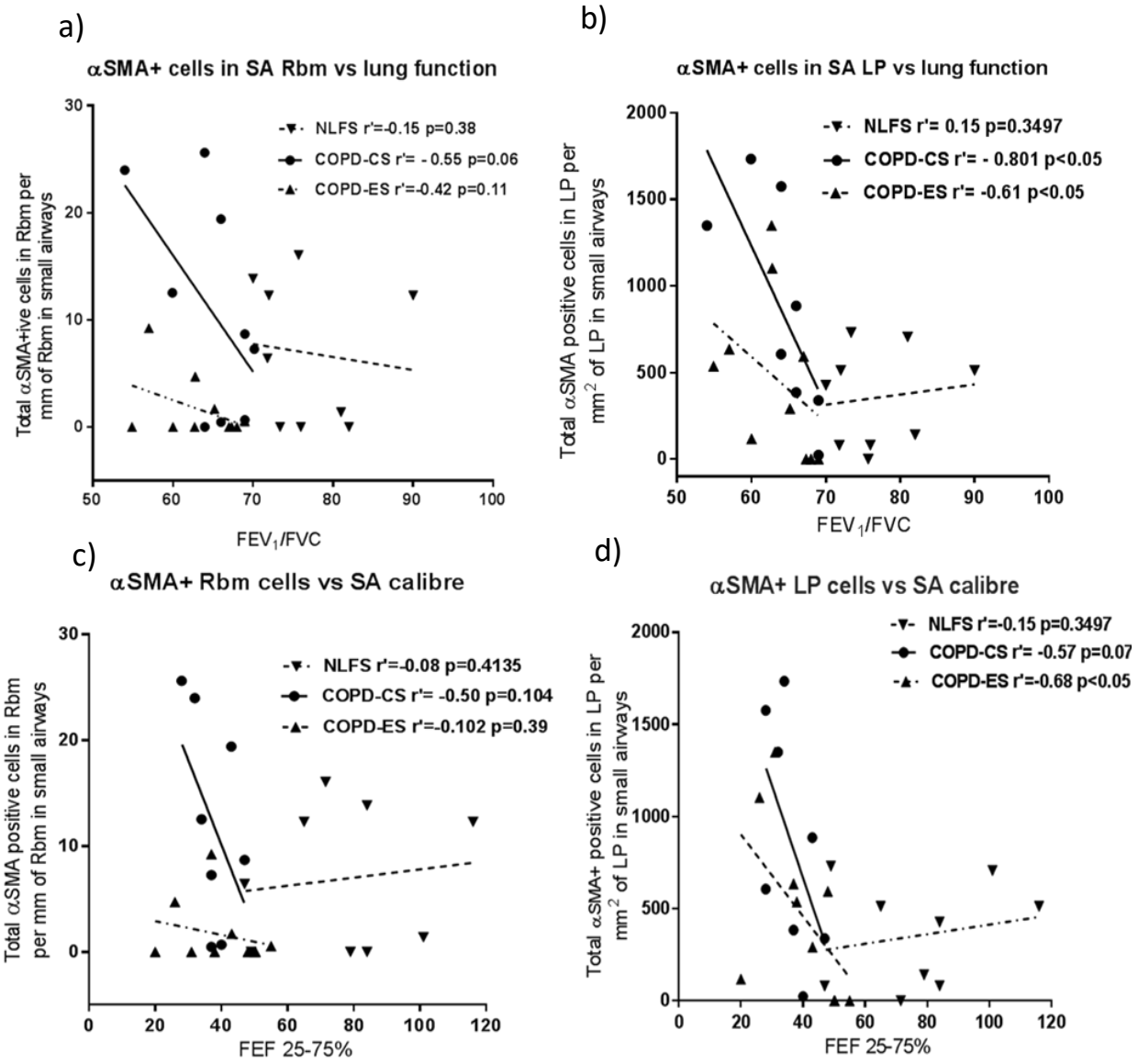

Figure 7.4 Correlation analysis between αSMA+ myofibroblast in the (a, c) Rbm and (b, d) LP of the three clinical group (NLFS, COPD-CS, and ES) and lung function parameters (a, b) %FEV_1/FVC and (c, d) FEF25-75%.

7.3.4 Correlation to αSMA with smoking history (pack-years) and small airway wall LP thickening

Regression analysis between increases in myofibroblasts SA wall LP thickness in all pathological groups (Figure 7.5) showed a very similar picture, i.e., quite strongly suggesting both smoking and COPD effect. There was also a positive correlation found between smoking history (pack-years) and density of αSMA positive cells in the small airway LP in COPD-CS and NLFS, suggesting some if not mostly a current smoking effect on this.

Figure 7.5

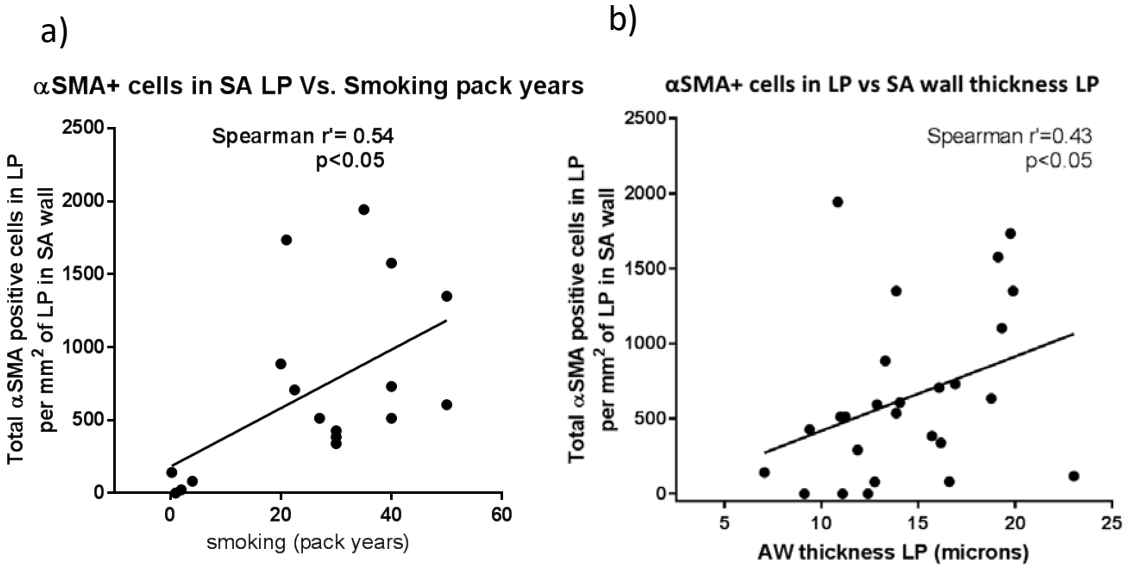

Figure 7.5 Correlation analysis between αSMA+ myofibroblast and a) smoking (pack-years) in NLFS and COPD-CS, b) airway wall thickness in the LP area of all clinical groups.

7.3.5 **ECM deposition in Small Airway**

This analysis provided here are percentage calculation based on objects per area of tissue staining (detailed in methods) in the specified (LP, Adventitia) region evaluated, however, does not take into account staining intensity which would have provided more information on how much protein deposition there was. Even so, there was an overall increase in the key “scar” ECM proteins, Collagen-1 and Fibronectin in the airway wall, with fibronectin changes being the most pronounced.

