

**CHARACTERISING HUMAN LUNG TISSUE FOR
BIOMARKERS OF EMT-FIBROSIS AND
FUNCTIONAL STEROID RECEPTOR
COMPONENTS IN CHRONIC OBSTRUCTIVE
PULMONARY DISEASE(COPD) PATIENTS AND
CONTROL SUBJECTS**

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ABBREVIATIONS

AAT- Alpha antitrypsin deficiency

AHR- Airway hyperresponsiveness

AP1- Activator protein

ATS- American thoracic society

BALF- Broncho alveolar lavage

COPD- Chronic obstructive pulmonary disease

CS- Current smoker

CCS- COPD current smoker

CEX- COPD ex-smoker

CLE- Centrilobular emphysema

CT Scan-computed tomography scans

ECM- Extracellular matrix

EDTA- Ethylenediamine tetra-acetic acid

EGFR- Epidermal growth factor receptor

EMT- Epithelial mesenchymal transition

EX- Ex-smoker

FEV1- Forced expiratory volume in 1 second

FVC- Forced expiratory capacity

FSP- Fibroblast specific protein

GOLD- Global initiative for chronic obstructive pulmonary disease

GR- Glucocorticoid receptor

GRE- Glucocorticoid receptor element

GR α - Glucocorticoid receptor-alpha

GR β - Glucocorticoid receptor-beta

HDAC 2- Histone deacetylase-2

hGR- Human glucocorticoid receptor

HAT- Histone acetylase

H & E- Haematoxylin & eosin

HPA- Hypothalamic pituitary adrenal axis

HNS- Healthy non -smoker

HSP- Heat shock protein

ICS- Inhaled corticosteroid

IHC- Immunohistochemistry

IPF- Idiopathic pulmonary fibrosis

JNK- c-Jun N terminal kinases

LBD- Ligand binding domain

LTB4-Leukotriene B4

MAPKs- Mitogen activated protein kinases

MMPs- Matrix metalloproteinase

NF κ B- Nuclear factor kappa light chain

NHS- National health service

NSCLC- Non- small cell lung cancer

PLE- Panlobular emphysema

SCLC- Small cell lung cancer

SMC- Smooth muscle cells.

STAT- Signal transducer and activator of transcription

p53- Tumour protein or phosphoprotein 53

PTM- Post translational modification

PYH- Pack year history

RAGE- Receptor advanced glycation end products

RBM- Reticular basement

RNS- Reactive nitrogen species

ROS- Reactive oxygen species

S100A4- Metastatin

S211- Serine 211

TGF β - Tumour growth factor beta

Th1- T-helper cells

TLRs- Toll like receptors

TNF- Tumour necrotizing factor

TNF α - Tumour necrotizing factor alpha

TPR- Tetratricopeptide repeats (Motifs)

TTC5- Tetratricopeptide repeat domain 5

VEGF- Vascular endothelial growth factor

ABSTRACT

COPD is a poorly reversible airflow obstruction commonly induced by cigarette smoke. Pathology is linked to a series of inflammatory and fibrotic events in the small airways and lung parenchyma; including fibrosis, emphysema and mucus plugging. This work focuses on the inflammatory and fibrotic aspects of COPD pathology. Processes of fibrotic Epithelial to Mesenchymal Transition (EMT) have been identified in heart and kidney disorders and we wished to evaluate if this is a contributing factor in COPD. Second, patient treatment options are limited. Steroid resistance is the major barrier for effective treatment and this study seeks to evaluate steroid receptor expression patterns in COPD patient and control subjects to better understand mechanisms of resistance. The aim of this study is to evaluate EMT (S100A4) and glucocorticoid receptor (TTC5 & S211) component expression in lung tissue of COPD patients and control subjects using immunohistochemical staining. The result obtained describes that S100A4 (EMT marker) was more highly expressed in active smokers (non-smoker and ex-smoker, versus current, $p < 0.0001$). TTC5 expression was higher in both ex-smokers and current smokers compared to NS ($p = 0.0022$). S211 expression levels were similarly raised in both ex and current smokers compared to NS ($p = 0.0078$). The conclusion is that raised S100A4 expression in active smokers indicates EMT and may play a role in fibrosis in COPD via a partially reversible process. Raised TTC5 and S211 in ex and current smokers indicate irreversible glucocorticoid receptor changes and may implicate a mechanism of steroid resistance in COPD patients, thus prompting further research in this area.

1. INTRODUCTION

Chronic Obstructive Pulmonary Disease represents a major health problem worldwide. The Global Initiative for Chronic Obstructive Lung Disease (GOLD) defines Chronic Obstructive Pulmonary Disease as preventable and treatable disease characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and lungs to noxious particles or gases. Chronic Obstructive Pulmonary Disease is a leading cause of morbidity and mortality worldwide with an overall prevalence (9-10%) in adults aged >40 years (Halbert et al, 2006).

1.1 EPIDEMIOLOGY OF COPD

In United Kingdom, 1.2 million people have been diagnosed with chronic obstructive pulmonary disease, making it the second most common lung disease in country after asthma. One patient dies every 20 minutes with COPD in England killing over 23,000 individuals each year. It is the 2nd most common cause of emergency admissions to hospital and one of the most costly inpatient conditions to be treated by the NHS (>£800 million each year). An important feature in the epidemiology of COPD is the high risk of under diagnosis (Miravitlles *et al*, 2009 & Hvidsten *et al*, 2010), therefore the above figure are likely to be an under-estimate and the mortality in the population is likely to be much higher. COPD is presently fourth leading cause of death worldwide but World Health Organization (WHO) estimates that by 2020, COPD will be the 3rd leading cause of mortality and the fifth cause of morbidity in the world. The primary cause of COPD is smoking, with 30-40% of smokers estimated to develop the disease (Lokke *et al*, 2006). Chronic Obstructive Pulmonary Disease (COPD) is an umbrella term and includes Chronic Bronchitis, Emphysema & Chronic

unremitting asthma which can all co-exist in some people with the disease.

1.1.1 EARLY HISTORICAL BACKGROUND

In 1679, Bonet described emphysema as “Voluminous lungs” and after century later in 1769 Morgagni described it as ‘turgid’ from air (Bonet, 1679; Morgagni, 1769, cited in Petty, 2006). The clinical understanding of the chronic bronchitis component of COPD was traced by Badham in 1814; he used the word catarrh to refer as ‘chronic cough’ and Mucus hypersecretion as cardinal symptoms (Badham, 1814, Cited in Petty, 2006).

The description of emphysema of the lungs was found in ‘*Treatise of diseases of the chest*’. Laënnec was the inventor of stethoscope and carried out study by careful dissections of patients lungs during his life. He recognized that emphysema lungs were hyper inflated and did not empty well. This combination was described as emphysema and chronic bronchitis (Laënnec, 1821, Cited in Petty, 2006).

The spirometer is a key to diagnosis and management of COPD. Spirometer was invented in 1846 by John Hutchinson by which only vital capacity was measured (Hutchinson, 1846). It took another 100 years for Tiffeneau to add the concept of timed vital capacity as a measure of airflow for spirometry to become a complete diagnostic instrument (Tiffeneau & Pinelli, 1947).

The great teachers of emphysema, Ronald Christie, suggested that “the diagnosis should be considered certain when dyspnea or exertion of insidious onset, not due to bronchospasms or left ventricular failure, appears in a patient who has some physical sign of emphysema together with chronic bronchitis and asthma” (Christie 1944). It is clear from this statement that Christie recognized that individual

components of COPD and relied on the history and physical examination for his diagnosis.

1.1.2 TWO LANDMARKS MEETINGS

The CIBA guest symposium in 1959 (Ciba guest symposium 1959, Donald 1971) and the American Thoracic Society Committee on Diagnostic standards in 1962 defined the components of COPD, which are the foundation for our definitions today (Committee on Diagnostic standards for Non-tuberculosis respiratory diseases, 1962). The American Thoracic Society (ATS) defined chronic bronchitis in clinical terms including chronic cough lasting at least three months for at least two years. By contrast, the ATS defined emphysema in anatomic terms of enlarged alveolar spaces and loss of alveolar walls. Asthma was described as a state of airway hyper responsiveness to a variety of stimuli. Asthmatic bronchitis was considered an overlapping condition (Committee on diagnostic standards for Non-tuberculosis Respiratory Diseases, 1962).

William Briscoe is believed to be the first person to use the term COPD in discussion at the 9th Aspen emphysema Conference. This term became established and today refers to COPD as the designation of this growing health problem (Briscoe & Nash, 1965).

COPD can be classified with respect to both phenotype and disease severity. It is a heterogeneous disease process that varies greatly from person to person with respect to lung pathology, natural history of disease and comorbidity. A result of this heterogeneity is that different researchers have championed alternatively hypotheses about COPD development over the past four decades. The British hypothesis stated that the presence of cough and sputum was the key factor in

COPD (Anthonisen, 2005), and the Dutch hypothesis pointed to the presence of increased airways responsiveness (Vestbo & Prescott, 1998). Less widely known hypotheses stressed the part of genetic factors (the Swedish hypothesis) and the role of impaired repair processes in the development of emphysema (the American hypothesis) (Rennard, 1998). All these hypotheses probably have elements of truth since COPD disease manifests increased airways reactivity, a characteristic response to infections, abnormal cellular repair and development of complications or comorbid conditions.

1.2 COPD FEATURES AND MECHANISMS

1.2.1 COPD PATHOPHYSIOLOGY

Chronic obstructive pulmonary disease is a common preventable and treatable disease though it is not curable. COPD is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. The main site of airflow obstruction in COPD is the small airways (Hogg & Timens, 2009). Airflow limitation in COPD may result of three different pathological mechanisms. 1) Luminal obstruction with mucus and plasma exudate as a result of inflammation, 2) Emphysema as a results in expiratory collapse for two reasons: Disruption of alveolar attachments permits small airways to close on expiration due to lack of elastic support and loss of lung elastic recoil decreases the intraluminal pressure, which also predisposes to small airways to collapse , particularly with forced exhalation, 3) Thickening and fibrosis of small airways (Chronic Obstructive bronchiolitis) which is presumed to be due to the effect of chronic inflammation. Fibrosis occurs around small airways and is thought to be a main factor that

contributes to the irreversible airway narrowing that is the characteristics of this disease (Hogg *et al*, 2004).

Chronic inflammation leads to the airways becoming narrowed, which reduces airflow. Cigarette smoke and other pollutants activate several inflammatory and structural cells of the lung to produce inflammatory and remodelling responses. An inflammatory cell plays a vital role in pathogenesis of COPD and is characterized by chronic inflammation throughout the airways, parenchyma and pulmonary vasculature. Macrophages, T lymphocytes (predominately CD8) and neutrophils are increased in various parts of the lung. Activated inflammatory cells release a variety of mediators including leukotriene B₄ (LTB₄) (Hill *et al*, 1999), Interleukin-8 (Yamamoto *et al*, 1997), tumor necrosis factor-alpha (Keatings *et al*, 1996) and others capable of damaging lung structures or sustaining neutrophilic inflammation. In addition to inflammation, two other processes thought to be important in the pathogenesis of COPD are an imbalance of proteinases and anti-proteinases in the lung and oxidative stress.

1.2.1.1 MUCUS HYPERSECRETION

Epithelial cells in human lungs are the first anatomical barrier between the tissue and inhaled air which are exposed in the airways. The upper lining of epithelial cells in the airways prevents toxins, inhaled foreign particles, allergens intruding and disturbing the sub-epithelial cell layers of airway walls. Ciliated epithelial cells play a major role in expelling the inhaled particles and mucus out of the lung in to the esophagus, where these possibly harmful factors are digested or otherwise exported from the body (Nordgren *et al*, 2014). Under chronic inflammatory conditions, this mechanism is disturbed and results in a destruction of the epithelial cell layer, which results in the subsequent loss of function as a barrier between inhaled air and sub-

epithelial tissue (Hessel *et al*, 2014).

Role of Mucin:

In normal airways, a thin layer of mucus covers the airway epithelium surface. Airway mucus is composed of 97 % water and 3 % solids (Wanner, 1996). Mucus maintains hydration and protects airways against toxic gases, inhaled pathogens via mucociliary clearance. Mucus transports antioxidants, antiproteases, antimicrobial substances by contributing to innate immune response but airway obstruction can be resulted due to abnormal mucus production. During such inflammatory conditions the epithelium that lines the airways becomes ironically inflamed leading to a breakdown in its integrity. Mucous glands become bigger, resulting in increased mucus secretion. The cilia that line the airways are destroyed and the ability of lung to remove mucus is impaired. Mucus hypersecretion results from an increase in mucin production & secretion from airway epithelial goblet cells and /or submucosal glands. Secreted mucins are high molecular weight glycoproteins synthesised in airway epithelial goblet cells and in mucus tubules of submucosal glands (Fahy & Dickey, 2010). The two main secreted airway mucins are MUC5AC and MUC5B (Fahy & Dickey, 2010; Rose & Veynow, 2006). Increased mucin stores are generally found in airway epithelium of proximal (Innes *et al*, 2006 & O'Donnel *et al*, 2004) and distal airways in COPD patients (Kim *et al*, 2008 & Saetta *et al*, 2000). Mucus hypersecretion in distal airways of COPD was further attested by the presence of mucus exudates obstructing airway lumens (Caramori *et al*, 2004 & Hogg *et al*, 2004). Many stimuli relevant to COPD induce mucin production in airway epithelial cells. One of them is cigarette smoke products like nicotine and acrolein (Borchers *et al*, 1998 & Gundavarapu *et al*, 2012). Cigarette smoke damages the airway epithelium leading to squamous metaplasia. This

abnormality is also seen in smokers without COPD, and likely does not contribute significantly to airflow obstruction. On a macroscopic level, epithelium layer thickness increases incrementally as disease severity worsens (Hogg *et al*, 2004).

Mucous Hyperplasia:

An important component of epithelial remodelling is mucous hyperplasia, a process in which mucus is overproduced in response to inflammatory signals (Williams *et al*, 2006). Mucous hyperplasia has been found in small airways of patients with COPD and significantly contributes to airflow obstruction (Hogg *et al*, 2004; Saetta, 2000 & Baraldo *et al*, 2003). This may develop from cigarette smoke exposure itself (Ebert & Terracio., 1975 & Deshmukh *et al*, 2005), acute and/or chronic viral infection (Holtzman *et al*, 2005), or inflammatory cell activation of mucin gene transcription (Burgel & Nadel, 2004). Chronic mucus hypersecretion accelerates lung function decline (Vestbo *et al*, 1996) and predisposes to hospitalization and infection (Prescott *et al*, 1995). Mucous metaplasia causes airflow obstruction by several mechanisms: increased mucus hypersecretion causing luminal occlusion, epithelial layer thickening encroaches on the airway lumen (James & Wenzel, 2007) and increased mucus alters surface tension of the airway, thereby predisposing it to expiratory collapse (Macklem *et al*, 1970).

Neutrophils:

Neutrophils are recruited in the airways of COPD patients (O'Donnell *et al*, 2004). Neutrophils may play multiple roles in mucus hypersecretion in COPD. Neutrophil proteases induce rapid and potent mucin secretion when neutrophils are recruited in close contact with airway epithelial goblet cells (Agusti *et al*, 1998 & Takeyama *et al*, 1998). Further, there is evidence of neutrophil elastase which induces mucin

synthesis *in vitro* in cultured airway epithelium cells (Shao & Nadel, 2005 & Kohri *et al*, 2002) and induces goblet cell metaplasia *in vivo* (Voynow *et al*, 2004).

Histopathological evidence of mucus hypersecretion in proximal airways of COPD patients has been closely associated with symptoms of chronic cough and sputum production (Mullen *et al* 1985 & Saetta *et al*, 1997). Chronic cough and sputum production are frequently present in (ex) smokers with COPD (Burgel, 2012). The distal airways of 19 COPD patients were studied by Kim *et al*, reported that mucin stored in epithelium increased with increasing airflow limitation (2008). Hogg *et al* (2004) have shown that obstruction of distal airways with mucus exudates is a major & independent determinant of airflow limitation in COPD patients. Importantly, chronic cough and sputum production are associated with increased rates of COPD exacerbations (Burgel *et al*, 2009), more rapid decline in lung function (Vestbo *et al*, 1996) and premature deaths (Ekberg-Aronsson *et al*, 2005).

1.2.1.2 EMPHYSEMA

Emphysema is a pathologic term defined as the abnormal permanent enlargement of airspaces by destruction of their walls and without obvious fibrosis (Piquette *et al*, 2000). Two main sub-types exist: Centrilobular Emphysema (CLE) and Panlobular Emphysema (PLE). CLE affects the lobules around the central respiratory bronchioles (Leopold & Gough, 1957), and is the primary pathologic subtype associated with cigarette smoke induced Obstructive Pulmonary Disease (OPD). This pattern of emphysema is typically more prominent in upper lung zones (Heard, 1958 & Thurlbeck 1963). PLE uniformly affects the entire secondary lobule (Wyatt *et al*, 1962). This subtype differs from CLE, as it is associated with α 1-antitrypsin deficiency and is more prominent in the lower lung zones (Thurlbeck, 1963).

One reason of obstruction is due to emphysematous changes where there is loss of elastic fibers in the alveolar walls and subsequent destruction of the alveoli (Black *et al*, 2008 & Merrilees *et al*, 2008). The chronic and abnormal inflammatory process induced by tobacco smoking contributes to increased extracellular matrix degradation and promotes the structural changes in lung parenchyma. Because of remodelling and narrowing of small airways and destruction of the lung parenchyma (pulmonary emphysema) there is consequent loss of the alveolar attachments of the airways (Morjaria *et al*, 2010). This results in diminished lung recoil, higher resistance in flow, and closure of small airways at higher lung volumes during expiration, with consequent air trapping in the lung. This leads to the sensation of dyspnoea and decreased exercise tolerance. Both small airway remodelling and pulmonary emphysema result from chronic peripheral lung inflammation (Caramori *et al*, 2015).

Alpha-Antitrypsin Deficiency (AAT):

AAT deficiency is a genetic cause of Chronic Obstructive Pulmonary Disease (Kelly *et al*, 2010). AAT is synthesised and secreted mainly in the liver by hepatocytes (Eriksson *et al*, 1978 & Koj *et al*, 1978) and also secreted from macrophages (Mornex *et al*, 1986) and bronchial epithelial cells. AAT is released by liver in circulation gets to the lung parenchyma where it buffers any excess in neutrophil elastase activity. Deficiency of AAT leads to protease/Anti-proteases imbalance, particularly during increased elastase activity such as acute disease exacerbations or exposure to inhaled irritants (i.e., tobacco smoke).

Proteinase and Anti-Proteinases Imbalance:

Proteinases-anti proteinases imbalance may play a central role in the pathogenesis

of COPD (Hogg & Senior, 2002). An important source of both proteinases and anti-proteinases within the lung is the alveolar macrophage, which produces Matrix Metalloproteinases (MMP1, MMP9 and MMP12) important for airway inflammation and the development of Emphysema (Shapiro, 1999 & Mocchegiani *et al*, 2011). Macrophages are the most abundant cell type found in BALF of COPD patients (Macnee, 2005). Cytokines such as IL-1beta and tumor necrosis factor (TNF) play crucial role in pathology of COPD (Chung, 2001). Bronchial damage through inflammation which causes tissue damage could contribute to emphysema. Thus, it is important to examine expressions of proteinases and anti-proteinases induced by inflammatory cytokines (Wilson, 2001).

1.2.1.3 FIBROSIS

During injury and repair, the boundaries of the tissue disintegrate and the protective architecture of the extracellular matrix (ECM) is disturbed, thereby exposing cells to drastic changes in the mechanical environment. Under this mechanical imbalance cells are exposed to an overwhelming cocktail of cytokines, initially derived from damaged cells, inflammatory cells and myofibroblasts which drive tissue repair by secreting collagen and reorganizing the ECM (Gurtner *et al*, 2008 & Hinz, 2010). Fibrosis is characterized by the massive deposition of Extracellular Matrix (ECM) as a reactive process initiated to protect the tissue from injury. Fibrosis is associated with the overgrowth, hardening and scarring of tissues and is frequently observed in chronic diseases of the lung, liver, kidney and heart (Guarino *et al*, 2009 & Wynn, 2008).

Fibroblasts and Myofibroblasts:

Lung fibrosis is characterized by progressive and irreversible destruction of lung architecture, disruption of gas exchange and death from respiratory failure (Wynn 2011). Fibroblasts and Myofibroblasts are the main cell types responsible for synthesis and secretion of the extracellular matrix (ECM) proteins and proteoglycans during tissue repair (Hinz *et al*, 2012, Klingberg *et al*, 2013). Fibroblasts are heterogeneous with respect to several phenotypic features. Under non-pathological condition, fibroblasts maintain homeostasis of the tissue (Powell *et al*, 1999 & Turner & Grose, 2010). Fibroblasts are spindle shaped cells found in stroma of tissues and during fibro-genesis, they get highly activated and possess the phenotypic characteristic of myofibroblasts. In adult tissue, myofibroblasts are activated by inflammation and are involved in maintaining the homeostasis and wound healing (Eckes *et al*, 2000, and Wynn, 2008). Myofibroblasts express α -smooth muscle actin (α -SMA) and differ from fibroblasts by the presence of cytoplasmic bundles of contractile microfilaments or stress fibers, which are similar but not identical to those in smooth muscle cells (Desmouliere *et al*, 2003). They are also characterized by an increased proliferation, migratory ability, production of cytokines and greater capacity to produce interstitial matrix (Desmouliere *et al*, 2003 & Guarino *et al*, 2009). However, due to the inability of myofibroblasts to regenerate tissue, they often create collagenous and stiff scar. This scar tissue frequently disrupts the function of intact residual tissues and alters the biochemical and biophysical microenvironment turning neighboring cells into fibrotic and dysfunctional cells (Hinz, 2009). Therefore, deregulated activity of myofibroblasts results in impaired tissue function and even organ failure (Hinz, 2009 & McAnulty, 2007). Myofibroblasts are prominent components of fibrosis in many tissues, including lungs (Schurch *et al*, 1998).