7.3.5.1 ***Collagen-1 deposition in LP and Adventitia.***

Increase in collagen-1 was observed in both the LP and adventitia. However, in comparison to percent collagen-1 expression in the LP (1.5 folds), the increase in adventitia were greater (5-6 folds) in smokers and COPD patients compared to NC. Compared to smokers and COPD-CS, in the adventitia the relative expression of collagen-1 deposition was reduced, though still higher than normals.

Figure 7.6

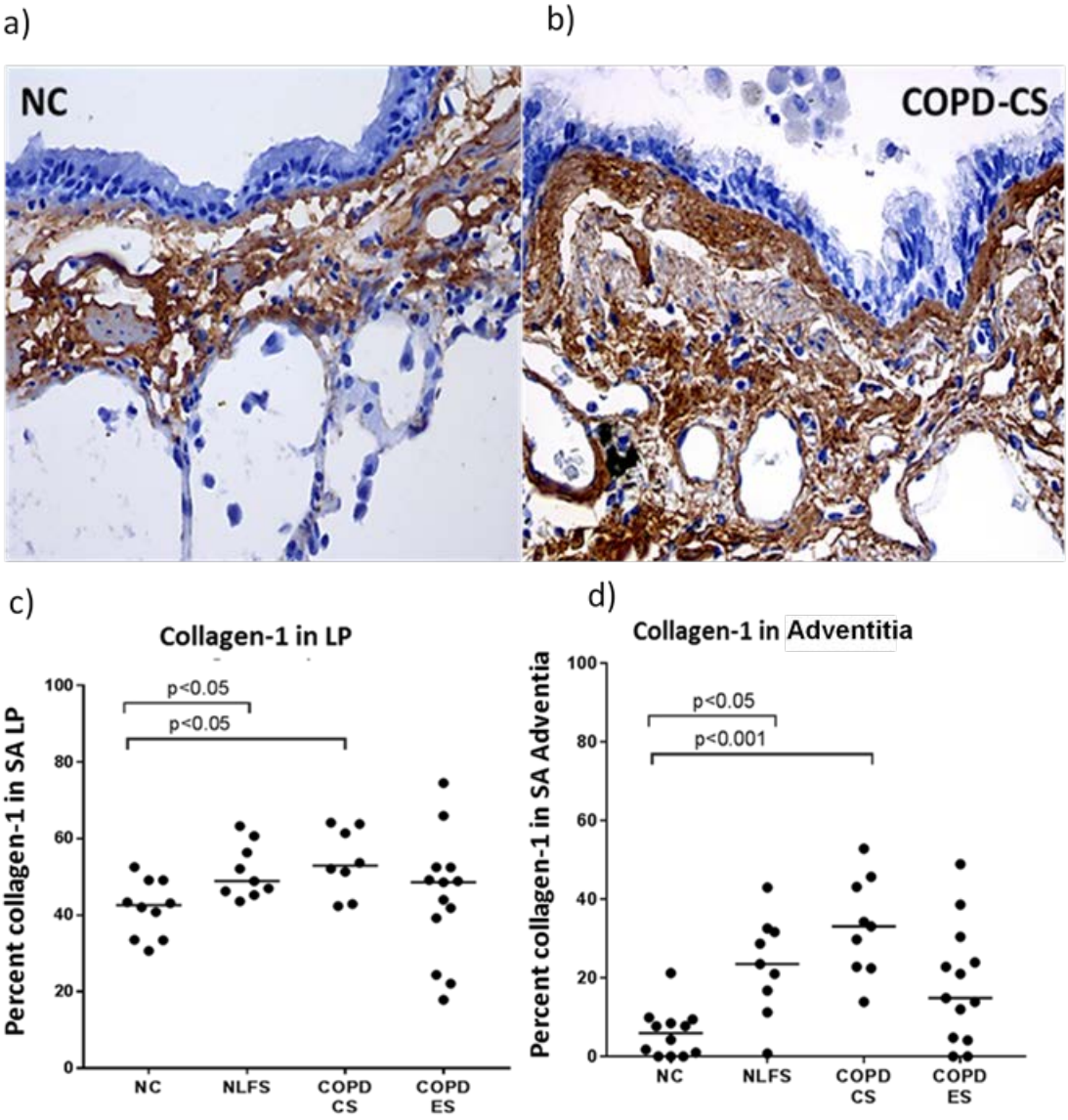

Figure 7.6 Representative images of collagen-1 deposition in the airway wall of a) NC and b) COPD patients, Increase in percentage collagen-1 expression observed in pathological groups in both the c) LP and d) Adventitia.

7.3.5.2 *Fibronectin deposition in LP and Adventia*

A significant and similar increase in percent fibronectin expression was observed both in the LP and adventia region of the small airway wall of smokers and COPD subjects. Again, similar to collagen-1 exprssion patterns, the levels in ex-smokers COPD were slightly lower compared to pathological groups, but were higher than normal controls.

Figure 7.7

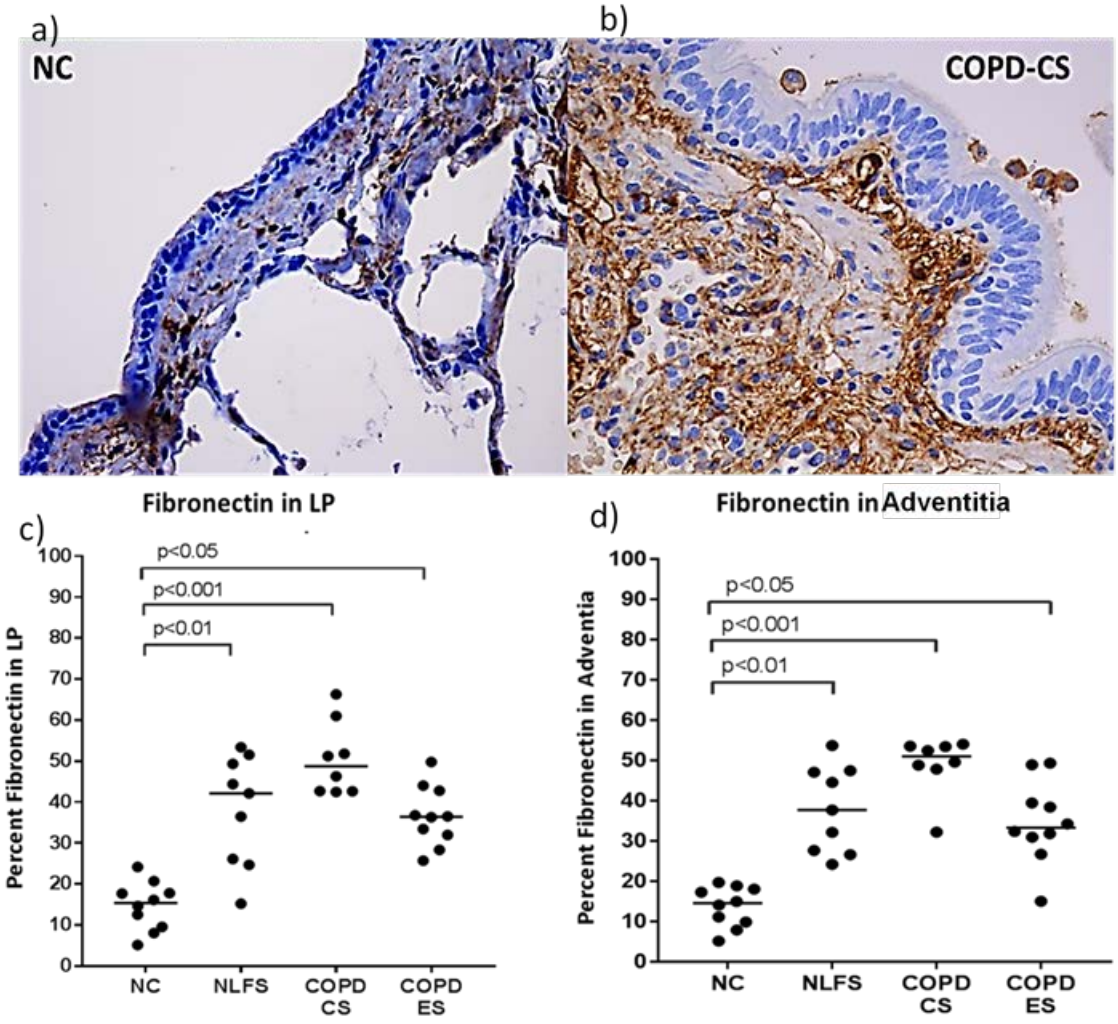

Figure 7.7. Representative images of Fibronectin deposition in the airway wall of a) NC and b) COPD patients. Increase in percentage fibronectin expression observed in pathological groups in both the c) LP and d) Adventitia.

7.3.5.3 *Total ECM index in small airway LP and Adventitia*

Interestingly and consequentially, on measuring the ECM (Collagen-1 and fibronectin) index, which is the product of total ECM protein change and thickness of the corresponding region of the small airway wall, I observed a significantly higher fold change especially with collagen-1 index in LP area and the adventitia. Similar, increase was also observed in fibronectin index deposition in all pathological groups in comparison to normal controls.

Figure 7.8

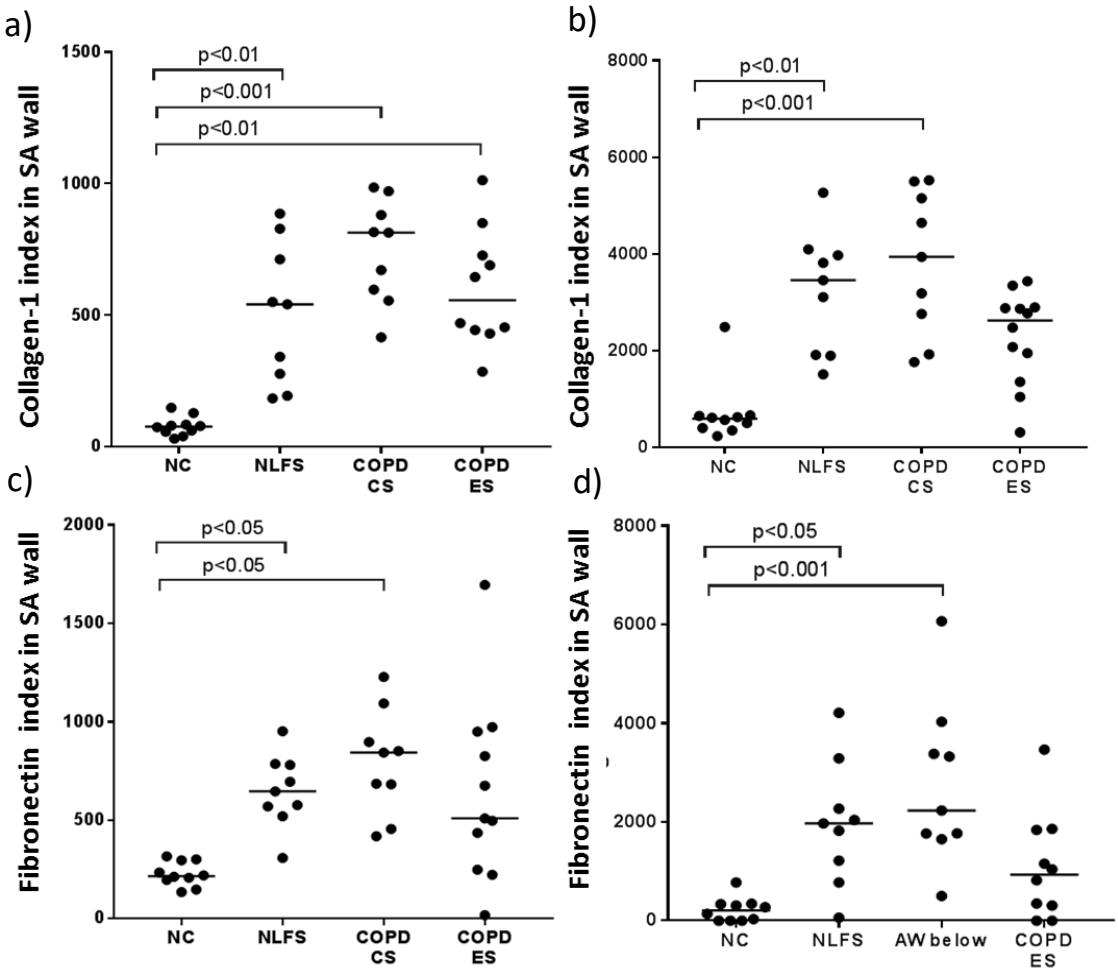

Figure 7.8. Collagen and Fibronectin index in small airway wall a, c) Lamina Propria and b, d) Adventitia

7.3.6 Correlation of percent Collagen-1 and Fibronectin to lung function, smoking history and SA wall LP thickness of pathological groups.

The percent by area tissue collagen-1 expression was significantly and positively correlated with LP thickness in COPD (both CS and ES) patients, but in contrast to the data given above in this chapter, this was not the case in the normal lung function smokers although their LP thickness was increased as I have shown (Figure 6.9 a). Notably, and contrary to collagen-1 expression, fibronectin percentage positively correlated to increasing smoker airway LP thickness only and not in the COPD groups (Figure 7.9 b).