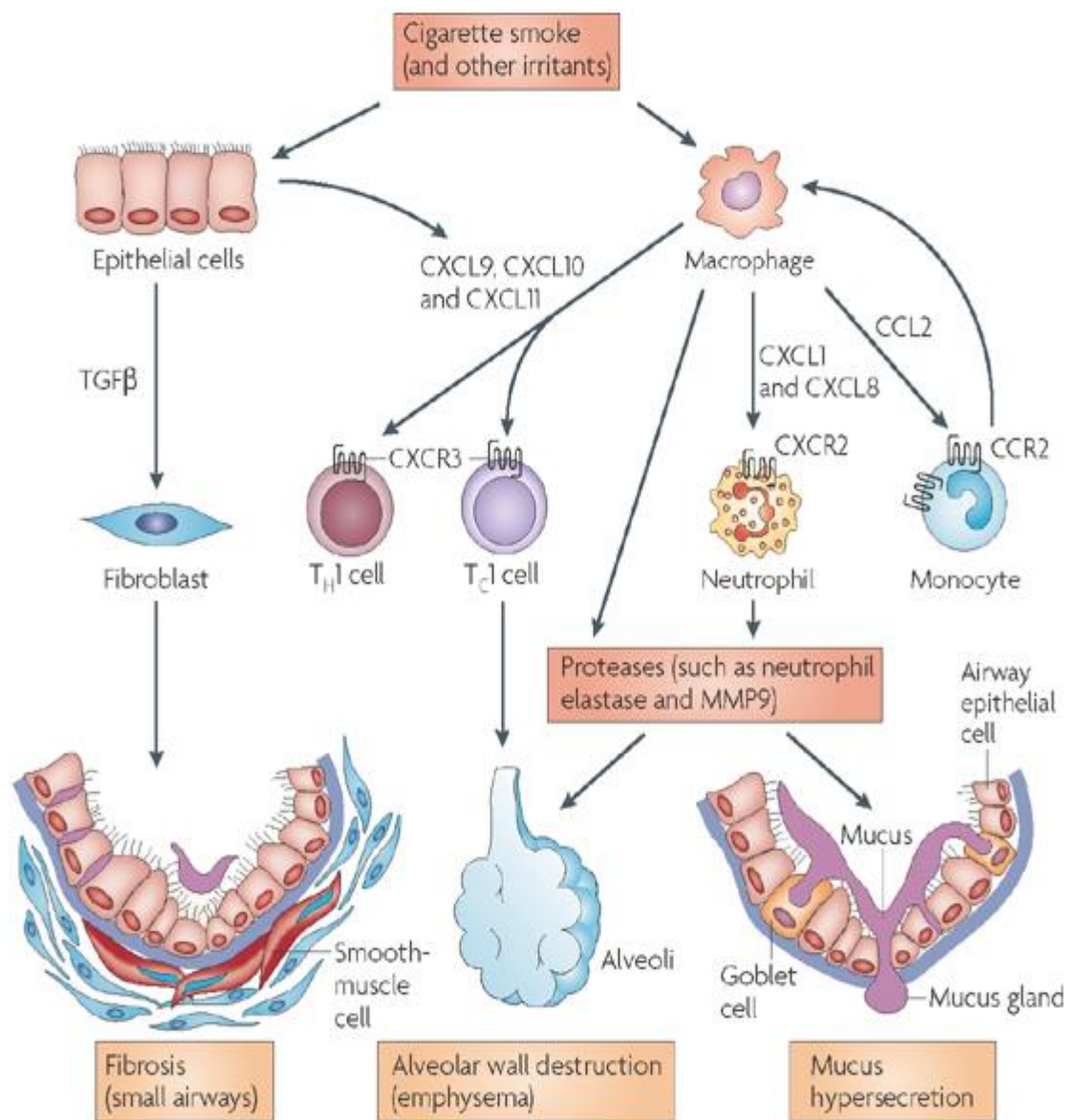
Myofibroblasts were originally believed to be generated by proliferation and activation of local fibroblasts (Barnes & Gorin, 2011 & Grillo, 1963). It has been considered, myofibroblasts can be derived from multiple sources (Abe *et al*, 2001 & Zeisberg *et al*, 2007). During pulmonary fibrosis, they are assumed to arise through trans-differentiation of resident pulmonary fibroblasts (Hashimoto *et al*, 2004). The circulating fibroblasts like cells which are derived from bone-marrow influx to the site of tissue injury are termed as 'fibrocytes' and possess fibroblast features (Abe *et al*, 2001 & Phillips *et al*, 2004). Fibrocytes are systemic source of contractile myofibroblasts in various lesions in lung as well as in keloids, sclerodema and kidney (Abe *et al*, 2001). Similarly, in liver fibrosis bone marrow derived hepatic stellate cells (HSCs) appear to be source of myofibroblasts (Baba *et al*, 2004). Myofibroblasts may be the effector cells in fibro proliferative diseases such as idiopathic pulmonary fibrosis (IPF) and COPD as well as the stromal reaction occurring in lung cancer (Lofdahl *et al*, 2011, De Wever *et al*, 2008 & Hinz *et al*, 2012). However, IPF falls under the category of interstitial lung disease. The distinct characteristics of IPF is the scarring of lung tissue which gets thicken and harden, making more difficult for a person to breathe whereas the destruction of alveoli and fibrosis of airway walls are characteristics of COPD which leads to the enlargement of air spaces. The fibrotic alternations in COPD, structural changes are observed primarily in the small airways (<2mm in diameter), and the severity of the disease appears to be determined by the thickening of the wall of the small airways caused by the fibrosis and infiltration of inflammatory cells (Jeffery, 2004). Airflow limitation is caused by tissue remodeling including reorganization of the extracellular matrix (ECM). In COPD lung ECM is degraded in alveoli and there is excessive deposition of ECM (fibrosis) in bronchi and bronchioles (Hogg *et al*, 2004). The elevated expression of ECM proteins are

associated to increased number of myofibroblasts, The sub-epithelial fibrosis encountered in airways is caused by a deposition of extracellular matrix (ECM) proteins like tenascin-c (Tn-C), collagens, fibronectin (Fn) and proteoglycans (Jones & Jones, 2000). The central airways and alveolar lung parenchyma contain distinct fibroblast populations. They possess different morphology, proliferation and ECM production (Kotaru *et al*, 2006 & Pechkovsky *et al*, 2010). This distinction is important to consider in COPD, as the ECM turnover is different in bronchi and alveoli. A study by (Hallgren *et al*, 2010) found differences in fibroblasts from COPD and control subjects. A lower potential proliferation was observed in COPD subjects compared to control subjects. The reason for this lower proliferation of fibroblast can be cigarette smoke (Nobukuni *et al*, 2002). Cigarette smoke can induce inflammation and directly damage the lungs (Wright *et al*, 1992). It is believed that this inflammation can lead to COPD. On the other hand, studies suggest that the tissue repair capacity of lung fibroblasts, which are responsible for the extracellular matrix regeneration and maintenance, appears to be decreased in COPD. One piece of evidence supporting the impaired repair theory is the reduced proliferation of fibroblasts seen in the patients with COPD compared to patients with normal lung function, despite a comparable smoking history (Holz *et al*, 2004 & Muller *et al*, 2006).

Airway smooth Muscle cells:

Airway smooth muscle cells have been believed to be a bystander of chronic inflammation in the airways of lung. In asthmatic patients, airway smooth muscle cells plays an important role in modulating the inflammatory response, which is characterized by thickening of airway walls and in COPD patients it has been assumed this to be a result of chronic inflammation. (Postma *et al*, 2014, Meurs *et al*,

2013 & Morgan *et al*, 2014). Studies have found airway smooth muscle mass to be increased in COPD (Bosken *et al*, 1990 & Kuwano *et al*, 1993). Chronic airflow inflammation from cigarette smoke causes constriction and hypertrophy of even normal airway smooth muscle (Baraldo *et al*, 2003). This serves to increase airway wall thickness and therefore cause greater luminal narrowing. In addition, the same degree of smooth muscle contraction in a thickened airway causes considerably greater airways resistance than in normal airways (Lambert *et al*, 1993). Several investigators have found direct correlations with the degree of smooth muscle mass and airflow obstruction in COPD (Hogg *et al*, 2004 & Finkelstein *et al*, 1995), supporting notion that smooth muscle hypertrophy not only is a significant pathologic finding but also that it contribute to the pathogenesis of COPD.



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FIGURE 1.1 PATHOPHYSIOLOGY OF COPD

Overview of the pathological processes that can take place in COPD. Pathology 1: Fibrosis occurs causing thickening of the respiratory membrane where O₂ diffuses into the blood. It is not yet fully understood why fibrosis occurs or where the fibroblasts come from, and this area is one of the key aims of this study. Pathology 2: Alveolar wall destruction is a cause of emphysema and linked with neutrophil and cytotoxic T cell activity. Pathology 3: Mucus over secretion is a key cause of obstruction in COPD and onset by monocyte and neutrophil activity. Adapted from Nat Revs Immunology Barnes 2008.

1.2.2 COPD EXACERBATIONS

COPD is characterized by episodic increases in respiratory symptoms, which are called exacerbations. The GOLD guidelines define COPD exacerbations as "an acute event characterized by a worsening of patient's normal day to day variations and leads to a change in medications (GOLD, 2011). Figure 1.2 shows the causes and risk factors for COPD exacerbation which include air pollution and most commonly bacterial and viral infection (GOLD, 2011, Peacock *et al*, 2011 & Sethi & Murphy, 2008). Bronchoscopic studies have shown that at least 50% of patients have bacteria in their lower airways during exacerbations of COPD (Sethi & Murphy 2008). During exacerbations precipitants worsen the bronchial inflammation, characteristic of stable COPD as evidenced by a rise in bronchial neutrophils and sign of oxidative stress (Drost *et al*, 2005 & Qiu *et al*, 2003). Oxidative stress is a key factor in the development of airway inflammation in COPD. Patients with severe exacerbations who needed hospital admission or assisted ventilation have evidence of increased large airway interleukin-8 (IL-8) levels and increased oxidative stress (Drost *et al*, 2005). Systemic inflammation increases when exacerbation is associated with bacterial and viral infection (Wedzicha *et al*, 2000). Several inflammatory markers increase at exacerbation, such as plasma fibrinogen and CRP that have been linked to increased cardiovascular risk. Respiratory infections have been associated with increased cardiac events (Smeeth *et al*, 2004) and thus COPD exacerbation if triggered by an infection, might increase cardiac morbidity. Inflammatory responses during COPD exacerbations result in cascades of increased lung inflammatory cell numbers and inflammatory cell mediator's release, enhancing the pathological process associated with lung function loss (Anzueto, 2010). A modest increase in the number of neutrophils, lymphocytes and eosinophils are seen

in the airways during exacerbations. Also, the airway inflammatory responses during COPD exacerbations cause airway oedema, bronchospasm, and increased sputum production, leading to worsening airflow limitation and development of dynamic hyperinflation (O'Donnell & Parker, 2006). So, markers of inflammation play a major role for assessment and thus investigation of sputum in chronic lung disease is focused on inflammatory mediators (Mayer-Hamlett *et al*, 2007, Roy *et al*, 2009 & Schmitt-Grohe *et al*, 2002).

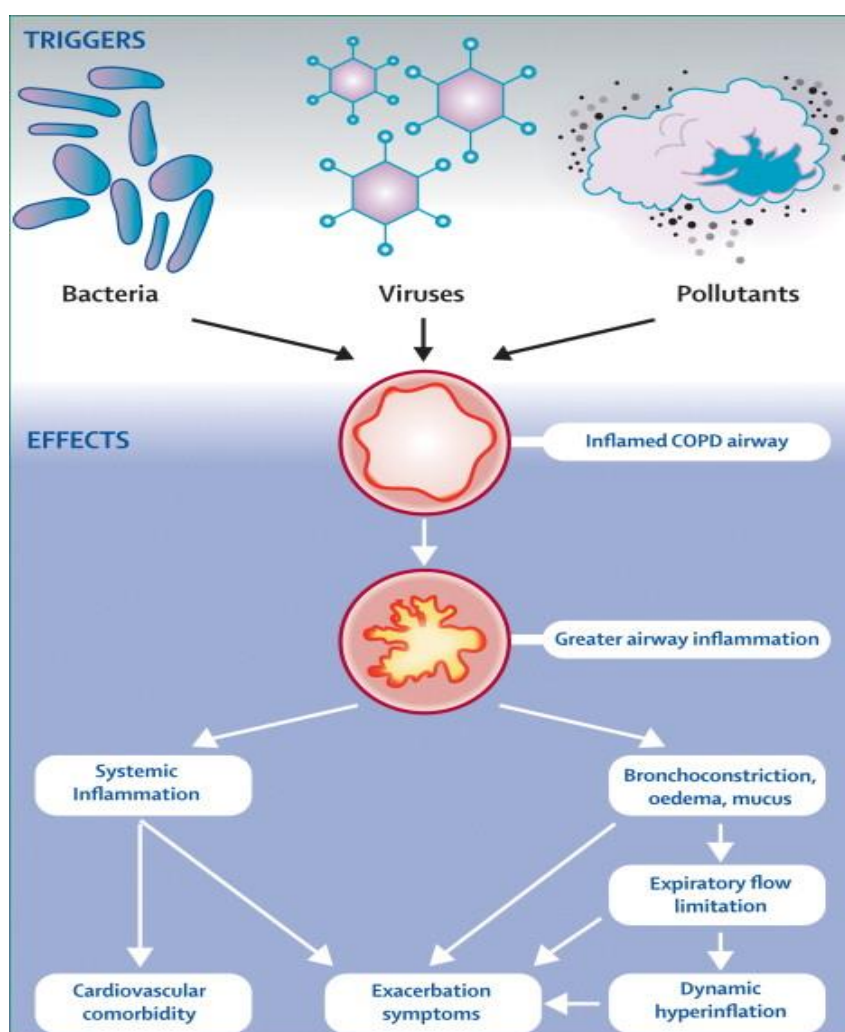


FIGURE 1.2 COPD EXACERBATIONS

Bacteria, viruses, pollutants trigger and cause associated symptoms leading to pathophysiological changes. Adapted from Lancet Wedzicha and Seemungal, 2007.

1.2.3 RISK FACTORS IN COPD

The role of cigarette smoke, exposure to outdoor and indoor pollutants, history of pulmonary tuberculosis (TB), asthma, and genetic factors are recognized as potential factors contributing to the development of COPD.

Cigarette smoke:

Cigarette smoke is the most common risk factors in COPD. Cigarette smoke contains harmful toxins that affect lung functionality. Toxins that are inhaled directly into the lungs over prolonged periods of time can lead to severe lung irritation, triggering the onset of COPD and eventually leads to inflammation and degradation in the lung (Brody and Spira, 2006).

Cigarette smoke causes an inflammatory process in the central airways, peripheral airways and lung parenchyma which is present even in smokers with normal lung function (Saetta, 1999). Bronchial biopsies of smokers have shown chronic inflammatory changes, with increased numbers of T-lymphocytes and macrophages in lung and structural remodeling resulting from repeated injury and repair (Saetta et al, 1993). It has been reported in studies that approximately 50% of smokers eventually develop COPD (GOLD 2008 and Lundback et al, 2003).

Indoor and outdoor air pollutants:

Globally, in the low income countries exposure to indoor air pollution is responsible for causing the COPD burden. According to world health organization (WHO), almost 3 billion people worldwide use biomass and coal as their main source of energy for cooking, heating and other household needs. Biomass fuel used by women for cooking account for high prevalence of COPD among non-smoking women in parts

of middle east, Africa and Asia.

Moreover, health risks from air pollution are greatest among elderly, children and those with chronic health conditions like asthma and COPD. The long term exposure to outdoor air pollution is thought to increase the risk of developing COPD. Also, the exposure to particulate matter air pollution makes COPD symptoms worse, resulting in an increased risk of death in people who have existing COPD. WHO estimates that urban air pollution causes 1 % cases of COPD in high income countries and 2 % in nations of low and middle income.

Pulmonary Tuberculosis:

As respiratory system is the most common site of active disease, Pulmonary TB may be the strongest risk factor in COPD in a TB endemic area (Hersh *et al*, 2007). Tuberculosis is characterized of chronic caseous granulomatous inflammation resulting in devastating tissue damage if left untreated (Elkingto *et al*, 2011). As the most common site of involvement, pulmonary TB can cause permanent obstruction or restrictive pulmonary function impairment, which in turn can contribute to the pathogenesis of COPD (Hnizdo *et al*, 2000, Chung *et al*, 2011 & Pasipanodya *et al*, 2007).

These comorbidities contribute to reduced health status, increased healthcare utilization and hospital admission and mortality (Decramer *et al*, 2012).

Asthma:

Asthma has been recognized to be a risk factor for developing COPD (Silva *et al*, 2004). More than 40% of patients with COPD report the history of asthma (Hersh *et al*, 2007). There are some striking similarities and differences in pattern of

inflammation between asthma and COPD. COPD affects both airways and parenchyma whereas asthma affects only the larger conducting airways (Barnes, 2000 & Jefferey, 2000). Both asthma and COPD are characterized by airway obstruction, which is variable and reversible in asthma but is progressive and largely irreversible in COPD. Despite differences in the casual agents, both COPD and asthma exhibit various degrees of inflammatory changes, narrowing of airways leading to airflow limitation and structural alteration of the pulmonary airways and vessels (Postma & Timens, 2006). In asthma, the deposition of collagen results in thickening of basement membrane termed as sub epithelial fibrosis whereas in COPD, collagen deposition is mainly around the airways termed as peribronchiolar fibrosis. Infiltrations of inflammatory cells in airways such as mast cells; granulocytes and macrophages show marked differences in both diseases. Mast cells plays a key role in asthma through the release of broncho-constrictors like histamines, release lipid mediators leukotriene (LT)C₄, LTD₄, LTE₄ with prostaglandin D₂ and also release cytokines linked to allergic inflammation, including interleukin (IL)-4, IL-5 and IL-13 (Galli *et al*, 2005). Asthma is most frequently diagnosed during childhood and is associated with atopy and eosinophilic inflammation, COPD is usually diagnosed during middle or later life and is associated with neutrophilic inflammation (Barnes, 2000). These reflect the differences in secretion of different chemotactic factors in these diseases. CC-chemokine Ligand (CCL) 11 is released mainly in epithelial cells in asthma. COPD shows the increased numbers of neutrophils in sputum along with the production of CXC-chemokine ligand (CXCL) 1, (CXCL) 8 in neutrophils and correlates with disease severity in COPD and asthma (Keatings *et al*, 1996). Macrophages are observed in both diseases. However, COPD shows far greater numbers of macrophages than in asthma. Macrophages amplify the airway

inflammation by releasing chemokines that attract neutrophils, monocytes, T-cells and proteases mainly MMP9 (Barnes, 2004). The pattern of inflammatory cells found in the respiratory tract therefore differs between COPD and asthmatic patients. Inhaled corticosteroids are effective against the inflammation in asthma but largely ineffective against the COPD inflammation (Haatela *et al*, 1991, Jefferry *et al*, 1992 & Keatings *et al*, 1997). Although asthma and COPD are two different diseases differential diagnosis is sometimes difficult and may be impossible in some older patients (Guerra, 2004), as response to pharmacologic treatment leads to reversibility of pulmonary obstruction in asthma and may decrease over time in some patients with long standing asthma, to the point of irreversible or only partly reversible airway obstruction.

1.3 DIAGNOSIS

Clinical Assessment:

A detailed medical history of a patients with symptoms suggestive of COPD should include exposure to risk factors (e.g., smoking and occupational or environmental exposures), family history of COPD or other chronic respiratory disease, patterns of symptoms, history of exacerbations, presence of comorbidities, medical treatments and patients quality of life. Diagnosis of COPD is based on a history of exposure to risk factors and the presence of airflow limitation that is not fully reversible, with or without the presence of symptoms. Since the early stages of COPD may manifest as chronic cough and sputum production, they may be present even in smokers without airflow limitation.

1.3.1 SPIROMETRY

Breathing test known as spirometry can be performed using a machine called a spirometer. This breathing test can determine the severity of lung function impairment. For the diagnosis and assessment of COPD, spirometry is the gold standard as it is the most reproducible, standardized and objective way of measuring airflow limitation. The important parameter measured during spirometry is called the Forced Expiratory Volume in 1 second and is abbreviated as FEV₁, this is the volume of air that can forcibly be breathed out in 1 second following a deep breath inwards. The Global Initiative for Chronic Obstructive Lung disease (GOLD) has introduced a five stage classification for the severity of COPD based on measurements of air flow limitation during forced expiration. Each stage is determined by the volume of air that can be forcibly exhaled in one second (FEV₁) and by the ratio of FEV₁ to the forced vital capacity (FVC) (Fig 1.3).

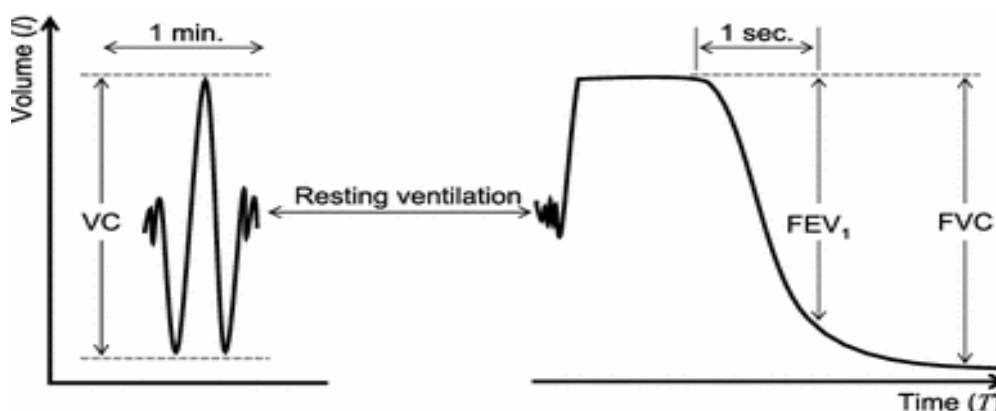


FIGURE 1.3 SPIROGRAM: RESPIRATORY MOVEMENT BY SPIROMETER.

Classification of severity of Airflow limitation in COPD according to the GOLD criteria

Table 1 Classification based on post bronchodilator lung function

GOLD 1 (mild)	FEV ₁ /FVC <0.70 and FEV ₁ ≥ 80% predicted
GOLD 2 (moderate)	FEV ₁ /FVC <0.70 and 80% > FEV ₁ ≥ 50% predicted
GOLD 3 (severe)	FEV ₁ /FVC <0.70 and 50% > FEV ₁ ≥ 30% predicted
GOLD 4 (very severe)	FEV ₁ <50% predicted plus chronic respiratory failure

Table 1 shows people with an FEV₁/FVC ≥0.70 and respiratory symptoms of chronic cough and sputum production are no longer included as a COPD stage (formerly GOLD stage 0). Patients with an FEV₁/FVC ≥0.70 but an FVC < 80% predicted meet spirometric criteria for restrictive process. Although this is not regarded as COPD, patients might present with several symptoms to these seen in COPD and these patients have an increased risk of deaths.

1.3.2 ASSESSMENT BY IMAGING

Airflow limitation in COPD is due to both small airway disease (obstructive bronchitis) and parenchyma destruction (emphysema), the relative contributions of which vary among patients. Thin-section CT has been used to quantify emphysema by detecting low-attenuation areas. Theoretically, thin-section CT depict the dimensions of airways as small as ~1 to 2 mm in inner diameter, suggesting that CT can be used to evaluate airway dimensions in a variety of disease (Coxson & Rogers, 2005). However, airflow limitation evaluated by FEV1 does not show a good correlation with the severity of emphysema as evaluated by CT (Baldi *et al*, 2001 & Omori *et al*, 2008) because small airway disease appears to contribute significantly to airflow

limitation (Hogg, 2006). Scintigraphic approaches may be used to assess COPD and to provide functional imaging. Ultrafine ^{133}Xe gas particles are being used for ventilation scintigraphy including single photon emission CT (SPECT) (Magnant *et al*, 2006).

Early stages of COPD can be confirmed using spirometry but other morbidities like lung cancer should also be taken into consideration. As tobacco smoking is one of the major risk factor in development of COPD, it is also associated with developing lung cancer (Bach & Ginsberg, 2002). The tobacco smoke stimulates both local and systemic inflammation and this inflammation play a casual role in both COPD and lung cancer (Barreiro, 2008). COPD diagnosis has been associated with a higher rate of lung cancer (Skillrud *et al*, 1986, Maninno *et al*, 2003 & Tockman *et al*, 1987), on the other hand several studies have shown that a common cause of death between those affected with COPD is lung cancer, mostly when COPD is mild and moderate (Manini *et al*, 2003). Skillrud *et al* and Tockman *et al* (2003), show that airway obstruction is associated with an increment 4 to 6 times in the risk to develop lung cancer, independently of tobacco smoking history. So for detection, differential diagnosis, staging and follow up of lung cancer imaging examinations like Computed Tomography (CT), Magnetic Resonance Imaging (MRI), Positron Emission Computed Tomography (PET-CT) play an eminent role.