Figure 7.9

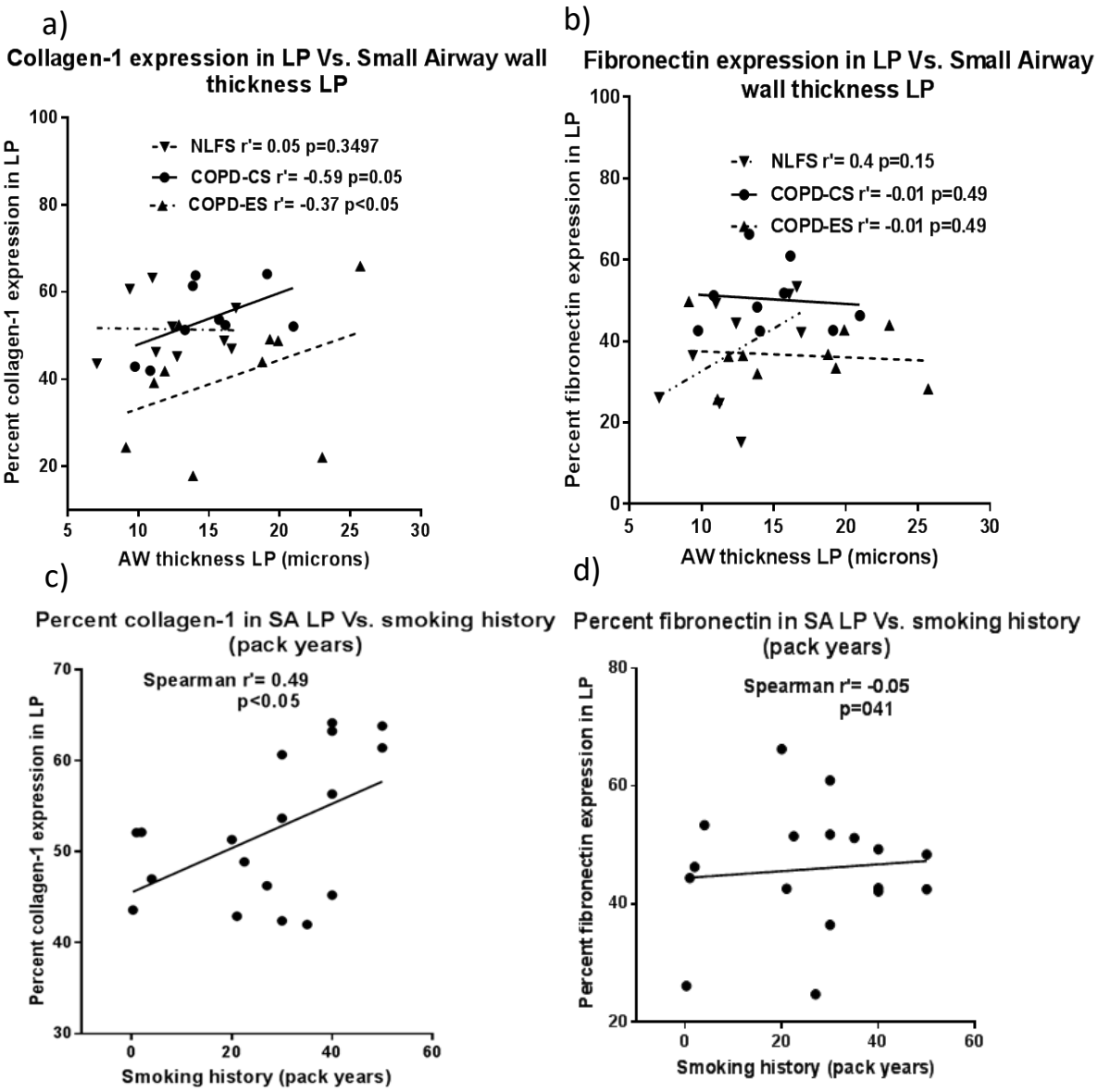

Figure 7.9 Correlation analysis between airway wall thickness and percent a) collagen-1 and b) fibronectin for all three-clinical group (NLFS, COPD-CS, and ES). An increase in percentage c) collagen-1 was found to correlate with smoking history pack years but not with d) fibronectin.

Further, there was a strong correlation between smoking history and collagen-1 percentage deposition in small airway LP, which was again absent with fibronectin (Figure 6.9 a, b).

A significant correlation was seen between collagen-1 deposition and lung function in the COPD-CS but was absent in for the ex-smoker group (Figure 6.10 a). I found no correlation between fibronectin deposition in small airway LP and either smoking history or lung function (Figure 6.10 b).

Figure 7.10

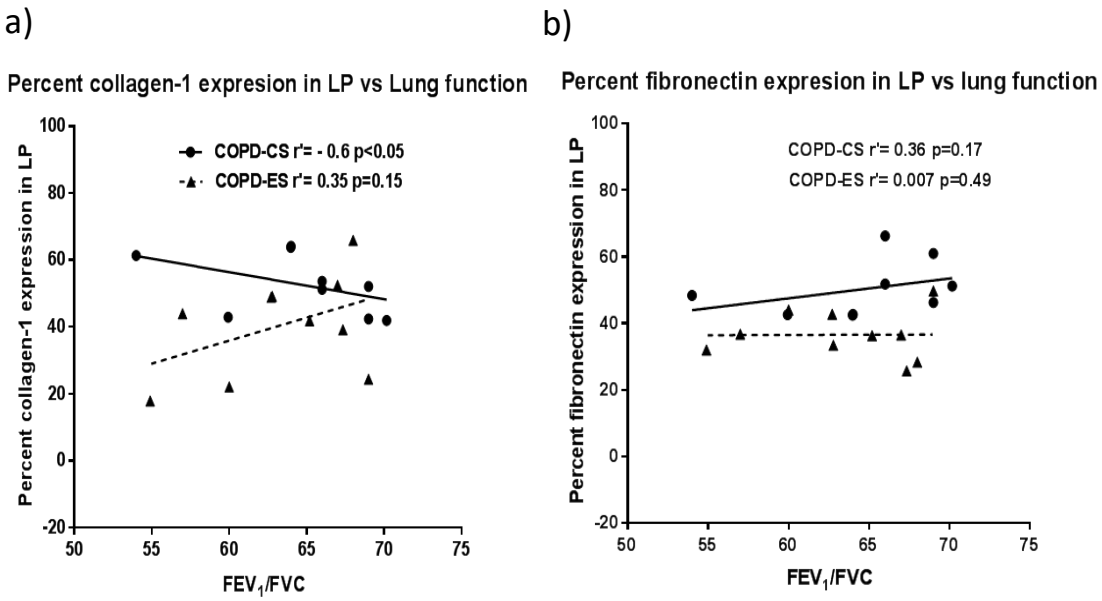

Figure 7.10 Correlation analysis between lung function and percentage a) collagen-1 and b) fibronectin expression in COPD-CS and COPD-ES.

7.3.7 **Correlation of αSMA positive my fibroblast to percent ECM changes in the SA wall of COPD group**

On evaluating the clinical groups, I found significant positive correlations between the density of αSMA+ myofibroblasts in both Rbm and LP with percent expression of both collagen-1 (Figure 6.11 a, b) but not with fibronectin (Figure 6.11 c, d) suggesting that myofibroblast could be the main contributor to collagen-1 deposition

Figure 7.11

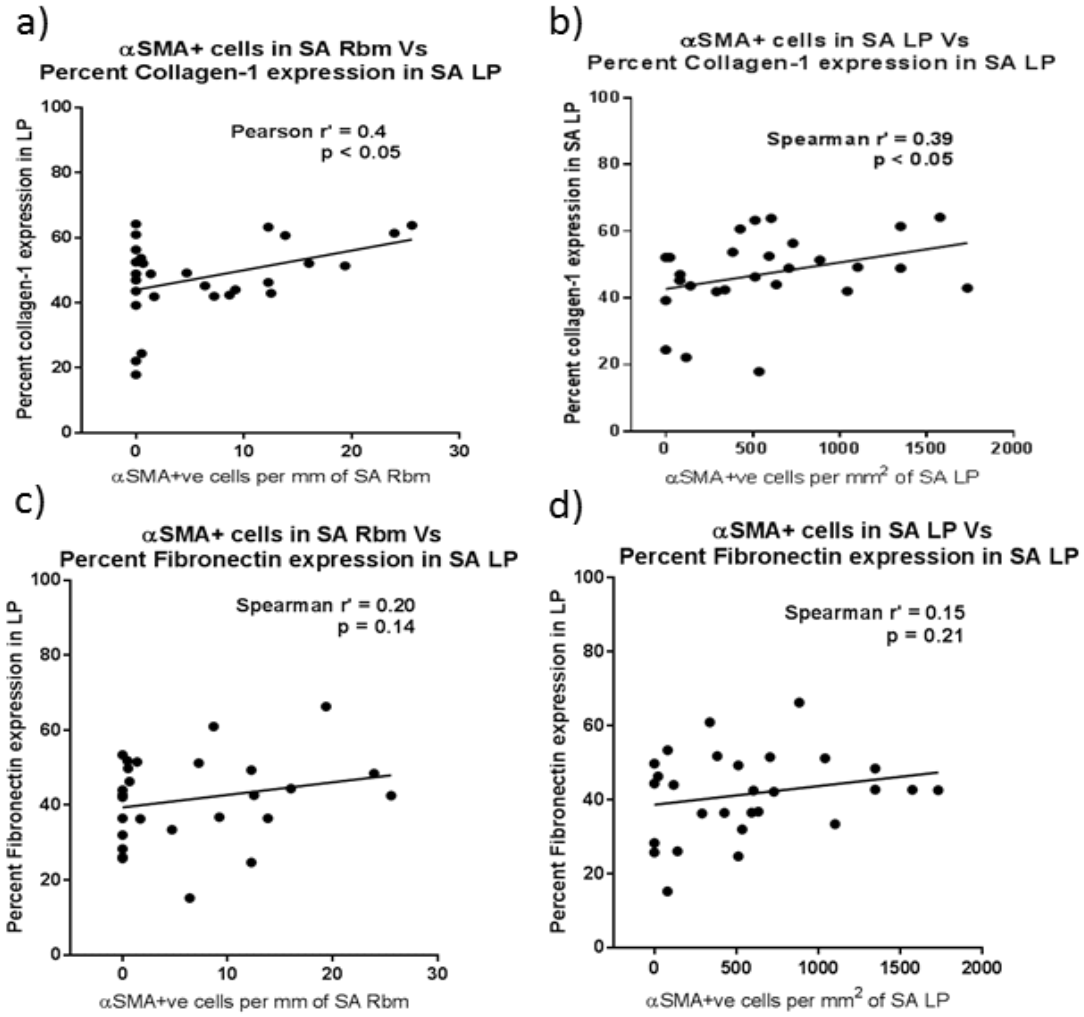

Figure 7.11 Correlation analysis between airway myofibroblast and percent collagen-1 and fibronectin expression in Rbm (Figure 7.11 a, c) and LP (Figure 7.11 c, d) for all three clinical group (NLFS, COPD-CS, and COPD-ES).

7.3.8 Correlation of EMT marker expression in the basal epithelium versus airway thickening in COPDs

I further evaluated whether changes in S100A4 positive cells as an index of EMT activity, expression of which has been previously shown by our group to be increased in both basal epithelial cells and Rbm cells (Mahmood et al., 2015). I further evaluated weather EMT marker expression are associated with αSMA positive myofibroblasts in the SA wall of smokers and COPD groups. αSMA cell expression is also thought to represent a late manifestation of the epithelium to mesenchymal transition. Notably, there was a strong positive correlation between the numbers of the two cell types, especially for basal epithelial cells against LP myofibroblast data.

Figure 7.12

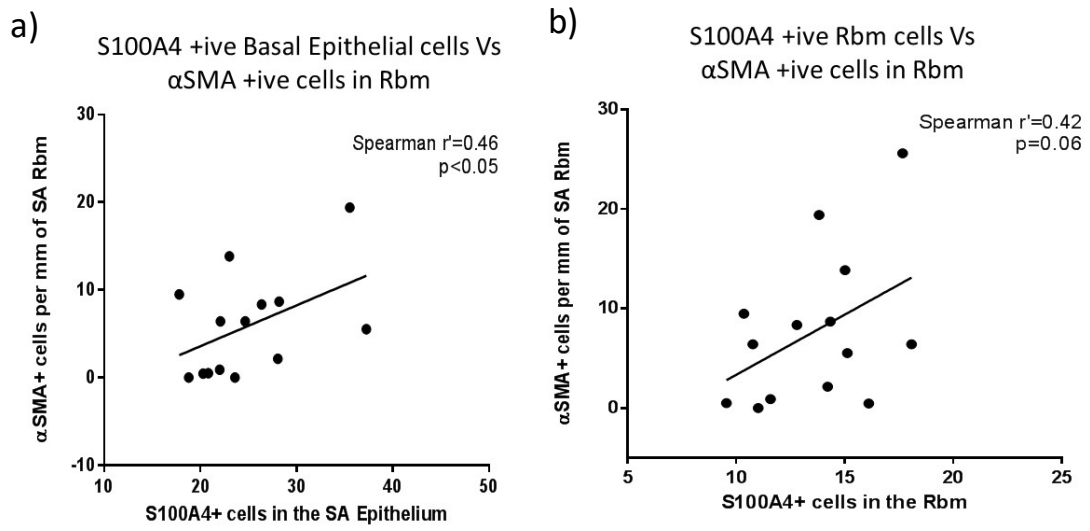

Figure 7.12 Correlation analysis between EMT marker S100A4 expression in the a) basal epithelial cells and b) Rbm with myofibroblast within the Rbm.

7.3.9 Correlation of EMT marker expression in the basal epithelium versus airway Lamina Propria thickening in COPDs

Further, a positive association was also observed between the increase in mesenchymal cell markers for both vimentin and S100A4 and increase in lamina propria thickness.

Figure 7.13

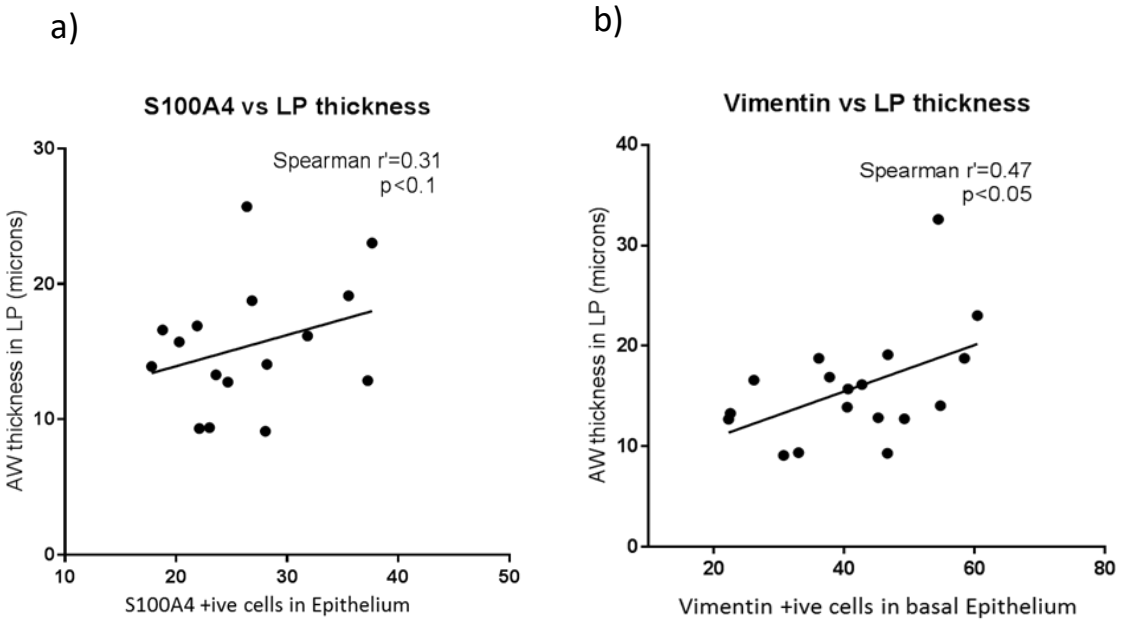

Figure 7.13 Correlation analysis between small airway wall LP thickness with EMT marker a) S100A4 and b) Vimentin expressed in basal epithelial cells.