1.3.3 ASSESSMENT OF AIRWAYS

Numbers of cytokines and chemokines are secreted in both asthma and COPD and are regulated by the transcription factor nuclear factor- κB , which is activated in airway epithelial cells and macrophages in both diseases and may have an important role in amplifying airway inflammation. Quantification and characterisation of

inflammatory changes are important assessments in diagnosis of COPD. These assessments can be performed by invasive and non-invasive methods. Bronchial biopsies, broncho alveolar lavage (BAL) or examination of surgical specimens are meant to be invasive methods whereas spontaneous and induced sputum is described as non-invasive. A different profile of inflammatory cells is obtained depending on the compartment of the lung examined by the techniques. The airway wall can be examined using bronchial biopsies and the peripheral airways using BAL (Maestrelli *et al*, 1995) whereas sputum analysis is performed for the examination of lumen of the airways (Alexis *et al*, 2000). Bronchial biopsies from asthmatic subjects reveal an infiltration of eosinophils, activated mucosal mast cells at the airway surface and activated T-cells whereas in COPD there is no evidence of mast cell activation but there is infiltration of T-cells and increased numbers of neutrophils, particularly in the airway lumen (Hogg, 2004). The release of mediators like histamine by mast cells in asthma may account for the variable bronchoconstriction as they are triggered by environmental allergens. On other hand mast cells do not seem to play a role in COPD which may explain the lack of variable bronchoconstriction. Also, presence of mast cells in airway smooth muscle is linked to airway hyper responsiveness (AHR) in asthma (Brightling *et al*, 2002). Several studies have suggested that asthma and AHR are important risk factors contributing to an increased rate of decline in FEV1 and thus to the development of COPD (Lange *et al*, 1998, Peat *et al*, 1987, Shernill *et al*, 2003, & Fletcher *et al*, 1976). The 18 years follow up study carried out by Peat *et al* found asthmatic subjects having greater rate of decline in FEV1 and a lower baseline lung function than in non-asthmatics (1987). Approximately 10% of patients with COPD have reversibility in lung function as assessed by forced expiratory volume in one second (FEV1), and

therefore behave more like asthmatics. These patients frequently have eosinophils in their sputum, an increase in exhaled nitric oxide and respond better to corticosteroid treatment, all of which are feature of asthma (Papi *et al*, 2000 & Brightling *et al*, 2005).

1.3.4 DIFFERENTIAL DIAGNOSIS

SPUTUM ANALYSIS:

Analysis of induced or spontaneous sputum has contributed to the identification of smokers susceptible to developing COPD, to the characterisation of the inflammatory process during exacerbations and to the effects of intervention with anti-inflammatory drugs or by smoking cessation. Abnormal inflammatory response of the lung can be driven by external drivers like cigarette smoke or noxious particles or gases. In COPD, the most common sputum changes in neutrophilia are increased products of neutrophil activation, including proteases, myeloperoxidase and elastase (Chung 2001, Kim & Nadel, 2004). Induced sputum shows a characteristic increase in the proportion of neutrophils that is much greater in patients with COPD than in smokers without obstruction (Stanescu *et al*, 1996). This suggests that sputum neutrophils or their products may be used as early markers of the manifestation of COPD. A study by Sunner *et al* (2013) identified sputum neutrophils and phlegm as important factors associated with cough in COPD patients, suggesting that molecules that target neutrophils (Barnes, 2007) represent interesting therapies to reduce mucus hypersecretion (Burgel & Wedzicha, 2013).

COPD is associated with other comorbid conditions, and patients who are admitted to hospital are more likely to have associated comorbid conditions such as ischaemic

heart disease, pneumonia and diabetes than patients without diagnosis of COPD. Pulmonary embolism also contributes to exacerbations, as approximately 25% of patients hospitalized for an acute exacerbation of COPD may have a pulmonary embolism (Rizkallah *et al*, 2009).

1.4 EPITHELIAL TO MESENCHYMAL TRANSITION

Respiration involves phasic movement of air through the conducting airways in and out of the gas-exchange portion of the lung i.e., the respiratory bronchioles, alveolar ducts, alveolar sacs and alveoli. Alveoli are microscopic thin-walled sacs that facilitate the exchange of the gases between the inspired air and the capillaries in the alveolar wall (Burri, 1997). The alveolar wall in the adult lung consists of a narrow connective tissue core that contains fibroblasts, pericytes and capillary endothelial cells and extracellular matrix components, most importantly those comprising the elastic fibres. The alveolar epithelium contains two main types of cells:

1) Type 1: large flattened cells (95% of the total alveolar area) which present a very thin diffusion barrier for gases. They are connected to each other by tight junctions (Crapo *et al*, 1982).

2) Type 2: (making up 5% of the total alveolar area, but 60% of total number of cells). These cells secrete 'surfactant' which decreases the surface tension between the thin alveolar walls, and stops alveoli collapsing when one breathes out, these cells are connected by tight junctions (Perez-Gil & Weaver, 2010).

Epithelial to mesenchymal transition is a biological process that allows a polarized epithelial cell, which normally interacts with basement membrane via its basal surfaces, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity,

invasiveness, elevated resistance apoptosis and greatly increased production of ECM components. EMTs are encountered in three distinct biological settings that carry very different functional consequences.

Type 1 EMT: EMT during implantation, embryo development and organ development

In embryogenesis, the initiation of placenta formation and implantation of embryo both processes are associated with EMT. A fertilized egg undergoes gastrulation. The first step is the formation of primitive streak which is generated in the epiblast layer (Hay, 1990). The epithelial like cells of the epiblast undergo programmed changes by specific expression of proteins associated with cell migration and differentiation (Thiery & Sleeman, 2006). This primitive streak generates the mesendoderm, which separates to form mesoderm and endoderm via an EMT by replacing hypoblasts cells, which either undergo apoptosis or contribute to the mesoderm layer via an EMT (Hay, 1995). Also, during embryonic development, a neural crest cells which expresses genes are formed by epithelial cells of the neuroectoderm and subsequently undergo EMT (Duband & Thiery, 1992). As a result, the cells become motile and disperse to form different parts of embryo by further differentiation. Hence, EMT programs are deployed during several subsequent phases of embryogenesis which shows involvement in later stages of organ development (Kalluri & Weinberg, 2009)

Type 2 EMT: EMT associated with tissue regeneration and organ fibrosis

In type 2, organ fibrosis in epithelial tissues is mediated by inflammatory cells and fibroblasts. EMT give rise to myofibroblast from epithelia to heal injured tissues, if the injury is mild or acute, the healing process is regarded as reparative fibrosis, but in ongoing inflammation, abnormal formation of myofibroblast cause progressive

fibrosis, therefore leading to organ parenchymal destruction by excessive deposition of extra cellular matrix (ECM) (Tenakoon, *et al*, 2016). The EMT knowledge has been accumulated using human pathological tissues and experimental animal models. The study of transgenic mice bearing germ line reporter genes whose expression was driven by epithelial-cell-specific promoters where evidence of formation of fibroblast from epithelial cells by EMT. In kidney fibrosis, myofibroblast are the main source of excessive ECM deposition. They are derived from renal epithelia/endothelia, interstitial fibroblastic cells or mesenchymal pericytes. EMT process from renal epithelia is regarded as the important pathway leading to formation of interstitial myofibroblast in diseased kidneys at advanced stages (Strutz, *et al*, 1995, Okado *et al*, 1997 & Rastaldi *et al*, 2002). Thus, "Type 2" programs begins as a part of a repair associated event that normally generates fibroblasts and other related cells in order to reconstruct tissues following trauma and inflammatory injury. Mainly wound healing, tissue regeneration and organ fibrosis are included. Though studies have shown EMT play a role in pulmonary fibrosis (PF) but its's aetiology is still unclear. In COPD, EMT type 2 could be responsible for destruction of small airways whereas in PF it leads to scarring of lung tissues and PF being the restrictive disease our aim here is to identify role of EMT in COPD (obstructive disease) where smoking plays an important risk factor in causing it.

Type 3 EMT: EMT associated with cancer progression and metastasis

"Type 3" EMTs occur in neoplastic cells that have previously undergone epigenetic changes specifically in the genes that favour clonal outgrowth and the development of localised tumors (Kalluri & Weinberg, 2009). Excessive epithelial cell proliferation and angiogenesis are hallmarks of the initiation and early growth of primary epithelial cancers (Hanahan & Weinberg, 2000). Many mouse studies and cell culture

experiments have demonstrated that carcinoma cells can acquire a mesenchymal phenotype and express mesenchymal markers such as α -SMA (smooth muscle actin), FSP1 (fibroblast specific protein-1), vimentin and desmin (Yang & Weinberg, 2008). The involvement of epithelial carcinoma cells in primary nodules transitioning to metastatic tumour cells in order to migrate through blood stream and in some cases, form secondary nodules in distant metastatic sites by mesenchymal epithelial transition (MET) (Zeisberg *et al*, 2005).

1.4.1 EPITHELIAL MESENCHYMAL TRANSITION AND COPD

Epithelial mesenchymal transition (EMT) has been well described in lung embryogenesis (Lee *et al*, 2006), metastatic malignant disease (Bjornland *et al*, 1999) and as a part of the repair process following tissue injury in renal disease (Yanez Mo *et al*, 2003). EMT has been recognized in the human lungs and airways during and after lung injury (Willis *et al*, 2006, Ward *et al*, 2005 & Hackett *et al*, 2009). Active EMT is indicated by the degradation of underlying epithelial cells into mesenchymal cells with migratory potential, such that they move away from the epithelium in which they origin into deeper tissue (Kalluri *et al*, 2003 & Rabe *et al*, 2007). In COPD there can be both types of airway disease like chronic bronchitis and destruction of lung parenchyma (Emphysema) (Barnes *et al*, 2003). One of the features of chronic airway disease including COPD is airway remodelling (Nakuno *et al*, 2002 & Chung *et al*, 2006). Remodelling involves the differentiation of airway epithelial cells to mesenchymal phenotype, with subsequent migration through reticular basement membrane (Rbm) to the subepithelial lamina propria, a process termed as epithelial mesenchymal transition (Jeffery, 2004 & Willis *et al*, 2006). In addition to being structural cells of the lung, airway epithelial cells are mediator producing cells that become very active in COPD. Epithelial cells are activated by

cigarette smoke to produce inflammatory mediators, including TNF- α and IL-8 and also important source of transforming growth factor (TGF)- β , which induces local fibrosis. It is proposed that in COPD airway epithelium is damaged and/or activated by irritants such as cigarette smoke constituents, and this stimulates the deposition of collagen from myofibroblasts in the lamina propria (Sohal *et al*, 2010). The sub-epithelial reticular basement membrane (Rbm) is variably thickened and markedly fragmented with clefts or elongated spaces within it. These elongated clefts in COPD contain cells. These cells positively express the mesenchymal markers S100A4, vimentin and MMP (Sohal, *et al*, 2011).

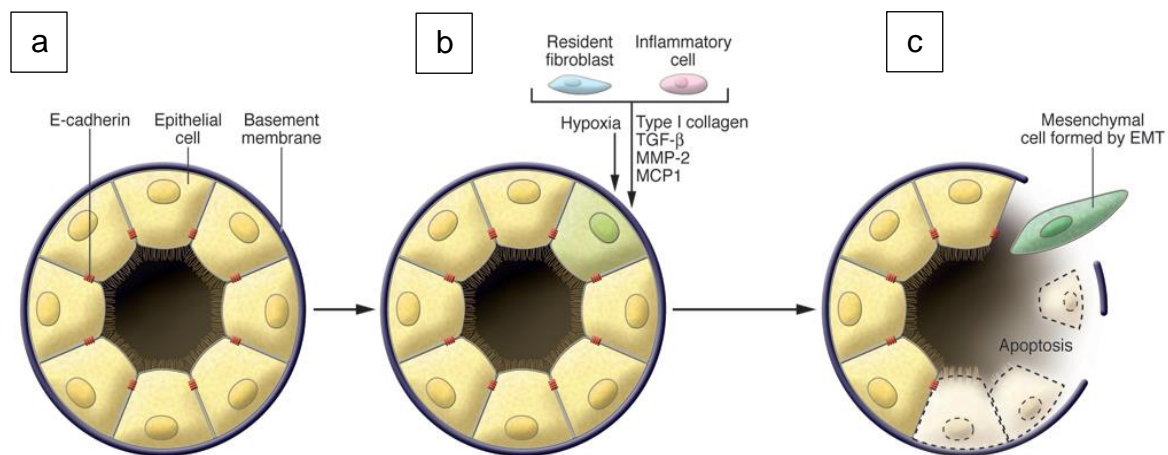


FIGURE 1.4 EPITHELIAL MESENCHYMAL TRANSITION AND FIBROSIS

During organ fibrosis (a), different types of inflammatory cells and resident fibroblasts (myofibroblasts) are generated which results in disruption and degradation of basement membrane (b). The epithelial cells lose polarity and either undergoes apoptosis or EMT (c). Adapted from (Kalluri and Weinberg, 2009).

Cells undergoing EMT have been characterised in a range of tissues including renal, heart and liver fibrotic conditions (Zeisberg *et al*, 2007 & Zeisberg *et al*, 2008). It is useful to characterise biomarkers associated with EMT to both characterise the mechanisms of how it occurs, and to be able to monitor and grade the severity of the

condition. The following proteins have been linked with the structural changes of the cells including Vimentin expression, S100A4 and α smooth muscle actin each serving an important role during EMT.

Vimentin serves to support intracellular structures in the cytosol of the cell. It is accepted that vimentin is a cytoskeletal component supporting cellular integrity. Typically, vimentin is expressed in mesenchymal cells. α -smooth muscle actin is another typical marker of myofibroblast formation and is involved with cell motility and movement; its expression has been linked with fibroblast contractile activity and is upregulated by TGF β expression (Boris *et al*, 2001).

1.4.2 THE ROLE OF S100A4

S100A4 has been identified as a protein linked with a range of cellular functions such as cell cycle progression and differentiation including EMT. The S100 gene family is the largest sub family of the calcium-binding proteins (Donato, 2001). S100A4 is calcium binding 101 amino acid polypeptide; upon calcium binding S100A4 undergo a conformational change in the canonical EF-hand resulting in the exposure of a hydrophobic binding pocket formed by helices' 3, 4, the hinge region (loop 2) and the C-terminal coil region (Figure 1.5 Garrett *et al*, 2006), thus allowing the protein to interact with different protein targets thereby explaining the broad range of intracellular and extracellular functions. Intracellular functions include regulation of cell cycle, cell growth, smooth muscle cell migration (SMC), phosphorylation and regulation of transcriptional factors. Extracellular function includes binding to cell surface receptors such as the receptor for advanced glycation end products (RAGE) and Toll-like receptors (TLRs) (Heizmann *et al*, 2002 & Donato, 2003). The interaction with non-muscle myosin proteins are well established and has been

demonstrated that metastasis-associated cellular motility is coupled to S100a4-NIIA interaction (Li & Bresnick 2006 & Garrett *et al*, 2006).

S100A4 is also known as metastatin (MtS1), fibroblast specific protein (FSP) 1, calcium binding protein placental homolog (APL1) and Calvasculin. The evidence connecting S100A4 and EMT comes from studies on renal fibrosis, in which S100A4 was identified as a FSP and renamed FSP1 (Strutz *et al*, 1995).

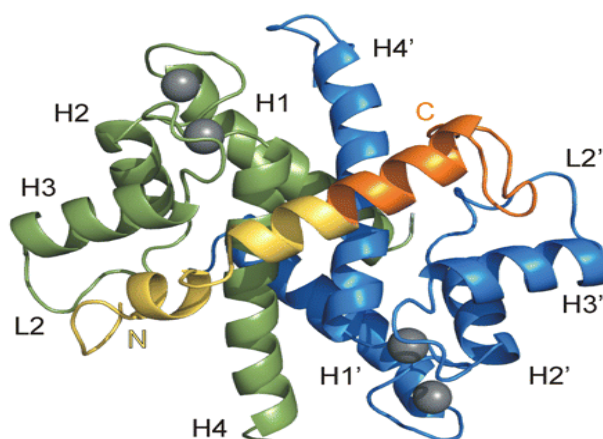


FIGURE 1.5 STRUCTURE OF S100A4-NONMUSCLE MYOSIN IIA INTERACTION

Nonmuscle myosin IIA complex structure of S100A4 with subunits A (Green) and B (Blue) are shown along with the two-fold symmetry axis of the 45-residue long dimer. Yellow peptides (residues 1893-1913), orange (residues 1914-1935) and Ca^{+2} ions are grey. (Adapted from Kiss *et al*, 2012).

Immunohistochemical analysis of human cancers shows significant S100A4 expression in breast, pancreatic, prostate, gall bladder, oesophageal, gastric, lung and thyroid carcinomas (Hefman *et al*, 2005). In these cancers, S100A4 expression is found at elevated levels compared with normal tissue suggesting that enhanced S100A4 contributes to manifestation of a metastatic phenotype.

With relevance to the current study, S100A4 acts as a key player in EMT induction by regulation of E-cadherin, β -catenin, Snail/Slug, matrix-metalloproteinases (MMPs)

and nuclear factor- κ B pathways (Keirsebilck *et al*, 1998, Brabletz *et al*, 2005, Stein *et al*, 2006, Moody *et al* 2005 & Grotterod *et al*, 2010). It is a prototypical fibroblast marker detecting EMT in breast cancer and fibrogenesis (Xue *et al*, 2003 & Iwano *et al*, 2002). In tissue fibrosis, most epithelial cells undergoing type 2 EMT express FSP1 early in transition to fibroblasts (Iwano *et al*, 2002). The pathological changes in the COPD are triggered by a chronic inflammation in lungs. Structural changes in pulmonary vascular remodelling include media hypertrophy, thickening of the intima with reduction of the lumen diameter and muscularization of small non-muscular arterioles (Barbere *et al*, 2004) and also the vasculature remodelling of lungs (in mice and humans) linked with cigarette smoking (Reimann *et al*, 2015). S100A4 plays a major role in the smooth muscle cells (SMC) migration and proliferation which is thought to be depended upon an autocrine or paracrine stimulation of the RAGE receptor (Lawrie *et al*, 2005).

Given the close association of S100A4 with EMT and EMT being a growing interest in COPD pathology, this study aims to investigate the expression of S100A4 in COPD lung tissue.

1.5 CURRENT TREATMENTS

Bronchodilation is the cornerstone of pharmaceutical treatment of COPD symptoms. Bronchodilators improve respiratory function, mainly by reducing the patient's hyperinflated state as COPD progresses, thereby improving symptoms like breathlessness and poor exercise capacity (Schermer *et al*, 2007, Appleton *et al*, 2006 & Van Noord *et al*, 2006). The GOLD guidelines have recommended a stepwise approach for management of COPD with bronchodilators as mainstay for the treatment. Bronchodilators help open the airways. Bronchodilator medication can be short or

long acting. Three long-acting inhaled bronchodilators in COPD have been approved: Tiotropium, Formoterol and Salmeterol. Currently available bronchodilators for COPD are categorized in three classes: anticholinergics, β 2-sympathomimetic agonists and methylxanthines. As anticholinergics and β 2-agonists both are equally effective, so GOLD guidelines do not have distinguished any difference. Impaired lung function in COPD is caused by structural narrowing of the airways, combined with the effects of cholinergic vagal bronchokonstrictive tone and decreased lung elastic recoil (Gross *et al*, 1989 & Barnes, 1999). Bronchodilators improve the airflow limitation observed in patients with COPD by producing airway smooth-muscle relaxation, although β 2-agonists and anticholinergics achieve this effect through different mechanisms. Anticholinergic bronchodilators (particularly Tiotropium) produce relaxation of airway smooth muscle through antagonism of acetylcholine at M3-muscarinic receptors on airway smooth muscle (Barnes, 1995), whereas β 2-agonists induce bronchodilation through stimulation of β 2-receptors, leading to an increase in cyclic adenosine monophosphate.

The differences in mechanisms between these two classes of inhaled bronchodilators are reflected in the relative utility of each for the management of COPD. Short acting β 2-agonists, such as salbutamol, have a more rapid onset but shorter duration of action than anticholinergics and thus are commonly prescribed as "rescue" medication to help relieve acute bronchospasm. Inhaled anticholinergic medications, such as ipratropium, have a slower onset and slightly longer duration of action. One consequence of the differences in their modes of action is that the effects of combining anticholinergic and β 2-agonists bronchodilators are additive, providing greater efficacy than either agent alone (Combiant Aerosol study group, 1994).

1.5.1 INHALED CORTICOSTEROIDS (ICS)

In asthma and COPD, inhaled corticosteroid (ICS) is commonly given as combination inhalers with long acting β_2 -agonists (LABA). ICS are recommended anti-inflammatory treatment for COPD patients with a forced expiratory volume in 1 second (FEV₁) of less than 60% predicted and who are prone to frequent exacerbations. Inhaled corticosteroids are highly effective in asthma, but only offer modest help in COPD patients (Keatings *et al*, 1997). Both diseases involve inflammation of the respiratory tract, but the nature of the inflammation differs, indicating that different regulatory mechanisms are likely to be involved.

Patients with asthma have a specific pattern of inflammation in the airways that is characterized by degranulated mast cells, infiltration of eosinophils and increased number of activated T helper cells (TH₂ cells). Suppression of this inflammation by corticosteroids controls and prevents these symptoms in the vast majority of patients. There is increasing evidence that structural cells of the airways such as epithelial cells, airway smooth muscle cells, endothelial cells and fibroblasts are major source of inflammatory mediators in asthma. Epithelial cells may be a major target for ICS as they are activated by environmental signals and release multiple inflammatory proteins including cytokines, chemokines and growth factors. Inflammatory proteins are regulated by increased gene transcription, which is controlled by pro-inflammatory transcription factors such as nuclear factor- κ B and activator protein 1 (AP-1) that are activated in asthmatic cells (Barnes & Adcock, 1998). At cellular level corticosteroids reduce the number of inflammatory cells in the airways, including eosinophils, T-lymphocytes, mast cells and dendritic cells. These remarkable effects of corticosteroids are produced through inhibiting the

recruitment of inflammatory cells into the airway by suppressing the production of chemotactic mediators and adhesion molecules and by inhibiting the survival in the airways of inflammatory cells, such as eosinophils, T-lymphocytes and mast cells. Thus, corticosteroids have a broad spectrum of anti-inflammatory effects in asthma with inhibition of multiple inflammatory mediators and inflammatory and structural cells.

Treatments tend to use long-acting bronchodilating agents in order to achieve maximum improvements; about 50% patients use these agents (Schermer *et al*, 2007). Patients with frequent exacerbations-preferably those with forced expiratory volume in one second (FEV1) <50%-receive additional treatments by inhaling corticosteroids, which decrease exacerbation rate, probably by reducing the inflammation of lung parenchyma and bronchi (Jones *et al*, 2003). Corticosteroid therapy and long acting bronchodilating agents are currently available combined in a single inhaler for more severe cases, and have a clear impact on symptom relief (Calverley *et al*, 2007).

Although combination therapy of bronchodilators with ICS is routinely used in COPD patients, many patients do not respond well to the treatments and still suffer greatly from their poorly treated condition. This has been reported at a clinical level but also at a cellular level. In vitro studies of human alveolar macrophages cultured with varying doses of steroid showed IL-8 release was not suppressed by Dexmethasone (Dex) in COPD patients, however showed better responses in healthy patients (Culpitt *et al*, 2003). Conversely, asthma although being an inflammatory lung condition is treated with similar drug combinations but these patients respond much better and have a better quality of life. Therefore there is an ongoing and urgent need to develop better therapies for COPD, and to further characterize which

patients may respond to steroid therapy and to establish if a subgroup of patients is 'good responders'.

In asthma oxidative stress is low (Montuschi *et al*, 1999), but in COPD oxidative stress is increased to a greater extent due, in part to a striking increase in the number of neutrophils and macrophages (Montuschi *et al*, 2000). This finding implies that histone deacetylase (HDAC) activity is likely to be much lower in COPD than asthma, resulting in steroid resistance. However, this is only one proposed mechanism and there may be others.

1.5.2 CORTICOSTEROID RESISTANCE IN COPD

COPD involves inflammation with co-ordinate expression of multiple inflammatory genes in the lungs. Inhaled corticosteroids show less therapeutic benefit in COPD. This may reflect that the inflammation in COPD is not suppressed by corticosteroids, with no reduction in inflammatory cells, cytokines or proteases in induced sputum even with oral corticosteroids. Furthermore, histological analysis of peripheral airways of patients with severe COPD shows an intense inflammatory response, despite treatment with high doses of Inhaled corticosteroids (Hogg *et al*, 2004). There is increasing evidence for an active steroid resistance mechanism in COPD, as corticosteroids fail to inhibit cytokines such as IL-8 and TNF- α that they normally suppress (Keatings *et al*, 1997 & Culpitt *et al*, 1999). In vitro studies show that cytokine release from alveolar macrophages is markedly resistant to the anti-inflammatory effects of corticosteroids, compared to cells from normal smokers and these in turn are more resistant than alveolar macrophages from non-smokers (Culpitt *et al*, 2003). This lack of response to corticosteroids may be by an inhibitory effect of cigarette smoking and oxidative stress on HDAC function, thus interfering

with the critical anti-inflammatory action of corticosteroids (Ito *et al*, 2001). Indeed, there is a correlation between HDAC activity and the suppressive effects of corticosteroids on cytokine release. It is likely that oxidative and nitrative stress in COPD specifically impairs HDAC2 (Ito *et al*, 2004) resulting in steroid resistance (Barnes *et al*, 2004). Although this is seen in all stages of COPD it is most marked in the patients with severe disease (Ito *et al*, 2005). The mechanisms of glucocorticoid sensitivity in normal asthmatic patient is shown in (Figure 1.6) and in patients with COPD who have stopped smoking the steroid resistance persists and these patients are known to have continuing oxidative stress (Figure 1.7).

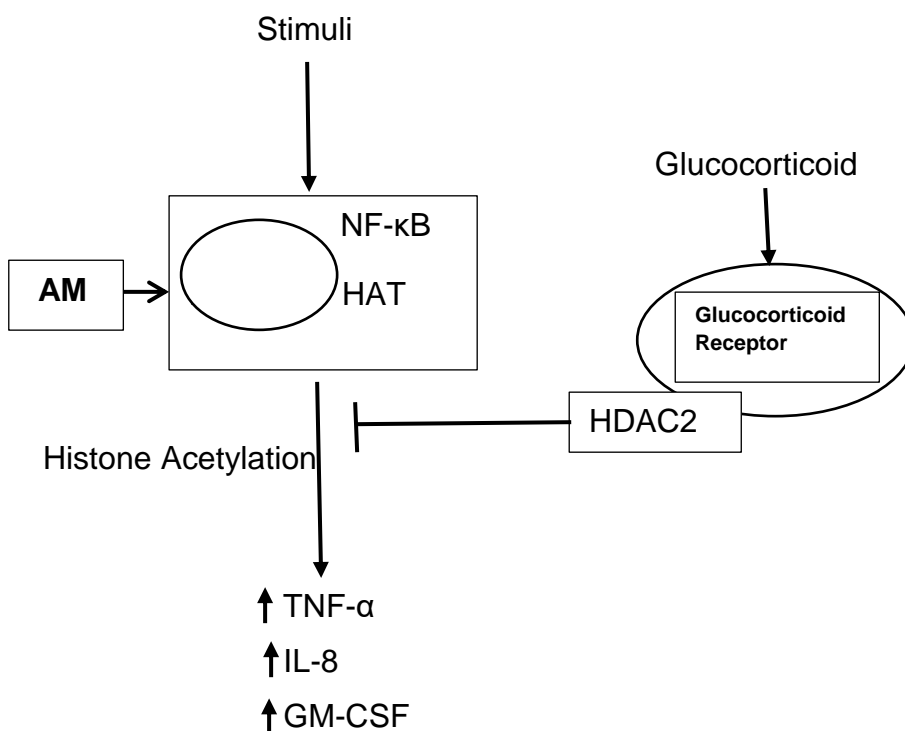


FIGURE 1.6 MECHANISM OF STEROID SENSITIVITY IN ASTHMA

In asthmatic alveolar macrophage (AM), nuclear factor (NF-κB) is activated following stimulation and switch on histone acetyltransferases (HAT), leading to histone acetylation and subsequently to the transcription of the genes encoding inflammatory proteins-tumour necrosis factor (TNF-α), interleukin (IL-8) and granulocyte macrophage colony stimulating factor (GM-CSF). Glucocorticoid reverses this by binding to glucocorticoid receptor and recruiting histone acetyltransferase (HDAC2). This reverses the histone acetylation induced by NF-κB and switches off the activated inflammatory genes.

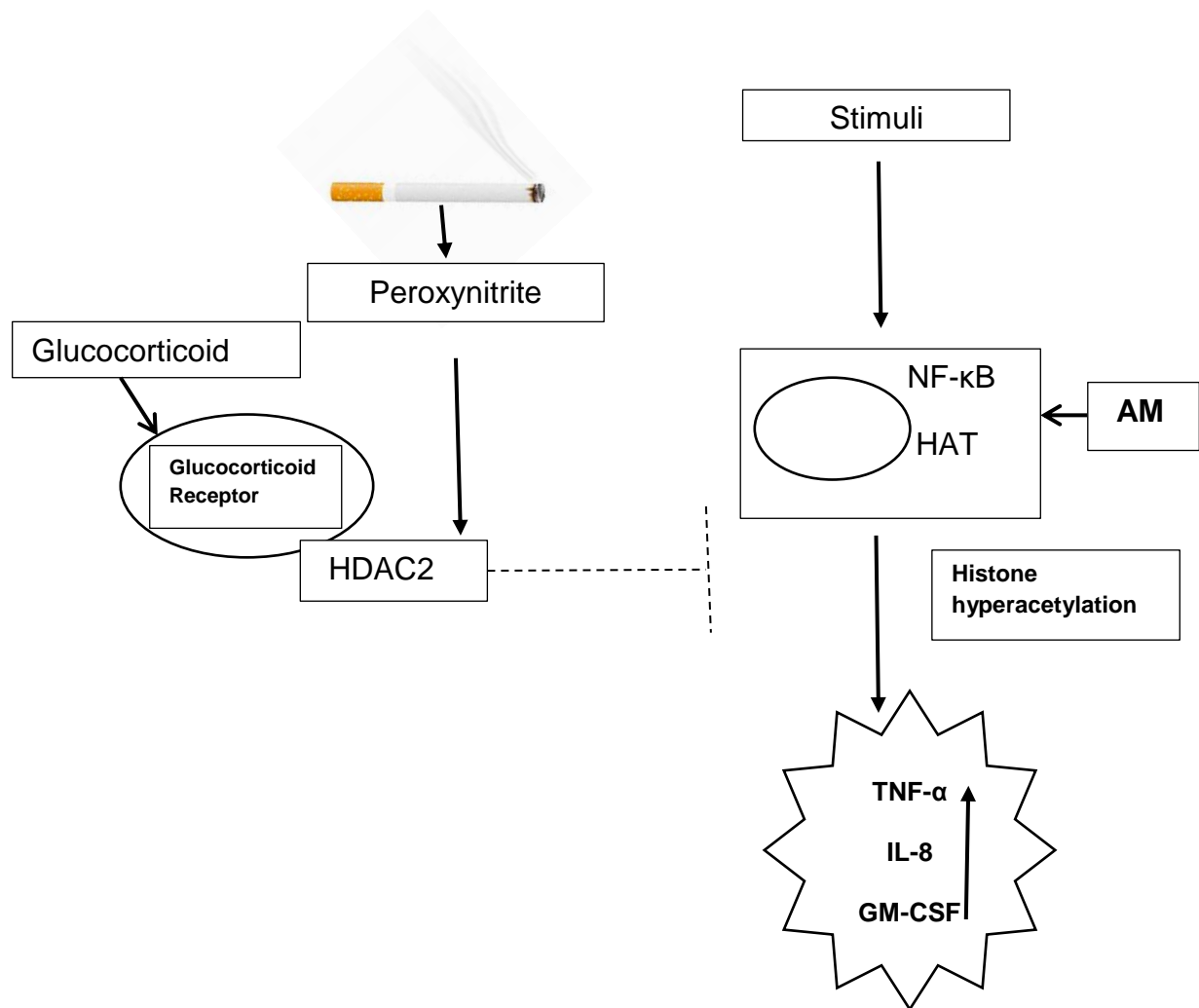


FIGURE 1.7 MECHANISM OF CORTICOSTEROID RESISTANCES IN COPD

In COPD and smoking asthmatic patient's alveolar macrophage (AM), nuclear factor (NF-κB) is activated following stimulation but cigarette smoke generate oxidative stress (acting through peroxynitrite) to impair the activity of HDAC2. This amplifies the inflammatory response to NF-κB activation by histone hyper acetylation, but also reduces the anti-inflammatory effect of corticosteroids, as HDAC2 is now unable to reverse the histone acetylation. (Adapted from Barnes *et al.*, 2004).

1.6 STEROIDS

Steroids occur naturally in human body and constitute an important class of hormones. Hormones are chemical compounds that are produced by specialized cells in the body and are released into the circulatory system. When these compounds reach their target cells, they elicit specific physiological responses. In steroid hormones it is achieved by regulating the expression of specific genes (Moss, 1989).

Steroid Receptors:



FIGURE 1.8 STRUCTURE OF STEROID RECEPTOR

Steroid receptor with its functional domain: amino terminal domain (NTD) lies towards the N-terminal also known as activation function-1(AF-1) whereas ligand binding domain lies towards carboxyl terminal also known as activation function-2(AF-2). DNA binding domain (DBD) along with hydrogen residues in between of both AF-1 and AF-2.

Steroid receptor consists of modulated domains with highly conserved centrally localized zinc-finger DNA binding (DBD) carboxyl-terminal ligand binding domain (LBD) and a divergent amino terminal domain (Figure 1.8). The amino terminal domain (NTD) is variable among receptors with exception of a region rich in negatively charged acidic amino acids. This region is known as either AF-1 (Activation Function-1) or tau-1, and its function is transcriptional regulation can be ligand independent. The AF-1 region has been shown to interact directly with basal transcriptional machinery and with many other co-factors that participate in

transcriptional regulation, moreover, disruption of AF-1 decreases reporter gene expression (McEwan *et al*, 1993., Dahlman-Wright *et al*, 1995).

1.7 GLUCOCORTICOIDS

Corticosteroids are classified as natural steroids and are of three types including glucocorticoids, mineralocorticoids and sex hormones. Glucocorticoids regulate many aspects of metabolism and immune function, whereas mineralocorticoids help maintain blood volume and control renal excretion of electrolytes. In humans, cortisol (Figure 1.9) is naturally occurring glucocorticoids from its precursor cortisone (Velden, 1998). The primary glucocorticoid and natural steroid hormone cortisol is secreted continuously by the adrenal cortex in diurnal pattern, but its release and effects are dynamic and increase dramatically in the setting of environmental stressors (Chrousos, 2000 & Hojilund *et al*, 2001).

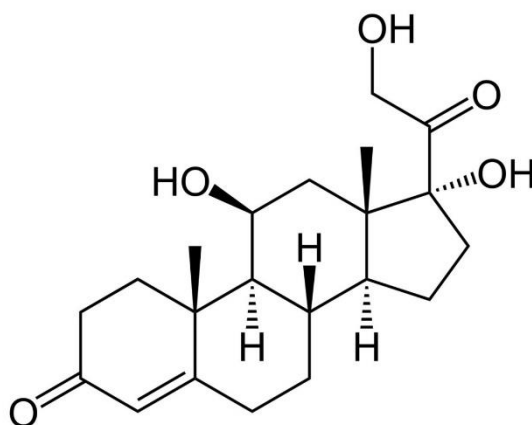


FIGURE 1.9 CHEMICAL STRUCTURE OF CORTISOL:

<http://organiceyourlife.com/mind-over-body-the-cortisol-effect/>

Cortisol synthesis in the adrenal cortex is controlled along the hypothalamic-pituitary adrenal axis (HPA) axis (Figure 1.9) through one of the two negative feedback loops, one direct and one indirect (Cowell & Buckingham, 2001, Morand & Leech, 2001).

During anti-stress effects of glucocorticoids in diminishing the elevated levels of particular serum cytokines it stimulate both the hypothalamus and anterior pituitary in

many pathological contexts, e.g. trauma, infection and other forms of physiological stress. Cortisol secretion is suppressed by classical negative feedback loops. When blood concentration rises above a certain threshold, cortisol inhibits CRH (corticotrophin-releasing hormone) secretion from the hypothalamus, which turns off adrenocorticotrophic hormone (ACTH) secretion, leads to a turning off of cortisol secretion from the adrenal gland. The combination of positive and negative control on CRH secretion results in pulsatile secretion of cortisol (ACTH) from the anterior pituitary.

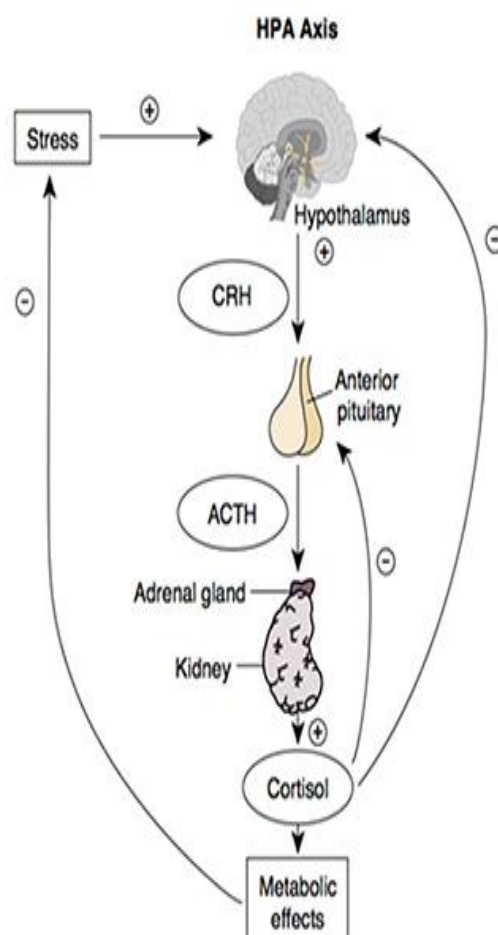


FIGURE 1.10 DIAGRAM OF HYPOTHALAMIC-PITUITARY-ADRENAL AXIS (HPA)
<https://www.onnit.com/academy/cortisol-heavy-metal-and-your-health/>

HPA axis controls the release of glucocorticoids. The hypothalamus produces corticotrophin releasing hormone (CRH) which stimulates the secretion of adrenocorticotrophic hormone (ACTH) from pituitary gland. ACTH then stimulates the adrenal cortex to produce cortisol. The negative feedback system of the HPA axis keeps glucocorticoid concentration within a normal range.

1.7.1 GLUCOCORTICOID RECEPTORS

Glucocorticoids are used as potent immunosuppressive and anti-inflammatory agents in the management of many inflammatory allergic, autoimmune & lymphoproliferative diseases. The actions of glucocorticoids are mediated by multiple isoforms of an intracellular receptor protein, the glucocorticoid receptor, which belongs to the steroid /sterol nuclear receptor superfamily, functioning as a hormone activated transcription factor that regulates the expression of a large number of glucocorticoid responsive genes (Kino *et al*, 2003).

The hGR gene consists of 9 exons and is located on chromosome 5 (Nicolaidis *et al*, 2010). There are 9 exons in the hGR gene: exon 1 (~ 116-981 bps) is a leader sequence, exon 2 (1197 bps) contains the coding sequence for AF-1 at the amino-terminal, exon 3 (167 bps) and exon 4 (117 bps) code for the first and second zinc-finger motif in the DBD respectively, exon 5 (280 bps), exon 6 (145 bps), exon 7 (131 bps), exon 8 (158 bps) codes for AF-2 and a large portion of the DBD, and exon 9 (4,108 bps) contains coding sequences for the two alternative carboxyl terminal of the LBD, α and β , and their respective 3' regions (Lu & Cidlowski, 2004) (Figure 1.8 and 1.11). In human cells total 777 amino acids are found which results from either posttranscriptional splicing of RNA or differences in start points. The main two types are GR α and GR β (Cidlowski, 2006).

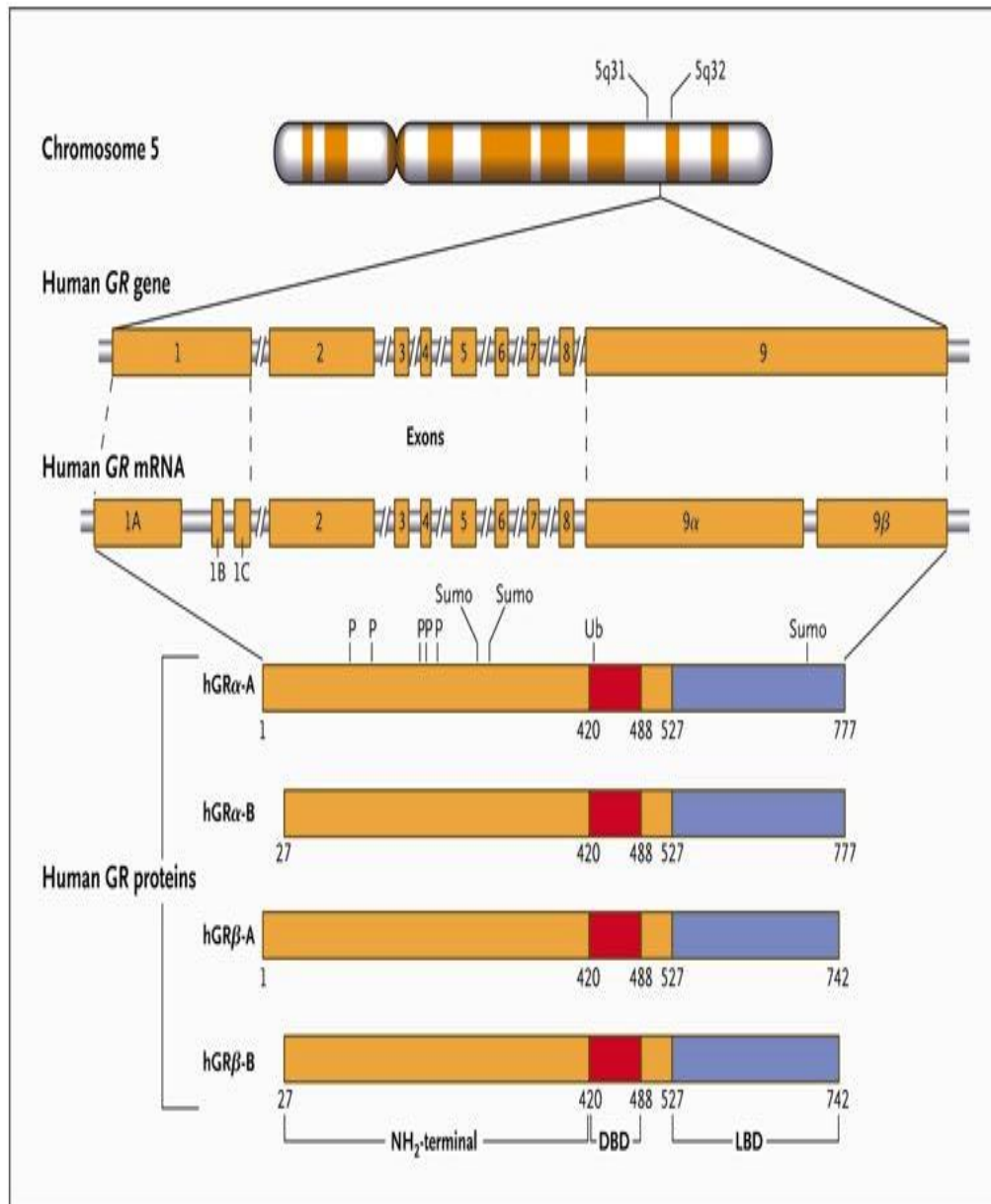


FIGURE 1.11 HUMAN GLUCOCORTICOID RECEPTOR GENE

The diagram represents genomic location and structure of human glucocorticoid receptor gene (hGR). Alternative splicing of primary transcript give rise to the two mRNA and protein isoforms: GR- α and GR- β and post translational modifications in the form of phosphorylation (P), ubiquitination (Ub) and Sumoylation (Sumo). DBD denotes DNA binding domain, LBD-Ligand binding domain and hGR human GR (Adapted from Rhen and Cidlowski, 2005).

GR α :

Human glucocorticoid receptor (hGR) α resides primarily in the cytoplasm of cells with several heat shock proteins (hsps) and represents the classic glucocorticoid receptor that functions as a ligand-dependent transcription factor. Glucocorticoid α isoforms undergo a conformational change when binding to hormone which results in dissociation of hsps, activation of their nuclear localization signals (NLS) and translocation into the nucleus (Kino & Chrousos, 2004 & Kino *et al*, 2003). Ligand activated GR α isoforms binds on to glucocorticoid responsive elements (GREs) in the regulatory regions of so-called co-activators and chromatin remodelling factors and influence transcription (Kino & Chrousos, 2004). Alternatively, GR α isoforms interact with other transcription factors, such as nuclear factor κ β and the activator protein 1 (AP1) to influence the transcription of genes responsive to these factors.

GR β :

GR β isoforms are identical to GR α but with a different c-terminal region, having 15 non-homologous amino acids at their c-terminal end (Hollenberg *et al*, 1985). Thus, GR β isoforms share the same N-terminal (NTD) and DNA-binding (DBD) domains with GR α , but possess unique "ligand binding" domain (LBD). GR β are located mainly in nucleus and generally fail to activate the transcription of glucocorticoid responsive gene (Castro *et al*, 1996). GR β function as dominant negative isoforms of GR α -induced transactivation of GRE containing glucocorticoid- responsive promoters and functions as natural inhibitor of glucocorticoid actions (Lu *et al*, 2004, Castro *et al*, 1996 & Bamberger *et al*, 1995). The ability of GR β to antagonize and moderate GR α effects suggests that GR β may play an important role in the regulation of target cell insensitivity to glucocorticoids. The expression of GR β is induced by cytokines and an increase of the GR β /GR α ratio has been reported in

patients with bronchial asthma, rheumatoid arthritis and chronic lymphocytic leukaemia (Vottero & Chrousos, 1999 & Lu *et al*, 2004).

In asthma, the ratio of both the isoforms vary as the glucocorticoid receptor GR α is linked with steroid sensitivity whereas the association between glucocorticoid insensitivity is due to increased glucocorticoid receptor GR β expression (Pujols *et al*, 2007). However, reports suggest the ratio of GR α and β does not vary in COPD patients (Korn *et al*, 1998). Therefore, does not provide a suitable explanation.