7.4 Discussion

Perhaps the major novel finding in the current chapter is an increase in some αSMA positive myofibroblasts in the SA wall of COPD patients and its direct association with an increase in the airway wall thickness in the small airway LP and a decrease in lung function reflecting airway caliber. These changes in the myofibroblast population were also directly related to pathological changes in ECM protein, namely collagen-1 and fibronectin. I have also been able to correlate my data with previous observations from our group, by finding that the increase in mesenchymal markers in basal epithelial cells and Rbm cells of COPD patients, interpreted as representing active EMT, were significantly correlated with both the increase in the number of airway wall myofibroblasts as well as the increase in airway wall thickness. It is reasonable to interpret these data, I believe, as strong evidence that EMT is “an” if not “the” driver of myofibroblast proliferation and activity; that the myofibroblasts are active in producing new and pathological ECM; that this, in turn, is related to airway wall thickening and so reduction in small airway luminal calibre and so airflow. This confirms EMT and these flow-on effects from that as having a central place in small airway damage, and ultimately destruction as the core pathology in COPD airway disease as we and others have previously suggested (Sohal et al., 2017a, Sohal et al., 2017b).

It has been accepted that one of the principal causes for airflow limitation in COPD is the airway wall tissue remodeling/scarring though re-organization of the extracellular matrix (ECM). However, as I have pointed out earlier, the evidence for that has not been strong. In the current study, I analyzed two important markers of ECM pathology, collagen-1, and fibronectin, both already described as co-localized to areas with increased proliferation of myofibroblasts (Harju et al., 2010). I also observed an increase in both collagen-1 and fibronectin in the airway wall of COPD patients compared to smokers and normal controls. Although significant differences were observed the percent expression of both these ECMs, there were more marked changes for fibronectin compared to collagen-1. However, since collagen-1 was profoundly expressed throughout the sub-epithelium, i.e., both in the LP and adventitia,

the overall relative collagen index that I have devised (to take into account thickening of the airway wall compartments; described earlier), was significantly higher for both collagen-1 and fibronectin, in the smoker/COPD pathological groups compared to normal controls. Interestingly, though I observed the relative differences greater in collagen-1 compared to fibronectin, on adding the airway wall parameters, which again is suggestive of collagen-1 being the main contributor to airway wall thickness.

My current observation of an increase in collagen-1 is in agreement with the earlier studies done by Harju et al., (2010) in small airway tissues where an overall increase in both collagen I and III subtypes in the early stages COPD (GOLD stage I and II) were observed in the small airway LP. At the same time, my data contrast to observations by Annoni et al. (2012) who suggested a decrease in collagen-1 deposition in mild-moderate COPD patients, although they too found an increase in fibronectin in the small airways of both smokers and COPD.

Interestingly, the excess deposition of collagen-1 in the LP, which related positively to the small airway LP thickness, was specific to the COPD groups, while remaining unchanged in smokers with normal lung function. This provides further evidence for pathophysiological relevance for collagen-1 changes in COPD. In contrast and interestingly, fibronectin changes were not related to airway thickness, smoking history or lung function in the way that collagen-1 was; this suggests that it is not involved in physical remodelling but that its cellular immune-modulatory roles may be more important. Furthermore, ECM changes in COPD in relation to lung function were only significant in current-smoking COPD and not ex-smokers. I found an increase in collagen expression in current smokers affected their lung function while remaining unchanged in ex-smokers suggesting a probable reversal in physiological functionality in the group.

ECM producing myofibroblasts, which may derive from fibroblasts, have a spindle-shaped morphology and are highly contractile. The contractile nature of myofibroblasts is directly related to their expression of αSMA myofilaments,

which form so-called “stress fibers.” Surprisingly, there have been few reports so far that the current data could be compared to. My finding of an increase in αSMA positive myofibroblast is in contrast to Karvonen et al., (2013) who showed a decrease in expression αSMA cells in the bronchioles of COPD patients compared to non-smoker controls. The differences in the findings are likely due to the counting strategy and area under consideration. Thus, while Karovnen et al., (2013) counted αSMA positive cells in the whole of the sub-epithelial wall, in the current study I took into consideration αSMA positive cells between the whole Rbm and the muscle layer, i.e., the true LP (Figure 6.1). Further, I also separately measured αSMA positive cells in the Rbm itself just below the epithelium, which could be suggestive of an EMT source. My finding, however, was similar to that of Harju et al., (2010) who provided a descriptive analysis of the tissue and evidence of co-localization of αSMA positive cells with collagen subtypes as well as mesenchymal marker such as vimentin in the SA wall. Their conclusion was similar; giving evidence that αSMA positive myofibroblast could be responsible for the increased accumulation of collagen-1 and fibronectin in the small airway LP of COPD patients. Indeed, myofibroblasts may well be responsible for airway wall thickening secondary to their activation and consequent excessive secretion and accumulation of ECM. Further, my observation of the decline in lung function and reduced airway caliber with the increased presence of myofibroblast suggest an active involvement of this cell type in airway remodeling and resulting pathological airway stiffness associated with COPD patients.

As mentioned, our group’s previous data has suggested that the underlying mechanism for airway wall remodeling is through the induction of epithelial-mesenchymal transition (EMT) (Sohal et al., 2010), with a strong relationship between markers of EMT activity such as S100A4 and vimentin with decreased lung function and airway obstruction (Mahmood et al., 2015, Sohal et al., 2010). EMT in COPD is potentially driven by the canonical TGFβ pathways via nuclear transcription factor such as pSMAD2/3 while reducing the inhibitory SMAD7 (Mahmood et al., 2017), with these SMADs strongly correlating with decreased lung function parameters and airway obstruction (Sohal et al., 2014). Interestingly, TGFβ1 is also known to play a crucial role in the development of

myofibroblast from fibroblasts, also through phosphorylation of the same SMAD pathway (Gu et al., 2007, Harris et al., 2013).

There is however some limitation to this study; there were fewer individual subject tissue samples available per clinical group at this stage through bio-bank attrition and lack of time to obtain and process more, though my findings seem statistically robust and consistent, without likely Type-1 or Type-2 errors. Further, for some end-points, especially the EMT markers, I took the opportunity of using sections already stained for these by a previous Ph.D. student (Dr. Malik Mahmood) as part of his work, but the overlap between his subjects and mine were more limited than would have been ideal. Also, as a generic problem, the current SA tissues were obtained from cancer patients and thus some confounding by this disease pathology in some way cannot be ruled out, but all analysis of the tissue was done well away from the cancer-affected areas, and this seems unlikely.

While the contribution of myofibroblasts was for me a central issue, in fact, my study lacks specific information on the origin of the fibroblast in the airway wall and the relative contribution of EMT, which I can only speculate on from indirect evidence. I had hoped to analyze fibroblast-specific markers with double-staining, such as NG2 for pericytes, and fibroblast/myofibroblast activation markers such as PDGFRα/β, CD44, and Thy-1 among others to further delineate this further, but essentially ran out of time.