Glucocorticoids induced gene transcription:

Glucocorticoids exert their effects by binding to a cytoplasmic GR, that has several domains, including ligand binding domain, a DNA binding domain and 2 domains that are involved in transactivation of genes once binding to DNA has occurred via association with other proteins (Karin, 1998). The GR is a ligand induced transcription factor, which regulates gene expression through binding to a specific response element on the DNA in an indirect manner by interacting with other transcription factors such as nuclear factor-KB (NF-kB), activator protein (AP-1), Signal Transducer and Activator of Transcription (STAT) and Nuclear Factor of Activated T cells (NFAT) (Tait *et al*, 2008). GR is expressed in almost all cell types, including bronchial epithelial cells, and is retained inactive in the cytoplasm by a multi-protein chaperon complex heat shock protein (hsps-90) (Howard & Distelhorst 1988, Adcock & lane, 2003 & Pujols *et al*, 2009). Upon hormone binding, GR conformation is modified by dissociation of hsps-90 complex and translocate to nucleus. In nucleus GR binds as homodimer to specific DNA motifs termed as Glucocorticoid Responsive Elements (GREs) and activates transcription of GRE containing genes (Dahlman-Wright *et al*, 1990 & Luisi *et al*, 1991). It can also

repress gene transcription via protein-protein interaction independent of DNA recognition (Yamamoto, 1995). Gene activation and transcription is facilitated when the helical structure of DNA is unwound, and so enabling rapid onset of transcription by RNA transcriptase activity. For this to happen the histone structures that the DNA is wound around need to be acetylated to enable an 'open' conformation.

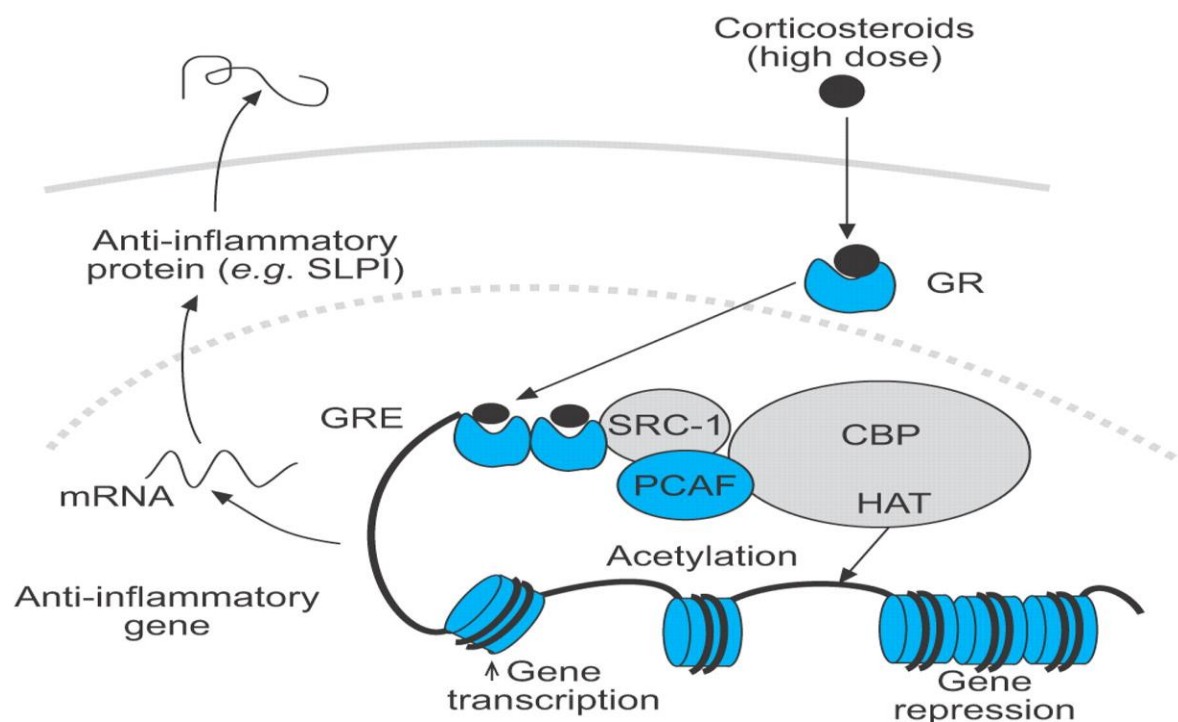


FIGURE 1.12 GLUCOCORTICOID INDUCED GENE TRANSCRIPTION

Glucocorticoid activation of anti-inflammatory gene expression. After ligand binding, activated glucocorticoid receptors (GRs) translocate to the nucleus where they bind to glucocorticoid responsive elements (GREs) in the promoter region of glucocorticoid responsive genes and also directly or indirectly to transcriptional coactivator molecules such as cyclic AMP response element binding protein (CBP). Coactivator molecule has intrinsic histone acetyl transferase activity (HAT) and cause acetylation of anti-inflammatory genes. SLPI-Secretory leucoprotease inhibition.

Conversely genes can be 'switched off' or deactivated by de-acetylating the histones so they can revert to the 'closed' conformation. (Figure 1.12). Glucocorticoid operates by binding to the cytosolic steroid receptor (GR) that enables the hormone

receptor complex to translocate to the cellular nucleus. In the nucleus the GR-hormone complex binds to glucocorticoid response elements (GRE) in the promoter region of steroid-sensitive genes. This leads to recruitment and activation of transcriptional coactivator molecules such as CBP, steroid receptor co-activator-1 (SRC-1), or other cofactors that have intrinsic HAT activity. This, in turn, results in acetylation of specific lysine residues on core histones proteins. Chromatin modification leads to local unwinding of the DNA structure, allowing recruitment of large protein complexes including RNA polymerase II. HATs cause acetylation of lysines on histone H4, which leads to activation of genes encoding anti-inflammatory proteins, such as secretory leukoprotease inhibitor (SLPI).

1.7.2 GLUCOCORTICOID RECEPTORS PHOSPHORYLATION

Glucocorticoid receptors are transcriptional regulatory phosphoproteins (Dalmann *et al*, 1988 & Bodwell *et al*, 1991). Post translational modifications of GR regulate target gene specificity and involve several cell signalling cascades (Davies *et al*, 2008). Phosphorylation, acetylation and SUMOylation of the GR are few examples of important modification of the GR after its translation. The GR undergoes several modifications following the mRNA translation into a polymer of amino acids. Such modifications not only play an essential role in determining the final structure of the GR, but also participate in establishing its function as a steroid dependent gene expression regulator (Holmstrom *et al*, 2008 & Brandl *et al*, 2009). Glucocorticoid receptors phosphorylation status can affect a range of GR functions, including DNA binding and transactivation potential, hsp90 interactions, subcellular localization and nuclear cytoplasmic shuttling (Adcock & Ito, 2005). This suggests that GR

phosphorylation may be one of the molecular mechanisms of corticosteroid resistance.

1.7.3 THE ROLE OF SERINE 211 (S211)

The most common sites for phosphorylation are serine. In human GR seven serine residues can be phosphorylation targets- S113, S134, S141, S203, S211, S226 and S404 (Oakley & Cidlowski, 2013). GR is usually phosphorylated at conserved residues suggesting a conserved function of phosphorylation. There are several kinases that phosphorylate the hGR α *in vivo* and *in vitro*. These includes 1) the yeast cyclin dependent kinase p34CDC28 (Ismaili, 2004 & Wang *et al*, 2002), 2) the p38 mitogen-activated protein kinase (MAPK) (Miller *et al*, 2005), 3) the CNS-specific cyclin-dependent kinase (CDK5) (Kino *et al*, 2007), 4) the glycogen synthase kinase 3 β (GSK-3 β) (Rogatsky *et al*, 1998 & Gulliner-Beckley *et al*, 2008), 5) the c-Jun N-terminal kinase (JNK) (Rogatsky *et al*, 1998 & Itoh *et al*, 2006). The 3 phosphorylation sites- Serine 203 (S203), Serine 211 (S211) and Serine 226 (S226) lies on N-terminal of human gene. The N-terminal transcriptional domain site S211 and S203 is targeted by CDK and p38 kinases (Bodwell *et al*, 1991 & Davies *et al*, 2008) whereas S226 is phosphorylated by JNK pathway (Kristic *et al*, 1997). GR becomes phosphorylated on activation in various amino acid locations at its N terminus (S203, S211 and S226) which affects their ability to interact with other proteins. Mutation in CDK genes in yeast reduce GR-dependent transcriptional activation, suggesting that phosphorylation of S203 and S211 is required for full GR transcriptional enhancement (Wang and Garabedian, 2003). Also, S211 acts as a substrate for p38 MAPK (Miller *et al*, 2005). In contrast, phosphorylation of S226 by JNK inhibits GR transcriptional activation (Rogatsky *et al*, 2003). This type of

phosphorylation results in inhibition of GR activity, possibly because of increase in GR nuclear export (Itoh *et al*, 2002). Studies have shown GR transcriptional activation is greatest when the relative phosphorylation of S211 exceeds that of S226 (Chen *et al*, 2008). Figure 1.13 show phosphorylated and non-phosphorylated sites for S211. The non-phosphorylation conformation exhibits random conformation of unmodified proteins whereas; the phosphorylated S211 induced a marked trend towards a more structured conformation with the peptide adapting a helical structure on both sides of the phosphorylation site.

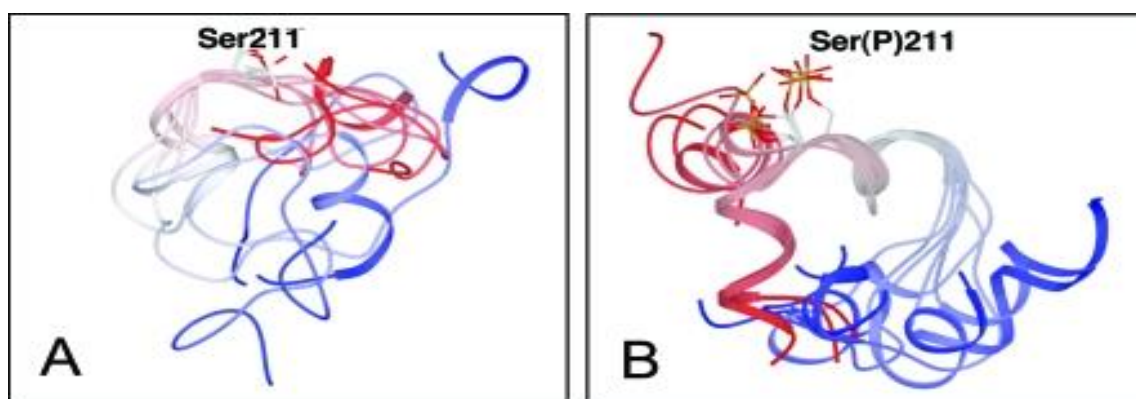


FIGURE 1.13 GLUCOCORTICOID PHOSPHORYLATION OF S211

Representative low-energy confrontations of: (A) Non-phosphorylated and (B) Phosphorylated (P-S211) peptide spanning the S211 site. Peptide is coloured blue (N-term) to red (C-term) and S211 is depicted. (Adapted from Mol. Endocrinology Chen *et al*, 2008).

1.7.4 THE ROLE OF CO-FACTOR TETRATRICOPEPTIDE 5 (TTC5)

Chronic obstructive pulmonary disease is an inflammatory disorder involving abnormalities in both innate and adaptive immune response. The process of inflammation, metabolism and immune response are regulated by glucocorticoids which are activated by glucocorticoid receptors (GR) (Wang *et al*, 2004). These activities are regulated at multiple levels including protein stability, post translational

modifications and cofactors interactions. During the transcriptional activity the hormone binds with the GR; translocate to the nucleus and binds to glucocorticoid response elements in the regulatory regions of its target genes. TTC5 is a stress responsive protein, also known as stress-responsive activator of p-300 (STRAP) and is present at GR regulated promoter region and can regulate the transcriptional activity of GR, acting as a co-activator or co-repressor in a gene specific manner. According to Davies *et al* (2011) findings in responsive stress manner, TTC5 interacts and stabilizes GR protein and regulates transcriptional activities. The regulation of GR by TTC5 is likely to contribute to the physiological role of the glucocorticoids.

TTC5 is composed of six protein-protein interactions tetratricopeptide repeat (TPR) motifs distributed throughout its sequence (Demonocas *et al*, 2001) and also contains one LXXLL (where L is leucine and X is any amino acid) nuclear receptor (NR) interaction motif between TPR 4 and TPR 5. However, mutation of LXXLL motif sequence and TPR motifs 2, 3 and specifically 6 not only reproduces alterations in interaction with receptors but also reduces the binding activity with GR (Davies *et al*, 2011) (Figure 1.14).

GR transcriptional effects are also modulated by co-regulators- TTC5 tetratricopeptide 5 which is thought to stabilize GR and alter its responses to cellular stress in a both positive and negative fashion. There is very little information about the expression of TTC5 in lung COPD tissue in comparison to healthy control patients; or how its regulation may be modulated by cigarette smoking (Davies *et al*, 2011).

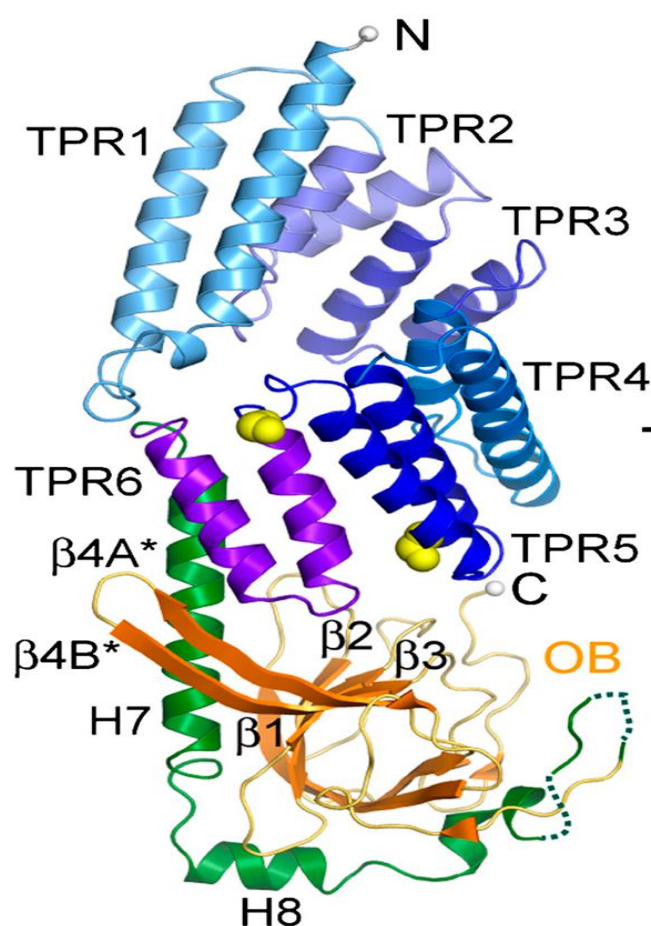


FIGURE 1.14 STRUCTURE OF TETRATRICOPEPTIDE 5 OR STRAP

Ribbon structure of STRAP (TTC5). It is elongated monomeric structure composed of two domains—a 250-residue N-terminal all α -helical domain comprising of 6 tandem TPR motifs and C terminal all β -sheet oligonucleotide/oligosaccharide binding (OB) fold. The domains are connected by a linker region containing a TPR-capping helix (H7) and a small perpendicular Helix (H8). The yellow spheres denote the phosphorylation sites.

1.8 AIMS AND OBJECTIVES

This thesis has the following research objectives:

1.8.1 To build on previous research from the fibrosis inflammation repair (FIR) group at Salford University to characterise EMT linked biomarkers in COPD and healthy control patients

- S100A4 was most strongly linked with smoking history and COPD development in previous studies, compared to other EMT related biomarkers. Therefore, this study aimed to further expand the patient numbers used for histochemical analysis of S100A4 expression in human lung tissue.

1.8.2 To build on nationally published data and Salford FIR group information on the properties of GR expression in COPD lung tissue.

- To characterise the degree of serine 211 (S211) phosphorylation expressions between the two subject groups, the hypothesis being there may be differential expression between COPD patients and control subjects.
- To characterise the degree of expression of GR-cofactor tetratricopeptide 5 (TTC5) in COPD patients compared to healthy controls; to address the hypothesis that differential expression between patients and control subjects may exist.

Collectively, this information will further the field of COPD research by offering insight into the mechanisms of EMT process in COPD, and the potential mechanisms of steroid resistance.

2. METHODS

2.1 SAMPLE SUBJECTS

Primary human lung samples were collected from patients suffering from chronic obstructive pulmonary disease recruited from Wythenshawe hospital. These patients had in most cases undergone resection surgery for tumour excision as part of their NHS treatment. The subject demographics were also obtained including their age, lung function, sex, medication and smoking status, although this data was blinded until statistical analysis was required preventing any influenced or biased data collection. All subjects gave written informed consent. The study was approved by the local research ethics committee (South Manchester).

Table 2.1 Patient Demographics:

VARIABLES	CONTROL SUBJECTS (N=27)			COPD SUBJECTS (N=19)	
	NON-SMOKER	CURRENT SMOKER	EX-SMOKER	COPD CURRENT SMOKER	COPD EX-SMOKER
SUBJECTS	5	8	13	8	11
SEX M/F	2/3	3/6	8/5	4/4	10/1
AGE	70±12.56	62.12 ±10.73	72.23 ±10.63	65.75 ±8.95	69.72 ±5.76
PYH	N/A	42.75 ±25.49	38.69 ±25.06	68.2 ±64.82	59.68 ±33.01
FEV% PREDICTED	97.4 ±5.17	75.37 ±24.42	85.53 ±17.58	65.12 ±19.03	65.42 ±19.54
FEV1/FVC RATIO	60.92 ±35.56	77.36 ±10.78	62.49 ±8.55	48.31 ±1.54	58.93 ±10.23

*Data are presented as mean SD. N/A - not applicable. PYH-Pack Year History=1 year smoking 20 cigarette per day. FEV1-Forced Expiratory Volume in 1 second. FVC-Forced Vital Capacity

2.1.2 PROCESSING OF LUNG BIOPSIES

Fixation was done as the first step to preserve the tissue substrate and render protein structure and other tissue components insoluble in all reagents exposed later in either processing or staining process. By fixation, autolysis and putrefaction can be halted and tissue substrates can be preserved. Formalin is mostly used as fixative agent because of its high penetration rate and preserve tissue for longer period of time.

After fixation, tissues were insufficiently firm and cohesive to allow thin section to be cut. This is why processing must be carried out. However, before the processing, about 3-5mm thick slices of lung tissue were obtained and put into tissue dek cassettes. The cassettes were then labelled for their laboratory accession number with lead pencils. Dehydration was then carried out to remove all water in tissue substrate so that water droplets will not trap and affect the specimen from being processed. Ethanol was used in dehydration process as it has least side-effects on tissue substrates compared to other dehydration agents. Water molecules were removed in ascending grades of alcohols to absolute alcohol.

Wax is insoluble in ethanol. Clearing agent that was miscible in both ethanol and wax are required. As xylene has least effect on tissue substrate, it was used in the experiment. However, xylene can harden the tissue, thus the immersion time cannot be prolonged.

After the clearing process, tissues were then infiltrated with wax. Additives such as bee's wax or synthetic resin were added to increase the hardness and stickiness of wax in order to give better cutting properties on tissue by altering the size of wax crystals. Temperature of the wax was kept 2° to 3°C above the melting point of wax

(~60°C) so that the wax will remain as liquid form throughout tissue infiltration process. However, it is important to make sure that the wax did not overheat as it would destroy plastic polymers and cause the cutting process to become more difficult.

In overall view, the tissue substrates were immersed in the reagents as shown:

- 1) 50 % Ethanol for 1 hour
- 2) 90% Ethanol for 1 hour
- 3) Absolute Alcohol for 1 hour (x2)
- 4) Xylene for 1 hour(x2)
- 5) Paraffin wax for 90 minutes (x2)

The whole process of dehydration, clearing and infiltration of wax was done on an automatic tissue processor (Leica, TP1020).

2.1.2.1 EMBEDDING

Before the cutting, processed tissue needs to be embedded in wax as a 'block' form to enable the section to be cut. Tissue cassettes molds method was applied as it did not require further trimming of wax around the tissue. For embedding process little wax was poured into the tissue molds and allowed to form at the bottom layer of the molds. Tissues were then picked with warm forceps and put onto the centre of the molds. Plastic tissue cassettes were placed over the mold. More wax was added into the molds if necessary. The molds were then moved to cold plate for rapid cooling and give fine crystalline structure to wax for better cutting properties.

2.1.2.2 MICROTOMY

As the sample moulds get solid the specimens were clamped into position on the microtome, (Leica, RM2125) by rotating the hand wheel clamp until the specimen clamp fit in the uppermost position and the handle locked in an upright position. The sample block could then be locked into the specimen clamp. By gentle turning the handle, thickest portion of the block started to trim, knife or blade was inserted in proper position so that the section obtained were trimmed at desired level of 5 μm thickness. The desired ribbon of tissue samples were mounted on to microscopic slides (Menzel-Glaser, Thermo Scientific, Braunschweig) for visualisation. Each sample was mounted gently transferring the section into the water bath set to 10°C below paraffin melting point of 50°C and then onto the microscopic slides, holding the slide half away in the water and gentle lifting out whilst the section began to stick, effectively pulling the section onto the slide. Tissues were air dried. Ensuring the handle wheel lock was in the upright position and the blade edge of the knife guard covered, the specimen could be removed. Using the coarse feed wheel a new specimen could then be loaded and new section ribbons were produced.

2.1.3 IMMUNOHISTOCHEMISTRY TECHNIQUE (IHC) on FFPE TISSUES

IHC is a powerful microscopy based technique for visualizing cellular components. The IHC technique was invented in 1940s (Coons, Creech & Jones, 1941) and is routinely used as an important tool in health care and pathology for e.g., diagnostic purposes to stratify patients for optimized treatment regimes. Figure 2.1 shows the process of binding primary and secondary antibodies with appropriate proteins.

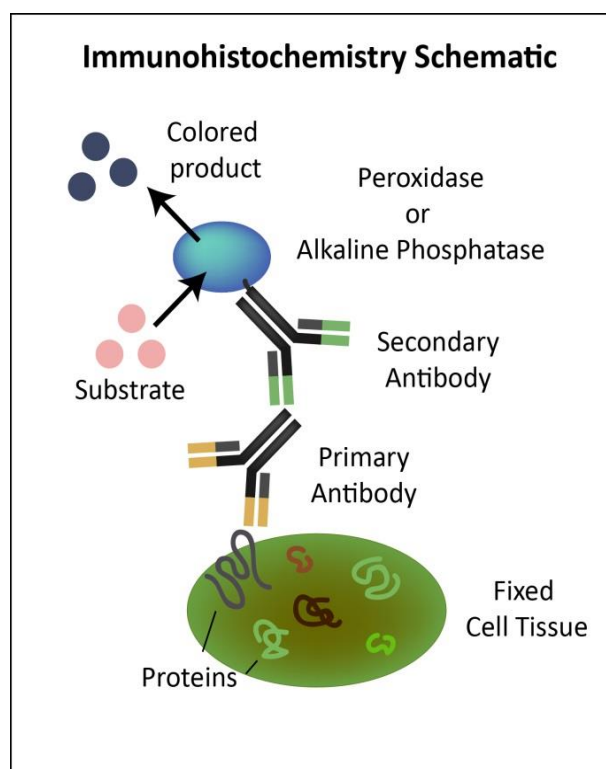


FIGURE 2.1 SCHEMATIC DIAGRAM OF IMMUNOHISTOCHEMISTRY.

2.1.3.1 OPTIMISATION

To confirm the antibody specificity and to achieve the best dilution, antibody optimisation was carried out. For this, different sets of dilutions were tested against given antibodies. The table 2.1 shows the optimal dilutions for antibodies required.

Table 2.2 OPTIMAL DILUTION

Primary Antibody	Monoclonal	Source	Cat no.	Dilution	Pre-treatment
S100A4	Rabbit	Dako	A5114	1:2500	N/A
TTC5	Rabbit	Dako	T3503-15	1:500	N/A
S211	Rabbit	Dako	4161100	1:1000	EDTA buffer

DAY 1:

Dewaxing of the samples through xylene for two sessions of five minutes each was performed, followed by rehydration through alcohol dilutions of 100% for 5 minutes, 90 % for three minutes, 75 % for two minutes and 50 % for one minute. The samples were then placed and run under water for five minutes, followed by pre-treatment. Pre-treatment involves the slides being placed in pre-treatment (PT) module and run for about 45 minutes per cycle. According to the antibody optimisation, S211 primary antibody needs a pre-treatment with EDTA buffer. 1mM EDTA was prepared by dissolving 0.37g of EDTA in 1000ml distilled water with pH 8.

Samples were then placed under running water for five minutes and then carefully each slide was dried with tissue paper, care was taken not to wipe away the sample, and for each slide the sample sections were drawn with a PAP wax pen, (Sigma, UK). Each sample was then blocked in 100µl of vector normal goat serum, (Elite Px, UK), 75µl in 5ml of TBS buffer, (Salford University Stores, UK), for thirty minutes at room temperature, followed by incubation in 100µl of primary antibodies S100A4 (1:2500) anti human rabbit antibody, S211 (1:1000) and TTC5 (1:500) for each COPD patients and control subjects (Elite, Px, UK).

The primary antibodies were prepared by using PBS; the samples were then incubated overnight at a room temperature in a humid box to prevent tissue dehydration.

DAY 2:

After overnight incubation in primary antibodies each sample was then washed in TBStween buffer for three sessions lasting three minutes each. TBS tween was

prepared from 500ml of 1xTBS (10x stock TBS, (Fisher, UK) diluted to 1:10 with distilled water) dissolved in 500µl of Tween-20. The samples were then placed in vector biotinylated secondary antibody, (Elite, Px, UK) for thirty minutes at room temperature. Vector biotinylated secondary antibody was prepared by adding 15µl of normal blocking serum to 1 ml TBS in a mixing bottle, then 5µl of biotinylated antibody stock was added to the mixture. Samples were then washed again in TBStween for three sessions lasting for three minutes each.

Each sample slide was then blocked of endogenous peroxidase by incubating in 40µl of 3% Hydrogen peroxide, (Fisher, UK) in methanol, (Salford University Stores, UK) for thirty minutes at room temperature. During this incubation period the avidin biotin complex (ABC), (Elite, Px, UK) was prepared as it was required to be prepared thirty minutes earlier before use. Preparation of ABC involved two drops of reagent A being added to 5ml of TBS, then two drops of reagent B added and mixed immediately standing for thirty minutes. After thirty minutes incubation at room temperature each sample was then washed in TBS tween for three sessions lasting three minutes each.

Following washing, each sample was then incubated in DAB substrate, (Vector, UK) until the sample turned visibly brown; this took approximately thirty minutes to an hour. A microscope was focused on each sample to stop the reaction following DAB stain has been reached. After this the samples were held under running water for a further five minutes. Counter stain was initially performed with Haematoxylin for 25 seconds and then samples were run under water. Dehydration back through alcohols occurred at dilutions of 50 % alcohol for one minute, 75 % for two minutes, 90 % for three minutes and 100 % for five minutes, each slide could be mounted with a cover slip containing DPX and visualized under the microscope.

2.1.3.2 MICROSCOPIC IMAGING

Digital microscopic images were obtained using a Leica microscope equipped with an imaging digital camera and Leica software. All images were stored as jpg and were cropped, where applicable, using Adobe Photoshop.

2.1.3.3 IMAGE ANALYSIS

Wayne S Rasband created a program named as Image J at NIH in 1987 (cited in Schneider *et al*, 2012). It is a free programme that can be downloaded from the website: <http://rsbweb.nih.gov/ij/download.html>

It is an open source image processing program for multidimensional image data with a focus on scientific imaging. It is incorporated with number of macros and plugins like Image processing, co-localization, deconvolution, registration, segmentation, tracking, visualization, cell counter and much more. The following Figure. 2.2 show the different stages of colour deconvolution.

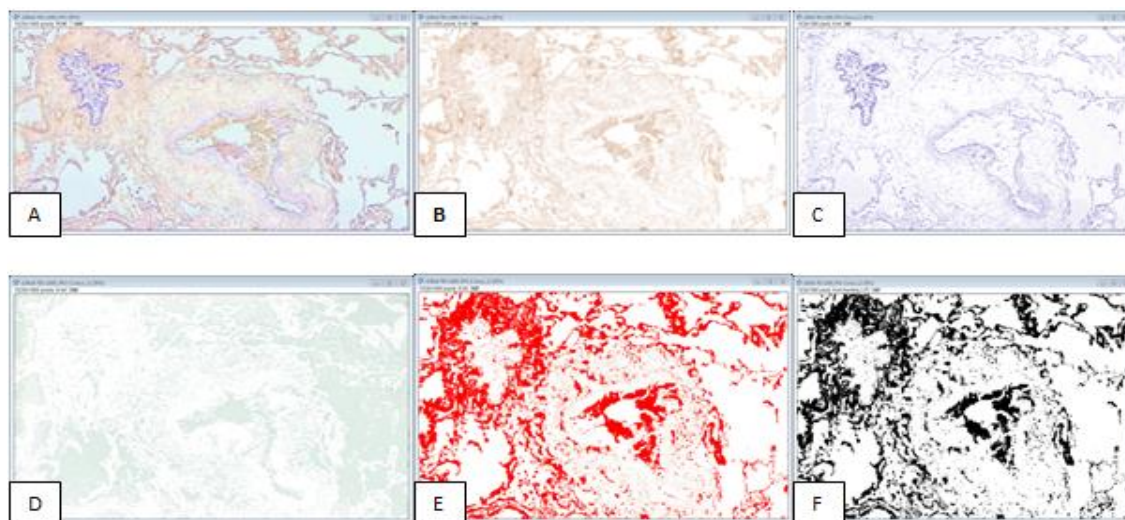


Figure 2.2 Images depicts the stages of colour deconvolution by Image J software. (Fig 2.2 A) shows normal Image with subtracted background, (Fig 2.2 B, C, D) shows 3 different

stained images by colour deconvolution, (Fig 2.2 E) depicts the threshold value and (Fig 2.2 F) shows the percentage area stained.

In Figure 2.2 for the first stage, the standard historical stain combination of haematoxylin & eosin (H&E) and 3, 3-diaminobenzidine (DAB) substrate with haematoxylin has been segmented by plugin deconvolution. In next stage, Image has been split into three components according to the colour stain used. Figure 2.2 B colour is brown because of DAB substrate, figure 2.2 C is haematoxylin, it is purple and figure 2.2 D is residue. Following deconvolution, thresholding was performed on figure 2.2 B to select the area of interest for measuring area stained and thus figure 2.2 E was obtained and in final stage the black area in figure 2.2 D shows the selected percentage area of the given stained image.

2.1.4 STATISTICAL ANALYSIS OF IHC IMAGES

Data were analysed in Graph Pad Prism 5 and statistics performed in Graph Pad Instat 3 (Graph Pad software, San Diego, CA, USA; <http://www.graphpad.com>). Immunohistochemistry data were normally distributed.

Non-parametric repeated One-way ANOVA (Kruskal-Wallis) test was performed to compare biomarker expression within the groups and when $p < 0.05$ subsequent unpaired tests t tests were performed to compared % area stained between groups. Unpaired t tests were used to analyse expression of biomarkers in healthy subjects (control) vs. COPD subjects.

Linear regression was performed to understand lung function (disease severity) and smoking history (pack year history) with biomarker expression.

3. RESULTS

3.1 ANTIBODY OPTIMISATION

In this experiment, fixation was the first step carried out for processing of lung biopsies to preserve the tissue. This fixation process during paraffinisation may create cross-linking of proteins. This may result in masking of the epitopes, resulting in weak or false negative staining. So, the process of antibody optimisation was carried out for determination of a particular epitope to expose at optimal pre-treatment for required lung tissue and to achieve the dilution for the antibodies relevant to pre-treatment condition.

The antibody for Epithelial Mesenchymal Transition marker was S100A4.

The selected antibodies for Glucocorticoid Receptors expressing co-factor antibody was TTC5 and Phosphorylated Glucocorticoid receptor antibody was S211.

According to the protocol provided by the antibody manufacturer, antibody concentration was tested on the COPD lung patient samples. Each antibody (S100A4, TTC5 and S211) was optimised on by doing serial dilution and with different buffers on lung patient 773. The conclusion of the antibody optimisation protocol (Figure 3.1) was that glucocorticoid antibody that detects glucocorticoid receptor phosphorylated at serine S211 required pre-treatment with EDTA buffer at pH 8 and the optimal antibody concentrations was 1:1000, for S100A4 and TTC5 was 1:2500, 1:500 respectively (Figure 3.2).

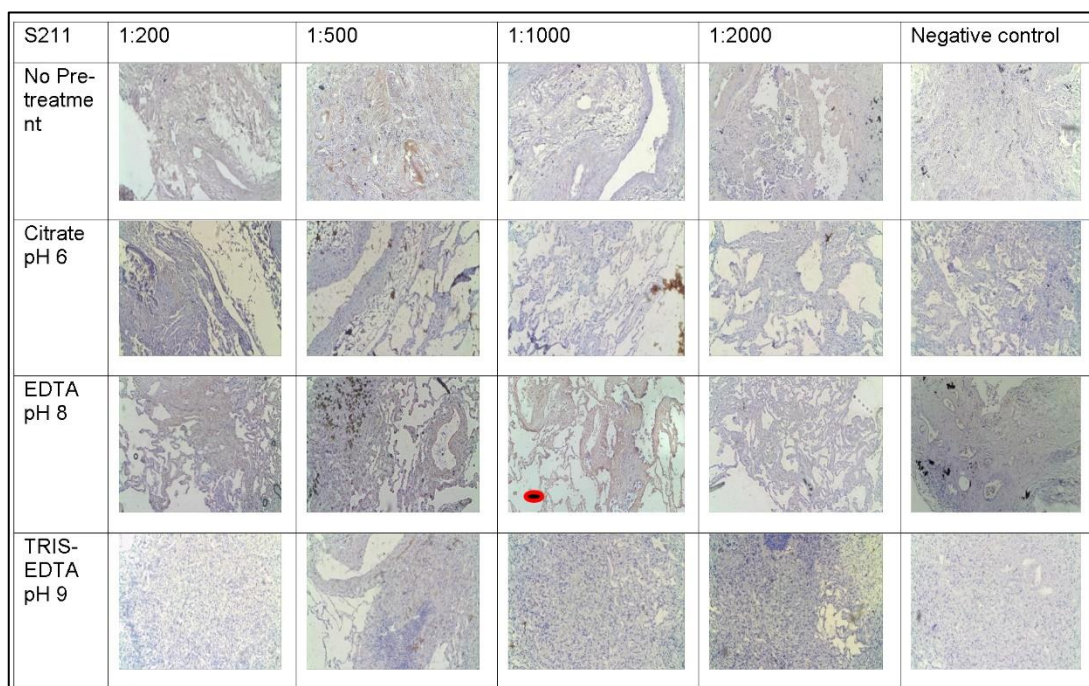


FIGURE 3.1 IHC OPTIMISATION OF S211

Lung sample 773 images depicts the staining that was achieved during S211 optimisation and used to determine the optimal dilution and pre-treatment condition (Red mark), for the samples used in the study. X100 magnifications.

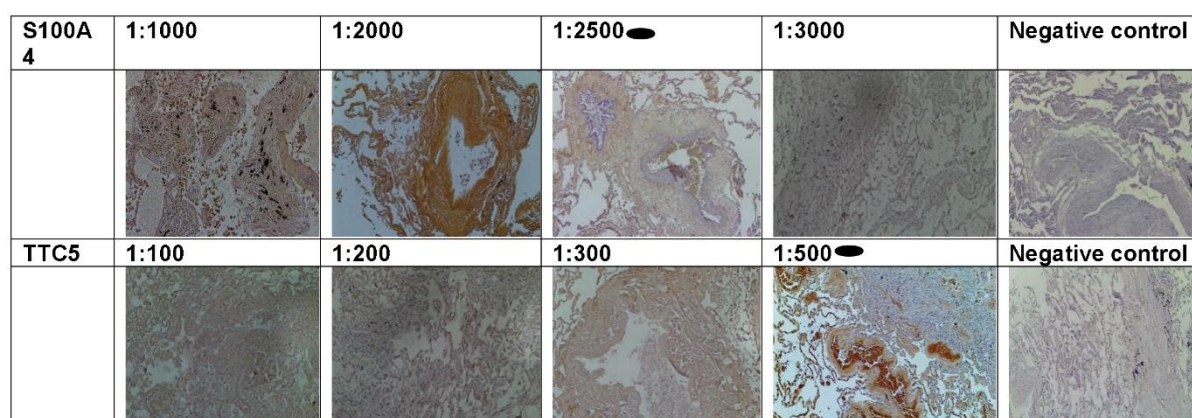


FIGURE 3.2 ANTIBODY OPTIMISATION OF S100A4 AND TTC5

Lung sample images show the optimal concentration for S100A4 and TTC5 antibodies (no pre-treatment). X100 magnifications.

The Figure 3.2 shows example of images of the antibody optimisation for S100A4 and TTC5 shown across the different ranges of dilutions. Both antibodies were treated with IHC pre-treatment buffers and different dilutions. During the experimental conditions, the result obtained suggests there was no need of pre-treatment as the clearest positive staining was observed without the pre-treatment.

For Epithelial Mesenchymal Transition marker S100A4 (1:2500 black mark) dilution was chosen, as the lower dilution shows heavy areas of staining and higher dilution shows no positive staining compared to the chosen one.

For TTC5, the Glucocorticoid receptor variant, lower dilutions barely show any stained areas compared to the chosen one (1:500 black mark) dilution which depicts the distinguishable structure of the lungs with positive staining.

3.2 QUANTIFICATION OF IMAGES

3.2.1 HISTOPATHOLOGICAL FINDINGS

The following (Figure 3.3) depicts the example of the control subjects in comparison with COPD subjects with antibody staining of the lung tissue. The control subjects show the intact structure and COPD shows the poor prognosis in compare to staining between all groups.

Figure 3.3 demonstrates the comparison between the control subject and COPD subject with appropriate antibody staining. (Figure 3.3 A) shows the bronchial epithelium (black arrows) with small basal cells sitting directly on basement membrane and upper layer covered with different epithelium cells. (Figure 3.3 B) shows the lumen of bronchiole (black arrow) within the lung parenchyma and pleura

with vessel (black arrow) within, these are little oedematous. (Figure 3.3 C) depicts the alveolar space is clear compare to the COPD subjects.

For COPD subjects (Figure 3.3 E) shows the accumulation of fine particles of carbon in the lung (Cigarette tar deposits). (Figure 3.3 F) demonstrates the thin alveolar wall surrounding the lung parenchyma, in (Figure 3.3 G) black arrows shows inflammation due to presence of lymphocytes, and black arrow shows disrupted alveoli.

Figure 3.3 D and H images depicts the slide stained without any primary antibody. (Figure 3.3 D) shows the intact structure with normal epithelial cells (black arrows) and (Figure 3.3 H) shows the disrupted and broken lung tissue (black arrows). There is a clear difference between the patient groups for epithelial marker S100A4 staining, glucocorticoid receptor markers TTC5 and S211 between control subjects and COPD subjects.

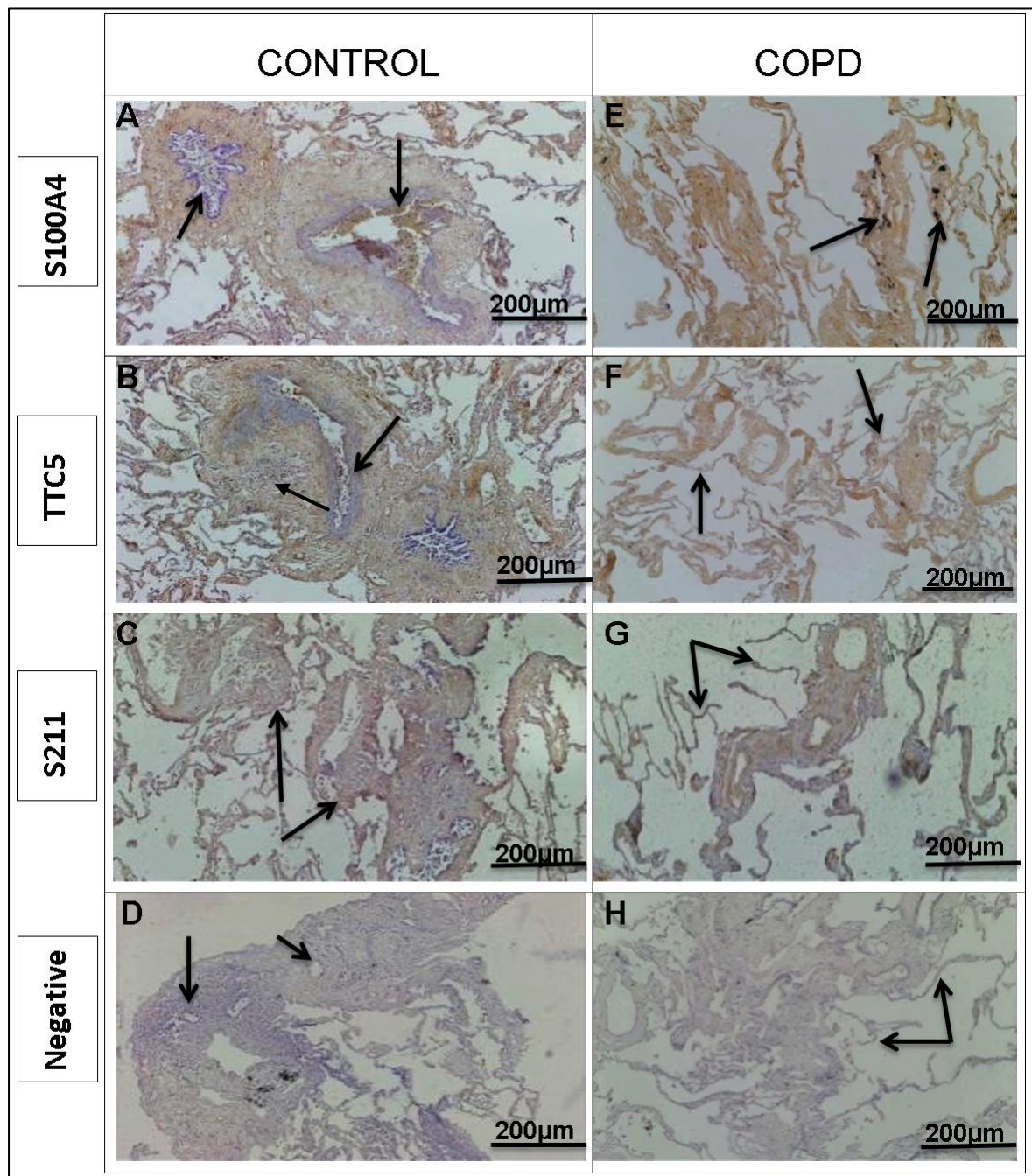


FIGURE 3.3 IHC OF CONTROL SUBJECTS VS COPD SUBJECTS

Examples of Epithelial Biomarker (S100A4), Glucocorticoid receptor (TTC5) and Phosphorylated glucocorticoid receptor (S211) were investigated *in situ* using immunohistochemistry in (A-D) healthy non-smokers vs. (E-F) COPD current smokers. A) Bronchiole, B) Lumen of Bronchiole, C) Alveolar spaces are clear, D) lymphocytes, E) Tar deposits, F) Thin alveolar wall, G+H) Disrupted alveoli. X100 magnifications.

3.2.2 PATHOLOGICAL FINDINGS

To analyse the immunohistochemical investigation of Epithelial Marker-S100A4 and glucocorticoid receptors connected markers-TTC5 and S211, a series of staining was performed between the patient demographics groups (Initially blinded) of healthy non-smoker, current smokers, Ex-smokers, COPD- current smokers and COPD Ex-smokers in comparison with the negative control. The microscopic images with appropriate antibody staining have been described.

The human lung tissue specimens from **healthy non-smokers** were found to be having an intact parenchymal structure. Small airways were visible with considerable amount of lymphocytes present. Bronchioles were covered with smooth muscle cells and connective tissue. Alveolar walls were intact. The sections in (Figure 3.4) show positive staining with S100A4. The bronchioles are clearly visible in (Figure 3.4 A) with infiltration of some lymphocytes and some positive cells stained for S100A4 in (Figure 3.4 B). Glucocorticoid receptor expressing components TTC5 and S211 both show some weak amount of staining compared to S100A4 with distinguishable features of the tissue. (Figure 3.4 C+D) shows infiltration of lymphocytes and positive brown cells for TTC5. Alveolar sacs were found in (Figure 3.4 E) and infiltration of positive cells in lumen of bronchiole which is beneath the epithelium (Figure 3.4 F). The negative control shows leucocytes (Figure 3.4 G+H).

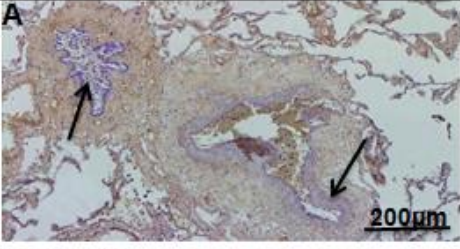
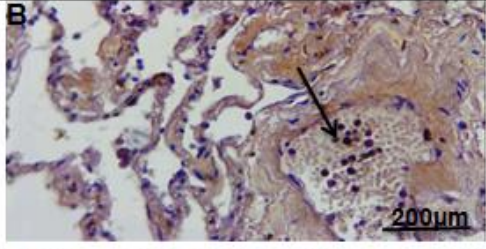
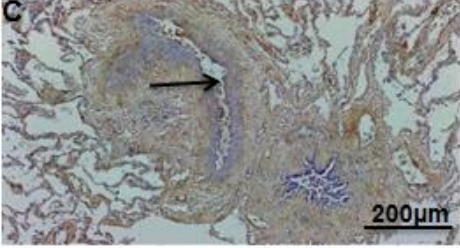
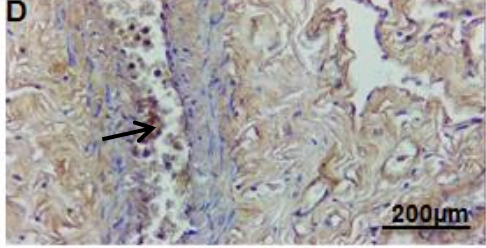
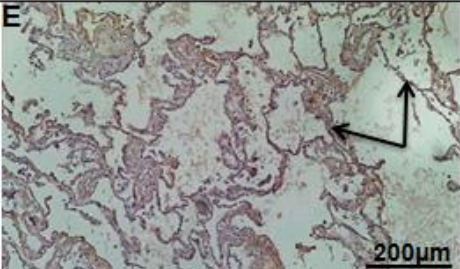
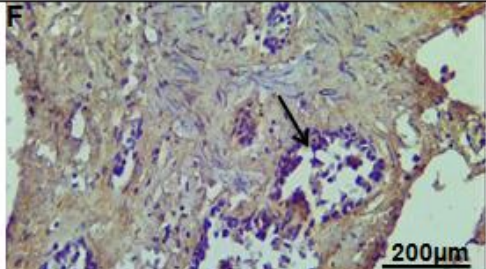
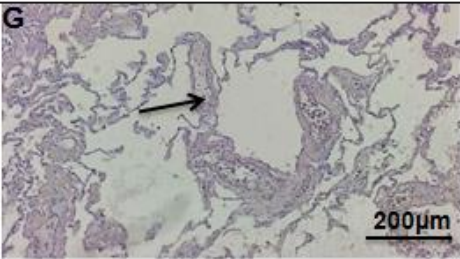
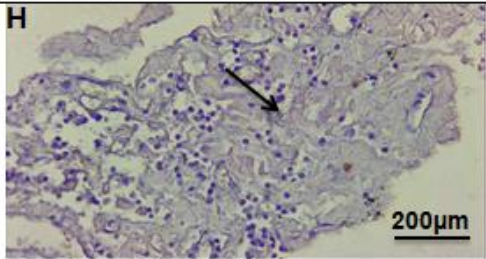
HNS	X100Magnification Column A	X400Magnification Column B
S100A4	A 	B 
TTC5	C 	D 
S211	E 	F 
Negative	G 	H 

FIGURE 3.4 IHC OF HEALTHY NON SMOKER

Human lung tissue specimen from healthy non-smoker stained for S100A4, TTC5 and S211 compared to negative control. Figure 3.4 **A** shows bronchioles (black arrows) & figure 3.4 **B** black arrow shows positive cells for S100A4. Figure 3.4 **C+D** black arrows indicate infiltration of lymphocytes and positive cells for TTC5. Figure 3.4 **E** detachment of alveolar ducts (black arrow) and figure 3.4 **F** black arrow show lymphocytes. In negative control figure 3.4 **G+H** black arrow points for leucocytes. X100 magnifications (column A) and X400 magnifications (column B).