Finally, regarding limitations, there is the whole issue of the airways in these COPD tissue samples being essential “survivors.” In other words, we now know from the work of Hogg (Kirby et al., 2017) that a significant number, even perhaps a majority, of airways will have been destroyed by the time the patients reached the degree of airflow obstruction. It is quite likely that the processes that are involved here do indeed represent this destructive process, but it seems even more interesting that the regression analyses were productive. My conclusion here reflects not only acute pathology relevant to this point in time that the airways were sampled but also in some profound way represent the whole history of the pathological process probably going back over many years.

The fact that there was so much pathology already present in apparently physiologically normal individuals would back this up very strongly.

7.5 Conclusion

In this chapter, I have provided comprehensive numerical estimates of the myofibroblast population in the small airways of COPD patients, and some estimate of their activity. Thus, their close (if in some ways complex) relationships to ECM deposition, airway remodelling and pathophysiology suggest that they do have an important and even a critical role in the core disease process in COPD airways and its progression.

Chapter 8
Summary and Conclusions

8.1 Overview of results

COPD is now known to be a globally devastating disease, mainly caused by cigarette smoking worldwide and certainly in more developed countries. The disease prevalence is 210 million persons affected worldwide, and it causes at least 3 million deaths annually, which corresponds to 5% of total mortality (Schluger and Koppaka, 2014). The disease is characterized primarily by slowly progressive airway narrowing with only partial reversibility at most. Vulnerable smokers develop in particular the core pathology of small airway fibrosis that leads to luminal narrowing and ultimately obliteration, accompanied by "bronchitis" and structural changes throughout the airway tree. Up to fifty percent of the patients go on to suffer also from varying degree of parenchymatous lung destruction (emphysema) which adds to airflow obstructions and associated symptoms.

Airway inflammation is widely said to be central to COPD airway pathology, but what this means is somewhat enigmatic and probably over simplistic. Indeed, over the years, the literature has reported increases in both innate and adaptive cells in the airway lumen in COPD with convincing increases in both bronchoalveolar lavage (BAL) and sputum samples in COPD (Pesci et al., 1998a, Rutgers et al., 2000b, Pesci et al., 1998b). However, there are substantial contradictions about the actual picture of inflammation, if it exists at all, in both the large and small airway wall (Di Stefano et al., 1998a, Di Stefano et al., 2001, Saetta et al., 1999). In my literature survey in chapter 2, I have provided a comprehensive analysis of the inconsistencies in the prevailing dogma about COPD at least as it applies to the crucial airway compartment. Thus, in this thesis I have provided a comprehensive cross-sectional reassessment of the total and differential inflammatory cells in the airway wall; it is this overall cellularity in the airway wall of COPD patients that is crucial. This has been detailed in chapter 4, and only the broad overview is discussed here. Further, in chapter 5, I provide a more in-depth analysis on the

understudied subtypes (M1 and M2) of macrophage, for both the airway wall and the lumen. In chapter 6, I evaluate the presence of another key inflammatory cell type, Mast Cells, in the small airway wall and provided evidence of dysfunctionality both specific to MCs but also a more generalized abnormality with a reduction in degranulation capacity of cells in the airway wall. In chapter 7, I assessed the myofibroblast population in the airway wall, studied their potential contribution to airway wall thickness and ECM changes, and further explored their relation to EMT activity in basal epithelial and Rbm cells. I also provide further evidence on the possible contribution of EMT to the fibrotic changes, airway thickness and remodeling, and presence of airway obstruction.

8.1.1 Assessment of airway wall cellularity and inflammation

In tune with my stated aims, I began by revaluating the cellular composition of the airway wall, and this followed on from our research group`s previous observations of likely hypo-cellularity in the large airway of COPD patients (Sohal et al., 2013a). Thus, in chapter 4, I have made a comprehensive assessment of cellularity in both the large and small airways. I observed that both large and small airways areas were hypo-cellular in COPD patients when compared to a normal control group. Interestingly, this decrease in airway wall cellularity in COPD patients reflects reductions in both innate and adaptive inflammatory cells such as neutrophils, macrophage, CD4 and CD8 cells in large airways, and in the small airway except CD8 lymphocytes. All cells types evaluated in the small airways were either reduced or at best remained unchanged from normal. Further, in this chapter, I have also questioned the use of CD68 expression alone as a marker for detecting macrophages after having observed that they also tag more fibroblastic looking cells. Finally, there was the novel contribution that classic inflammatory cell types alone constituted only 20-30% of the overall cellularity, which formed the basis of further investigation on the role of other cell types in the airway wall, which I believe, are largely stromal cells (Figure 8.1).