The pathological findings of **current-smokers** are illustrated in Figure 3.5 for epithelial marker S100A4, Glucocorticoid receptors expressing variants TTC5 and S211 in comparison with negative control. A partially condensed structure was notified in the specimens obtained for current smokers. A moderate amount of tar deposits was observed. Thickening and remodelling was observed in (Figure 3.5 A+B). The reaction with TTC5 shows a positive reaction indicating the presence of GC receptors. Presence of brownish cells within the respiratory bronchioles is shown (Figure 3.5 C+D). Slightly weak reaction was observed for S211 antibody having terminal bronchiole continuing with the respiratory bronchiole, which extends into the alveolar ducts and ultimately the alveoli (Figure 3.5 E+F). In (Figure 3.5 G+H), the presence of brownish pigmented indicates the macrophages within the respiratory bronchioles and alveolar ducts may be associated with Peri-bronchiolar chronic infiltrate of lymphocytes.

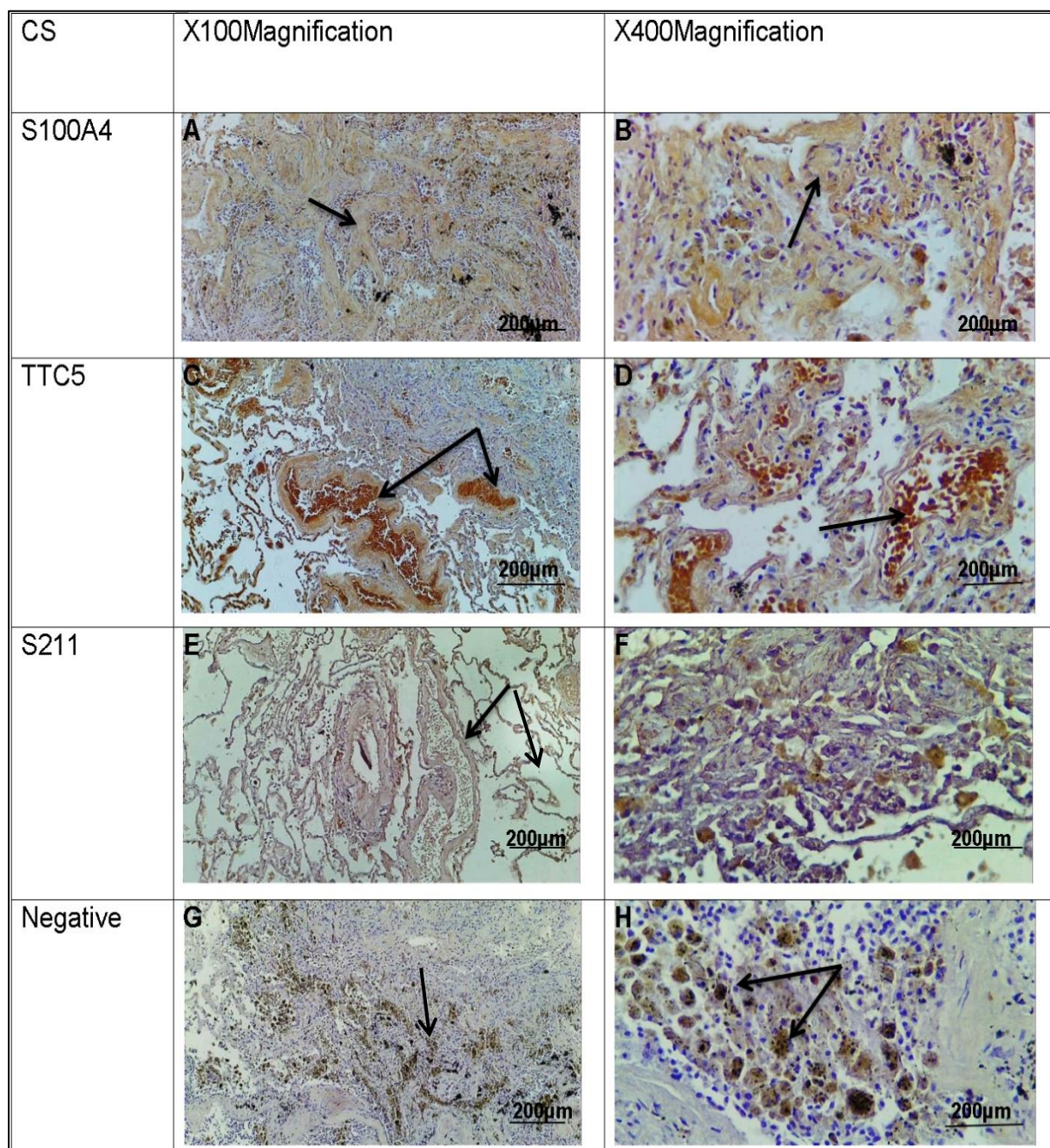


FIGURE 3.5 IHC OF CURRENT SMOKER

Human lung tissue specimen from current smoker stained for S100A4, TTC5 and S211 compared to negative control. Figure 3.5 A+B indicates thickening of tissue (black arrows). Figure 3.5 C+D black arrows shows positive cells for TTC5. Figure 3.5 E shows alveolar ducts/alveoli (black arrows) and in figure 3.5 G+H black arrow points towards macrophages. X100 magnifications (column A) and X400 magnifications (column B).

The pathological findings of **Ex-smokers** are illustrated in (Figure 3.6) for epithelial marker S100A4, Glucocorticoid receptors related markers TTC5 and S211 in comparison with negative control. The presence of tar deposits was obvious because of the cigarette smoke in the given patient tissue sample. The architecture of lung parenchyma is in intact form. Lower number of macrophages and lymphocytes was detected. The epithelial marker S100A4 shows reduced expressions with considerable number of lymphocytes (Figure 3.6 A+ B). Ex-smokers show high reactivity with TTC5 expressed in epithelial cells, (Black arrows in Figure 3.6 C+ D). There was an inadequate expression of S211 in tissue sample of Ex-Smokers (Figure 3.6 E+ F). The negative control also depicts the thickness of the epithelial cell lines and lymphocytes in (Figure 3.6 G+ H).

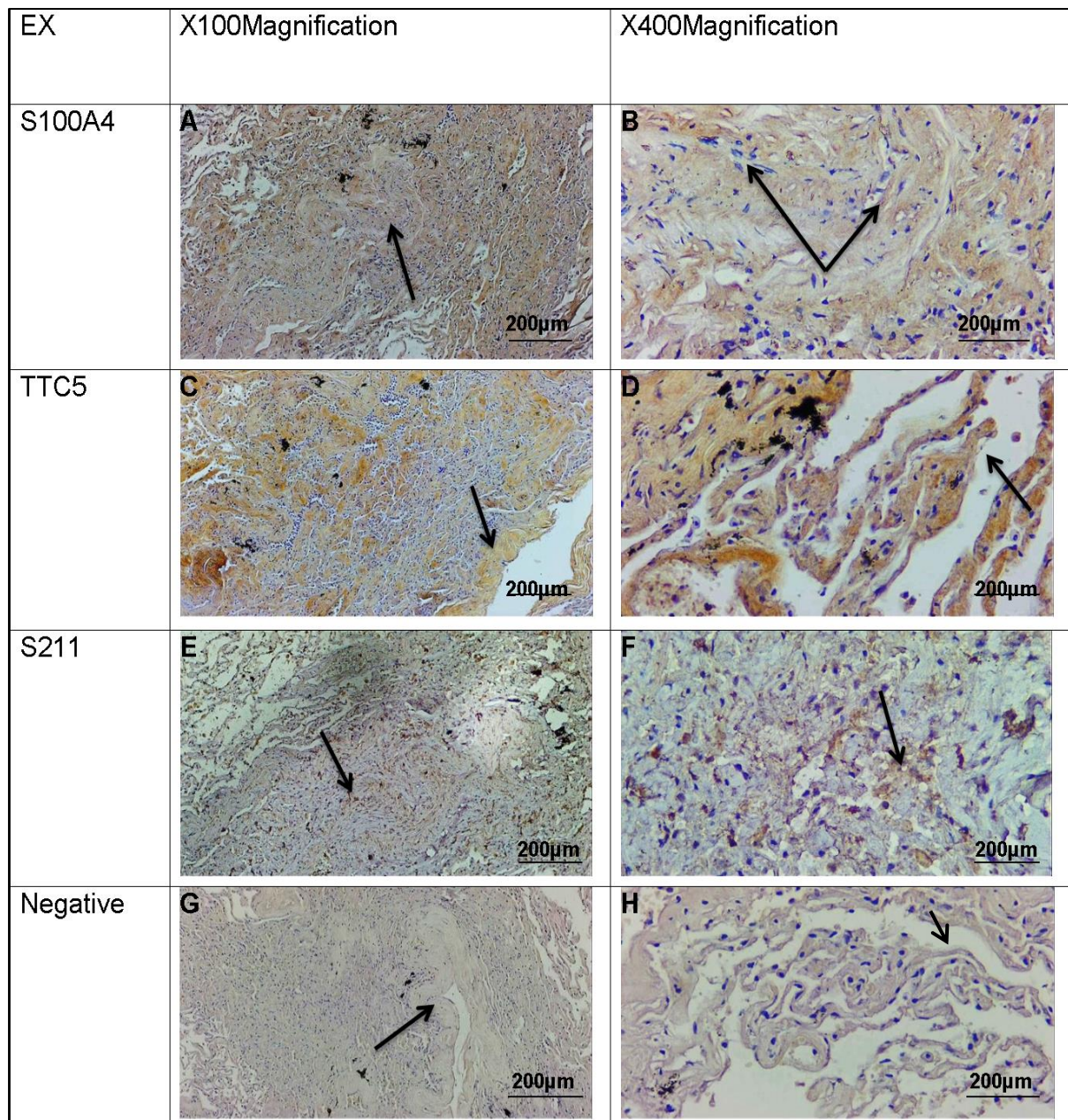


FIGURE 3.6 IHC OF EX-SMOKER

Human lung tissue specimen from ex-smoker stained for S100A4, TTC5 and S211 compared to negative control. Black arrows in figure 3.6 **A+B** shows presence of lymphocytes. In figure 3.6 **C+D** black arrows indicates the thickening of epithelial linings. Figure 3.6 **E+F** shows minimal positive cells for S211 (black arrows) and figure 3.6 **G+H** depicts the thick epithelial lining. X100 magnifications (column A) and X400 magnification (column B).

Figure 3.7 + Figure 3.8 shows the immunohistochemical staining for epithelial marker S100A4, Glucocorticoid receptor markers TTC5 and S211 in **COPD Current smokers (CCS)** and **COPD Ex-smokers (CEX)** respectively. In CCS, partially condensed structures and airway remodelling were observed in lung tissue samples. S100A4-epithelial marker was expressed positively in (Figure 3.7 B). Deposition of tar and capillaries are present in (Figure 3.7 A). Glucocorticoid receptor markers TTC5 and S211 were also expressed positively in the lung tissue of CCS. Infiltration of lymphocytes and macrophages are present in (Figure 3.7 C). Pigmented macrophages are also seen in (Figure 3.7 D). Alveolar detachment and spindle shaped cells in the epithelial cell lines may indicate the presence of fibrosis in the patients with CCS (Figure 3.7 E+ F). Negative control in (Figure 3.7 G+ H) also indicates the presence of infiltration of macrophages, spindle shaped cells and deposition of tar in airways.

COPD ex-smokers also indicated the presence of airways thickening and squashed structure of lung parenchyma. (Figure 3.8 A+ B) shows the expression of epithelial marker S100A4 and black arrows pointing towards the capillaries present in the tissue section with positive brown cells in the epithelium. GC receptor expressing variant TTC5 in (Figure 3.8 C+ D) shows the presence of type ii pneumocyte (black arrows) and presence of lymphocytes in epithelial layer. GC receptor expressing variant S211 in (Figure 3.8 E+ F) shows precise positive reaction by expressing in the upper layer of epithelial cell lines and shows the presence of large number of macrophages. Negative control shows the infiltration of macrophages (Figure 3.8 G) and lymphocytes indicating inflammation (Figure 3.8 H).

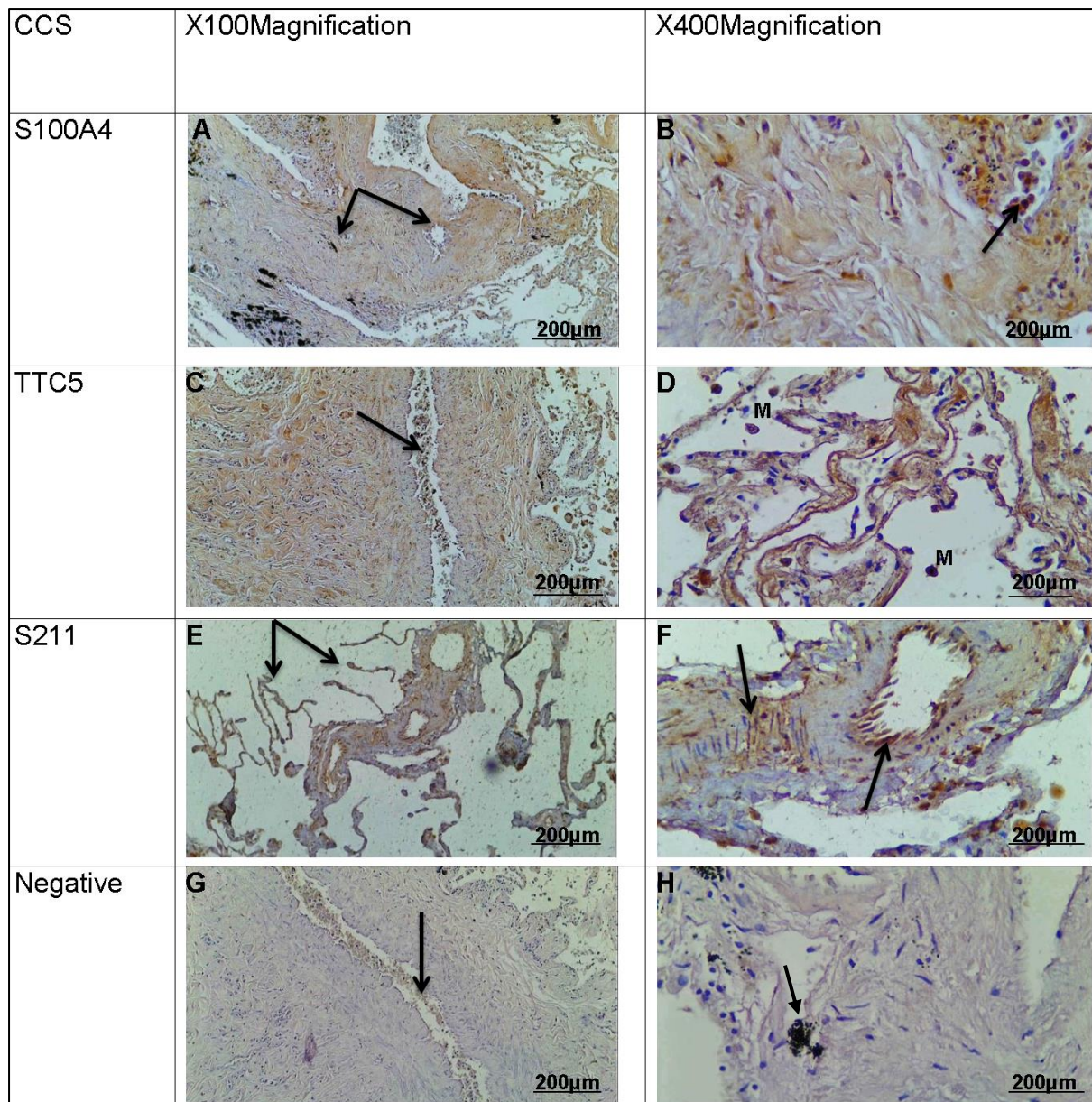


FIGURE 3.7 IHC OF COPD CURRENT SMOKER

Human lung tissue specimen from a COPD current smoker stained for S100A4, TTC5 and S211 compared to negative control. Figure 3.7 **A+B** shows presence of tar deposits and capillaries (black arrows). Figure 3.7 **C** indicates infiltration of lymphocytes and figure 3.7 **D** shows pigmented macrophages denoted by M. in figure 3.7 **E+F** alveolar detachment and spindle shaped brown cells are seen (black arrows). In figure 3.7 **G+H** black arrows indicates the deposition of tar + infiltration of macrophages. X100 magnifications (column A) and X400magnifications (column B).

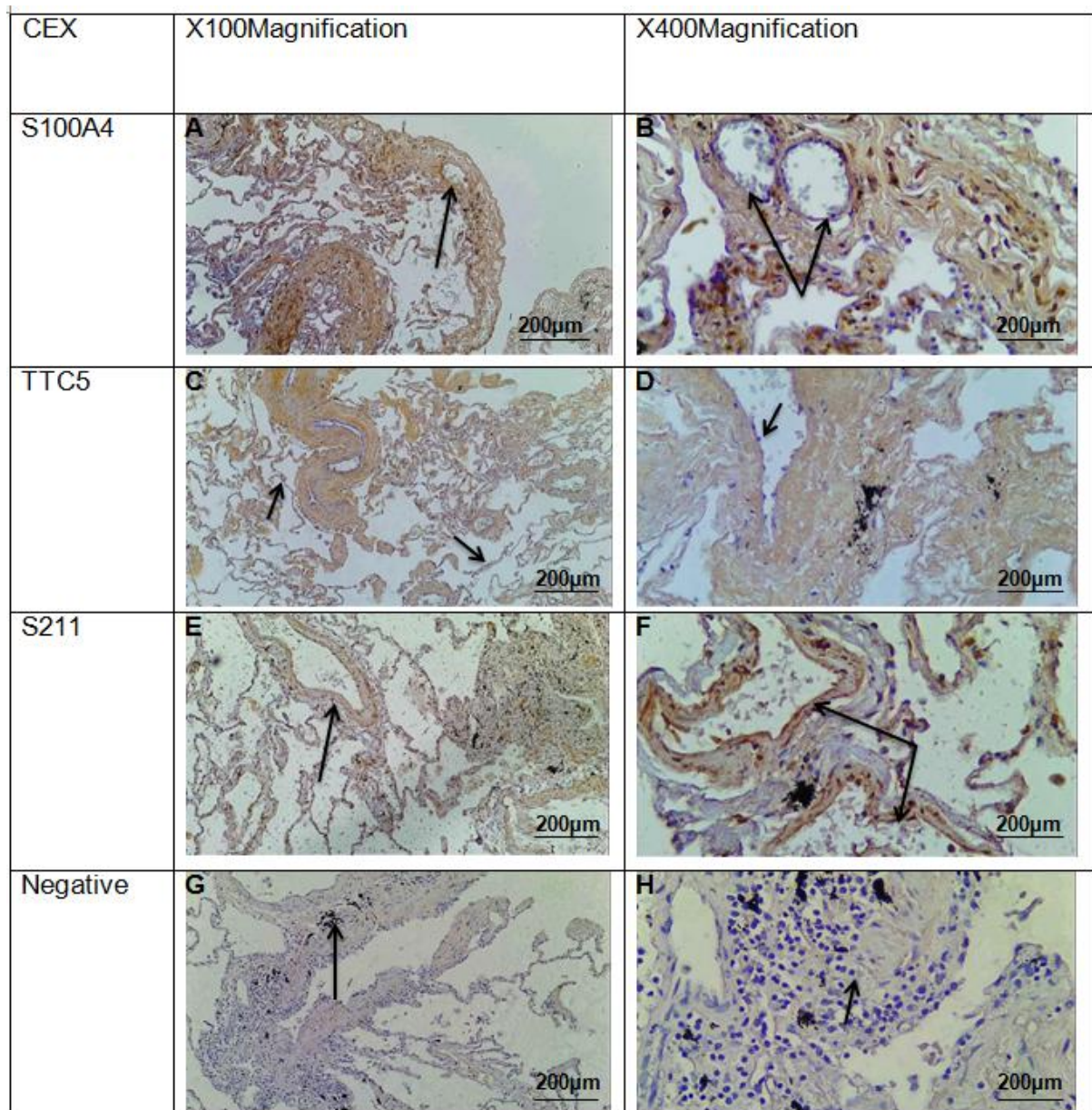


FIGURE 3.8 IHC OF COPD EX-SMOKER

Human lung tissue specimen from a COPD ex-smoker stained for S100A4, TTC5 and S211 compared to negative control. Figure 3.8 **A+B** shows positive cells for S100A4 and capillaries (black arrows). Figure 3.8 **C+D** shows type ii pneumocytes (black arrows). In figure 3.8 **E+F** black arrow indicates thicker epithelial cell line with positive cells for S211. In negative control figure 3.8 **G+H** shows infiltration of macrophages and lymphocytes. X100 magnifications (column A) and X400 magnifications (column B).

3.3 STATISTICAL ANALYSIS

A comparison study was conducted to assess the study groups between control subjects and COPD subjects. A non-parametric one-way ANOVA (Kruskal-Wallis) test was performed between the individual study groups (Non-Smoker, Ex-Smoker, Current Smoker, COPD Current Smoker and COPD Ex-Smoker). Also, to assess the effects of current smoking and previous smoking patients were carried out by splitting up Current (COPD and Control) smokers, Ex (COPD and Control) smokers and Non-Smokers for data analysis.