Figure 8.1

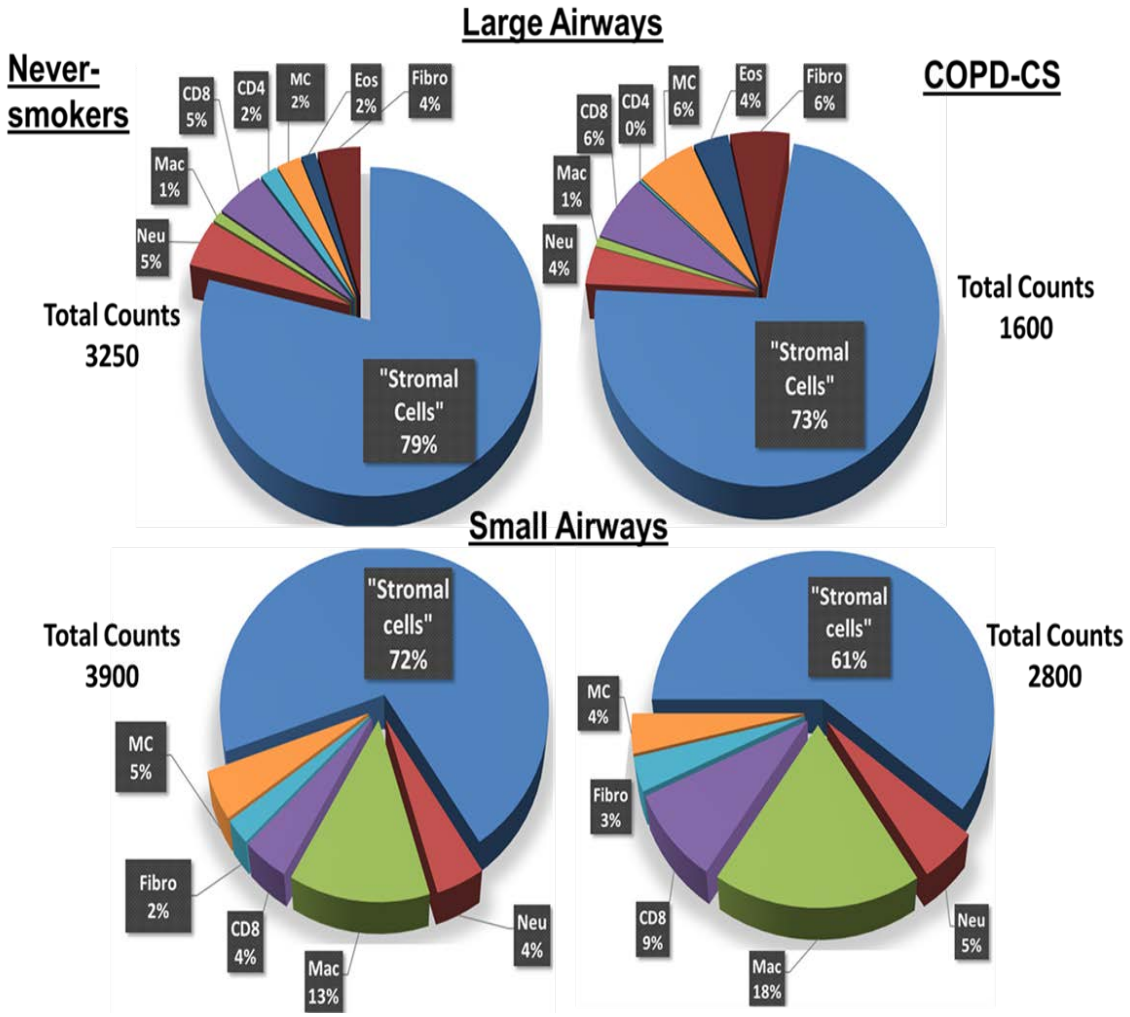

Figure 8.1 Summary of percent inflammatory cell component to the total cells in the large and small airways of non-smokers (NC) and COPD-CS (Neu- Neutrophils, Mac- CD68+ Macrophages, Fibro (CD68+fibroblast like cells), MC- Mast cells, Eos- Eosinophils, CD4 and CD8 cells).

8.1.2 Abnormal switching in the M1 and M2 macrophage phenotypes in small airway wall and the lumen of COPD patients (Chapter 5)

Another novel and somewhat paradoxical finding were that macrophages in the airway lumen are skewed towards an anti-inflammatory/ pro-fibrotic phenotype while M1 pro-inflammatory macrophage is more common in the airway wall. Indeed, in my work, this was the only evidence that there may be an inflammatory process in this compartment. This is the first time that such phenotype switching has been described and to that extent, at least, this change may underlie downregulation of macrophages function that has been described in luminal cells, e.g., in efferocytosis. Further, I provide evidence that this switching of macrophages may be influenced by the luminal microenvironment of the region, which again was found to be skewed towards an M2/TH2 phenotype. Another notable observation was the generalized excessive expression of the enzyme arginase-1 in the airway wall of COPD current smokers both in their epithelium and sub-epithelium areas. Such an increase of arginase-1 is known to tilt the balance of tissue homeostasis by again promoting a more fibrotic phenotype through inducing collagen formation and thus airway stiffness. This change from an iNOS dominance is also likely to explain why in smokers and COPD nitric oxide production from the respiratory tract is reduced, which has been known for some time; in turn this my may underlie a defect in microbial killing.

8.1.3 Evaluation of mast cells, degranulated mast cells and degranulated cells in the airway wall of COPD patients

In chapter 6, I also evaluated the presence of mast cells and their degranulating capacity in the airway wall of COPD patients. Indeed, as all other inflammatory cells described earlier, I found that there was a decrease in the number of mast cells in the sub-epithelium of COPD patients. This was again in contrast to the previous report from our group which found that an increase in mast cell in a similar area as in the large airways. Further, this is the first reported use of LAMP-1, a lysosomal marker to evaluate degranulation capacity in mast cells. While the only slight decrease was observed in the degranulating mast cells,

the overall degranulating capacity of the cells in COPD patients was found to be significantly lower in when compared to normal control. This could thus suggest a decrease in the overall functionality of the cells. However, the description of the degranulation provided in this chapter is generalized and thus warrants more investigation, specific to individual cell types.

8.1.4 Extensive evaluation of small airway fibrosis

This is the first time than anyone has systematically “dissected,” using immune-pathological methods on resected lung tissue, the dimensions of the small airway wall in smokers and COPD patients compared with normal controls. Given that we know that the pathophysiology in COPD airways is focused in this area, this has been a remarkable gap in respiratory research. The core finding related to COPD is of thickening of all compartments in the small airway wall, i.e., Rbm, LP, ASM layer, and adventitia, but the changes in LP are the strategically the most specific to COPD, while other changes may be more of a smoking effect. Indeed, the degree of pathology that was found in apparently physiologically “normal” smokers was quite striking. All of these clinical tissues came from cancer resections and it is possible that they represent the end of the normal lung function smoker range, but they are still not being picked up by conventional spirometry airflow methods.

Myofibroblast in the airway wall, specifically in the LP, were dramatically increased in COPD and statistically related to airway thickness. Similarly, the airway wall depositions of collagen-1 and fibronectin, the ECM proteins related to fibrosis that I measured, were increased, but only the collagen was closely related to airway wall thickening and to airflow obstruction. Driving all these changes is likely to be EMT activity in the basal epithelial cells. Given the relationships found, this is likely due to their transition directly into myofibroblasts, though it is conceivable that the myofibroblast change could be indirect through EMT-related activity stimulating stromal cells, pericytes or endothelial cells to transition; this, however, is highly speculative at this stage, and a direct effect of EMT is most consistent with the data now provided.

8.2 Concluding remarks

I believe that this work presented here advances the understanding of the core pathology in smokers and COPD substantially and somewhat by directly adding to the early work of Macklem and colleagues who describe the small airways as the site of both increase airway resistance in smokers and probably enhanced “fibroblast” activity as the likely pathology.

The focus of most pathogenic research in COPD over the past 40 years has been on airway luminal innate immune activation (“inflammation”) and the drivers of this (and emphysema) including disturbance of protease/anti-protease balance and oxidant/anti-oxidant balance. There has been limited follow up on the seminal work of pathologists Lynne Reid and Michael Dunnill describing abnormal epithelium in COPD. Only until recently work by Ronan Crystal`s group demonstrated the significance of the abnormality in basal (stem) cell genetic programming in the airway epithelium and their consequence to airway pathology. Parallel to this was our group’s observation that one of the consequences of such reprogramming in these basal cells is active EMT. I have taken this observation a significant step further by showing the EMT activity in the epithelium is strongly related to myofibroblast expansion in the sub-epithelial LP of the airway wall and what reasonably seem to be consequential changes in airway thickness, ECM changes and airway obstructive changes

The finding that the airway wall in COPD is essentially NOT an inflammatory milieu but is indeed hypo-cellular and fibrotic is especially important. At the expense again of slightly exaggerated language, my picture of the airways in COPD is one of a “dying tissue,” which fits in well with the vital observations by the Hogg’s group of the disappearance of small airways being such a dramatic early feature of this disease process.

Finally, I have made some rather more preliminary and provisional observations: on macrophage phenotype switching in the airways which may well underlie important known functional changes in this cell population; on a

switch from iNOS to arginase dominance in the airway tissues in COPD; on dysfunction in degranulating cells in the airway wall; and indeed even more preliminary observations of likely protein-handling/lysosome and autophagy abnormalities in the COPD epithelium. This latter has not been included in the thesis because they are not yet sufficiently mature, although recently presented at national and international conferences. I mention this feature now only because these new observations open potentially new avenues for COPD research and potential advances in integrative pro-fibrotic (and pro-malignant) understanding of fundamental COPD pathogenesis which have been sorely absent for much of the last 40-50 years, with some crucial exceptions which I have tried to highlight.

Chapter 9

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