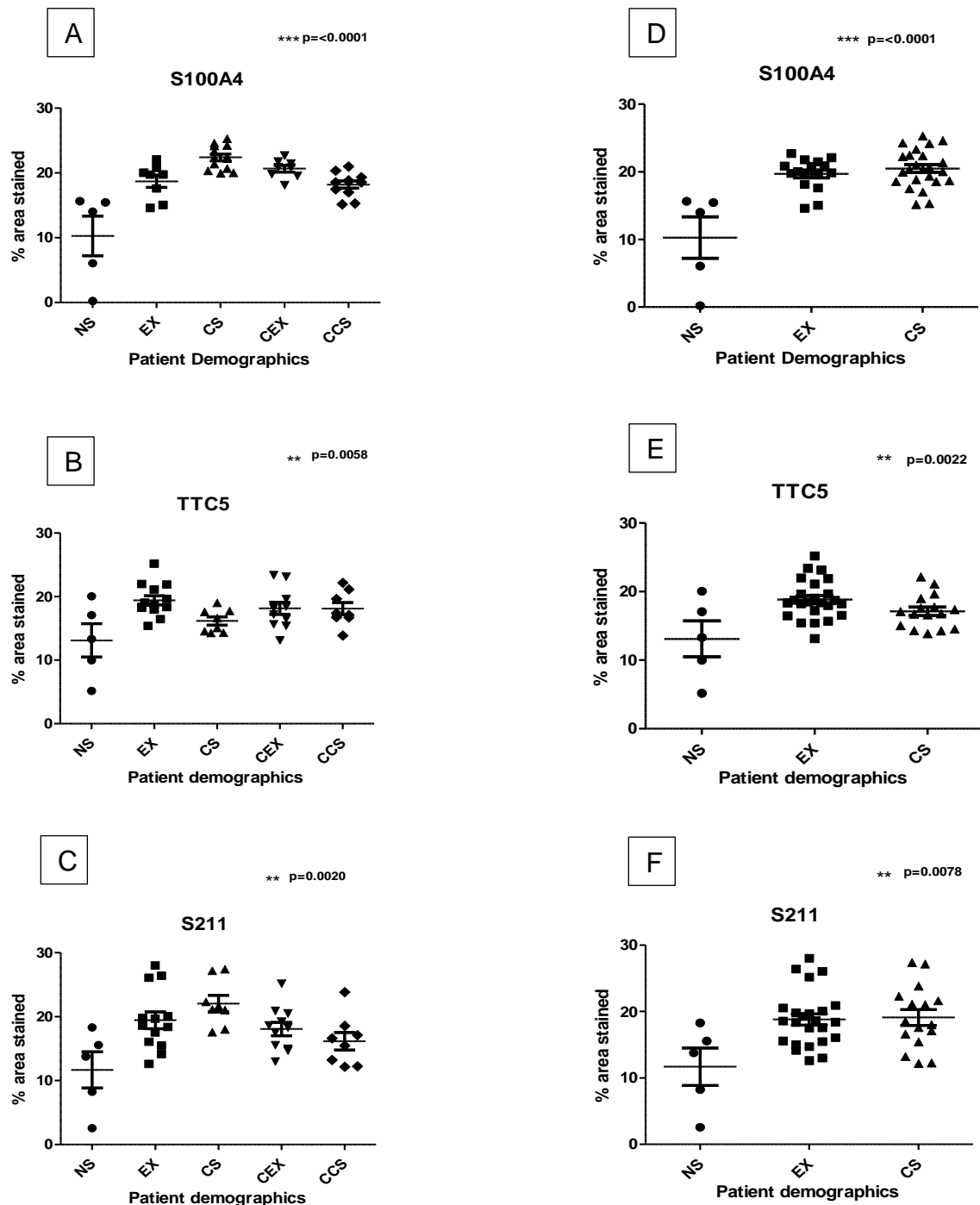


FIGURE 3.9 COMPARISONS OF BIOMARKERS BETWEEN NON-SMOKER (NS), EX-SMOKER (EX), CURRENT SMOKER (CS) WITH COPD EX-SMOKER (CEX) & COPD CURRENT SMOKER (CCS).

A-C: Comparison of positive percentage area stained for S100A4, TTC5 and S211 between the separate patient groups. D-F: Comparison of positive percentage area stained for S100A4, TTC5 and S211 between the split into smoking status groups (regardless of if the subjects has progressed to COPD). Data are presented as mean % area stained (\pm s.e). Following ANOVA unpaired t-tests were performed to determine significance between groups. $P < 0.05$ was taken as significant.

3.3.1 COMPARISON OF BIOMARKERS Vs ALL PATIENTS SUBJECTS AND SMOKING STATUS SUBJECTS

A one-way (Kruskal-Wallis) ANOVA was performed for all patient groups including healthy (NS) non-smoker (n=5), (EX) ex-smoker (n=13), (CS) current smoker (n=8) with (CEX) COPD ex-smoker (n=11) and (CCS) COPD current smoker (n=8). The graph shows extremely significant difference ($p<0.0001$) in EMT marker S100A4 expression in lung tissue between then healthy NS, EX, CS and COPD (CCS + CEX) (Figure 3.9 A). The significance in expression is clearly observable as rise in expression in the ex-smokers compared to never smokers. These raised levels remain high in current smokers and ex and current smokers with COPD.

In (Figure 3.9 B), the ex-smoking groups (EX and CEX) show the highest percentage TTC5 expression compared to never smokers (NS) with a very significant difference ($p=0.0022$). Levels of TTC5 expression remain high in current smokers (CS and CCS) regardless of COPD development or not. The graph represents very significant difference ($p=0.0020$) in S211 expression among the EX, CS, CCS and CEX compared to NS (Figure 3.9 C). Ex-smokers (EX and CEX) show the significant difference compared to never smokers and remains high in current smokers (CS) and COPD current (CCS) and ex-smokers (CEX).

In smoking status group, there is also an extremely significant difference ($p<0.0001$) of mean between non-smokers (NS) and ex-smokers (EX) in EMT marker S100A4 (Figure 3.9 D) and it shows high percentage of marker present in current smokers compare to lower percentage in never smokers. S100A4 expression levels are similar between ex-smokers and current smokers. One way ANOVA with post –test was also performed between non-smoker (NS) and ex-smoker (EX), the results

shows extremely significant difference between both the groups ($***p<0.05$) data not shown. This indicates the smoking elevates the expression of S100A4 in smoking individuals.

The TTC5 expression levels (Figure 3.9 E) among the patient groups shows very significant difference as ($p=0.0068$). Ex-smokers show the highest expression levels compared to current smokers and non-smokers. Performing the one way ANOVA with post-test for TTC5 represents very significant ($**p=0.0023$) difference between non-smokers (NS) and ex-smokers (EX). But comparing both the ex-smokers (EX) and current smoker (CS) doesn't show any difference ($P>0.05$) data not shown.

The S211 expression levels shows very significant difference ($p=0.0078$) among the smoking patient groups (Figure 3.9 F). Ex-smokers and current smokers show the high percentage expression of marker compared to never smokers. By comparing non-smoker (NS) and ex-smoker (EX) with one way ANOVA post-test, it shows very significant difference ($**p=0.0037$) data not shown but no difference was found between ex-smoker (EX) and current smoker (CS).

3.3.2 COPD PATIENTS VS CONTROL SUBJECTS

The following analysis was performed to evaluate if biomarker expression levels were different between control subjects (including never smokers, current smokers and ex-smokers) compared to those subjects who had gone on to develop COPD (including current and ex-smoking patients).

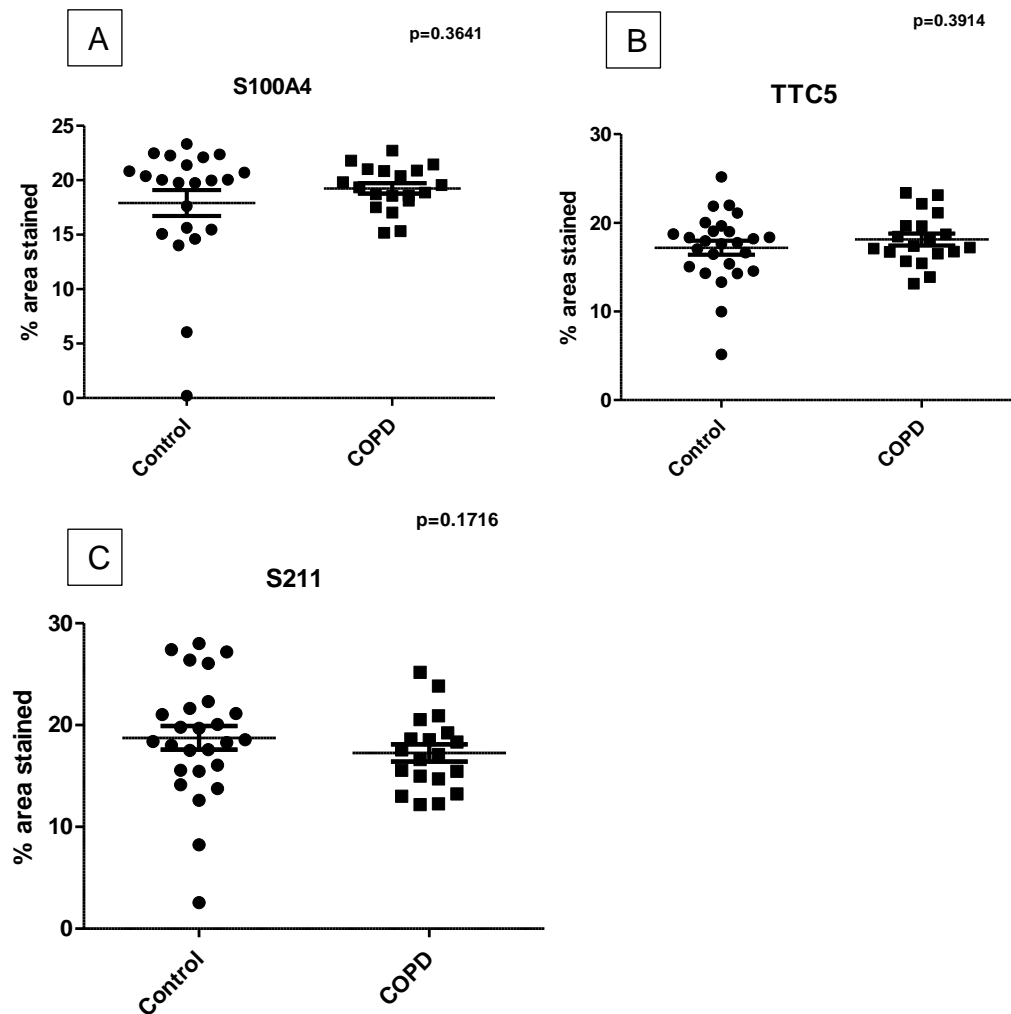


FIGURE 3.10 COMPARISON BETWEEN COPD AND CONTROL SUBJECTS

Comparison of Epithelial Mesenchymal Transition marker S100A4, Glucocorticoid Receptors co-factors TTC5 and phosphorylated Glucocorticoid receptor S211 between COPD and control subjects.

The Mann-Whitney test was performed to determine the total percentage area between Control (Never smokers, Current smokers and Ex-smokers) subjects and COPD current and ex-smoker's subjects.

Figure 3.10 A, the expression of biomarker S100A4 show high level in COPD patients group compared to control subjects with no significance difference ($p=0.3641$).

TTC5 expression levels in Figure 3.10 B show high percentage in COPD patient group compared to control subjects but with no significant difference ($p=0.3914$).

In Figure 3.10 C, the comparison of S211 expression among the control subjects show higher level compared to COPD with no significant difference ($p=0.1716$).

Overall, all three biomarkers (S100A4, TTC5 and S211) in control subjects and patients there is no notable difference in biomarker expression shown by $p>0.05$ for each of the three comparisons. This again supports the evidence that smoking alone raises biomarker expression regardless of lung obstruction and the development of COPD.

3.3.3 BIOMARKER EXPRESSION CORRELATION WITH LUNG FUNCTION

A linear regression was performed for the complete data sets to evaluate the expression of S100A4, TTC5 and S211 in relation to lung function (percentage predicted FEV1, FEV1%) and smoking. Graphs confirm all 3 biomarkers, that expression did not alter significantly due to development of obstruction (reflected by declining lung function performance %predicted FEV1).

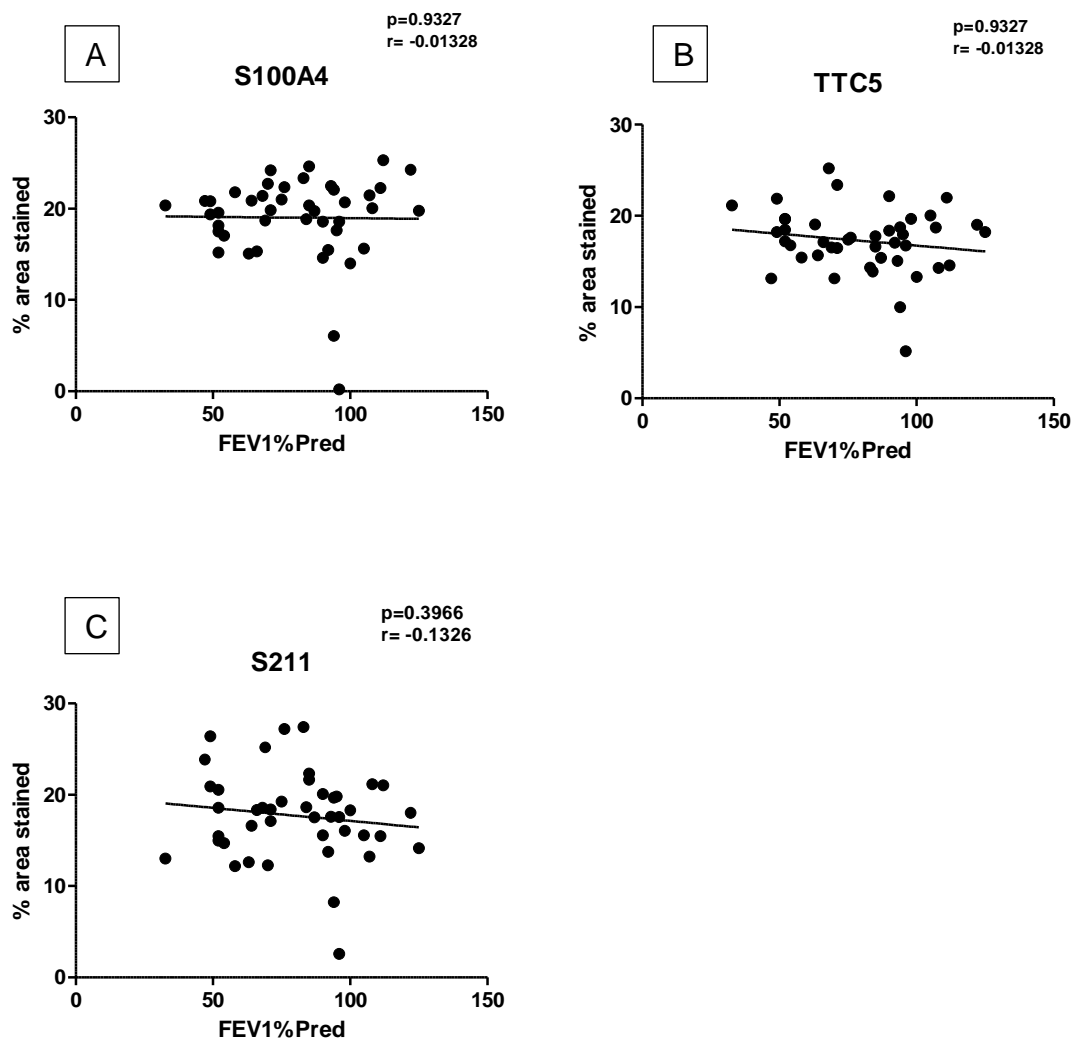


FIGURE 3.11 LINEAR REGRESSIONS OF BIOMARKERS AND LUNG FUNCTION

Linear Regression of lung function FEV1% Predicted and % area stained for Epithelial Mesenchymal Transition marker S100A4, Glucocorticoid receptor co-factor TTC5 and phosphorylated glucocorticoid receptor S211.

3.3.4 PACK YEAR HISTORY

Conversely, analysis of smoking effects shows S100A4 and S211 (significantly) have clear trends of positive correlation; indicating rising expression levels with clinically assessed smoking pack year history. This trend is not evident for TTC5.

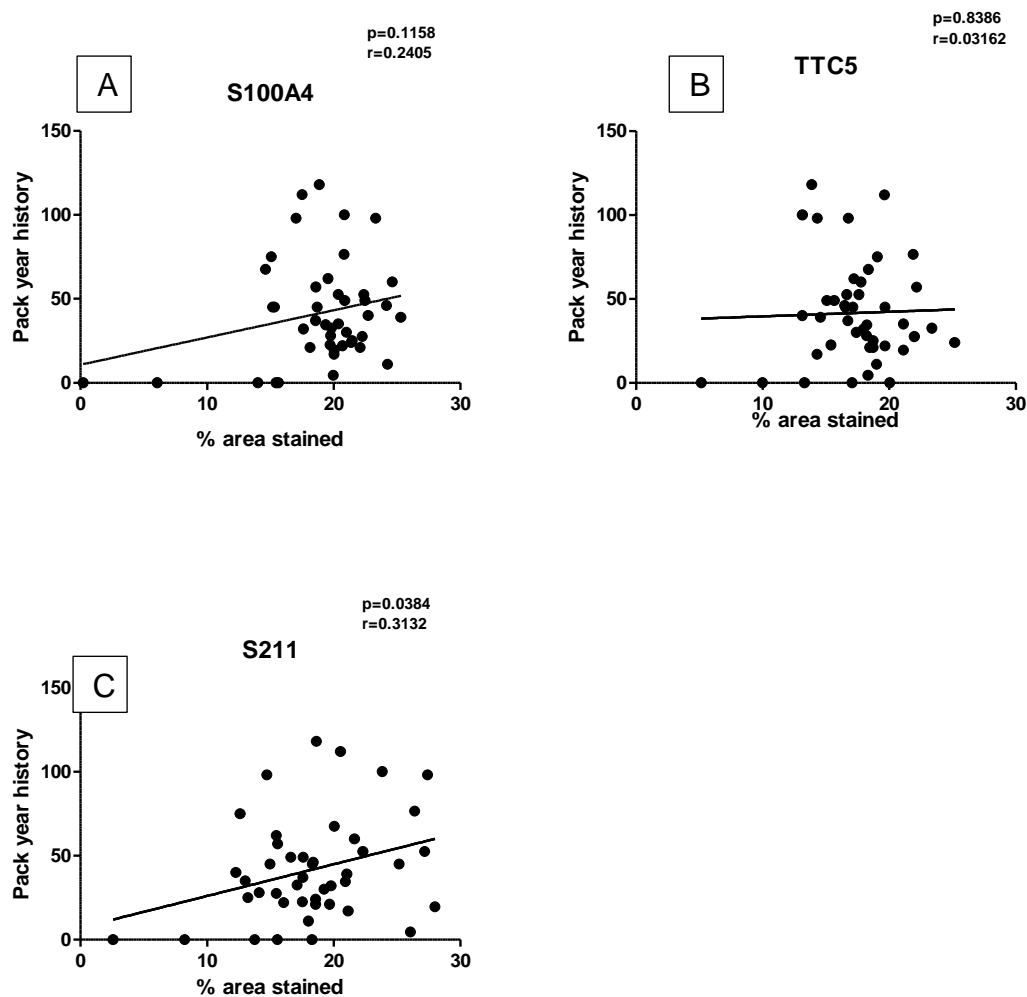


FIGURE 3.12 LINEAR REGRESSIONS OF BIOMARKERS AND PYH

Linear Regression of pack year history and percentage area stained for A) EMT marker S100A4, B) GC receptor co-factor TTC5 and C) Phosphorylated GC receptor S211.

4. DISCUSSION

The investigation and characterisation of fibrosis and glucocorticoid resistance in COPD was carried out with human lungs resections from different patient categories ranging from never smoked to COPD smokers with different biomarker antibodies and was verified by immunohistochemical staining (IHC) and antibody optimisation. Staining and optimization of the epithelial biomarker S100A4 (no pre-treatment required), GC receptor co-factor TTC5 (no pre-treatment required) and phosphorylated GC receptor site expressing biomarker S211 in EDTA buffer pH 8 were effectively conducted with 1: 2500, 1:500 and 1:1000 dilutions respectively . The quantification and statistical analysis of microscopic images was done via GraphPad prism and InStat 3. in 1:2500 in 1:500 in 1:1000

EPITHELIAL MESENCHYMAL BIOMARKER S100A4:

The results obtained for S100A4 from IHC findings and statistical analysis represent airway remodelling in current, COPD current smokers and COPD ex-smokers. Higher expression rises in active smokers and continue to be high in the ex-smokers and COPD smoker's subjects.

S100A4 is a specific marker of both fibroblasts and the EMT that epithelial cells undergo in response to injury (Iwano *et al*, 2002, Okado *et al*, 1997 & Zeisberg *et al*, 2007). The injury induced inflammation mediates EMT which in turn results in fibrosis (Thiery& Sleeman, 2006). From these observations, S100A4 may play a central role in fibrotic pathology.

Our findings show similar aspects to the research work conducted by Sohal and group on airway biopsies of COPD current smokers (n=17), COPD ex-smokers

(n=15), healthy smokers (n=16) and non-smoking control subjects (n=15). A thickened and fragmented epithelium membrane with elongated clefts show positive expressions of mesenchymal biomarkers in cells with S100A4, Vimentin and MMP-9. This represents EMT may be actively present in active smokers and COPD smokers. (Sohal *et al*, 2010).

A study on primary epithelial cells from non-smokers (n=5), smokers (n=12) and COPD smokers (n=15) suggests that EMT is present in small bronchi of smokers and patients with COPD. EMT expressions were raised in human bronchial epithelial cells (HBEC) and cigarette smoke extract (CSE) promotes EMT through the activation of intracellular ROS, the release of TGF- β , the phosphorylation of ERK1/2 and Smad3 as well as by the downregulation of cAMP, which may contribute to the thickening of the wall of the small airways, caused by fibrosis (Milara *et al*, 2013).

Historically, S100A4 has been expressed as fibroblast specific protein-1 (FSP1) marker in kidney fibrosis and it has been verified that TGF- β 1 and epidermal growth factor induces the expression of S100A4 in renal tubular epithelium undergoing EMT *in vivo* (Iwano *et al*, 2002). Similarly, role of S100A4 has been observed in other diseases including liver fibrosis where S100A4 expressing cells increase fibrosis by EMT in hepatocytes (Zeisberg *et al*, 2007). In cardiac hypertrophy (cardiac fibrosis in mice) growth and regeneration of injured heart is regulated by EMT involving S100A4 induced by TGF- β and is reversible by BMP-7 (Inamoto *et al*, 2000, Zeisberg *et al*, 2007 & Storm *et al*, 2004). However, Strutz *et al* did not observe staining of endothelial cells, nor of any cells in the heart and liver of mice (1995). The explanations that account for such observations may be due to species-specific differences in S100A4 mRNA and protein expression or S100A4 may be post-

transcriptionally regulated (Ambartsumian *et al*, 1998 & Davies *et al*, 1995) implying that the expressions of S100A4 mRNA and protein may not correlate.

Mahmood *et al*, 2015 work focuses on small vs large airways of smokers with chronic airflow limitation (CAL), wherein large airways showed characteristics of Type –3 EMT and small airways predominates TYPE-2 EMT and is thought to be especially profibrotic and might be more related to small airway obstruction and obliteration. The relationship seen between the fibrotic marker S100A4 and airflow obstruction would certainly fit with this.

Our research shows a significant raised expression of S100A4 in active smokers (Control subjects) and EMT is evident in smoker's airways but also high levels are found in ex-smokers it's an irreversible process. Cigarette smoke is the highest risk factor for causing COPD disease. Cigarette smoke generates large number of free radicals (ROS) which in turn activates the oxidative stress originated from neutrophils and macrophages (Ya & Rahman, 2011). ROS derives directly from mainstream smoke in gaseous form and activate ERK-% (BMK-1) via C-Src Kinase, which is thought to be an activator kinase of HIF-1 α (Jung *et al*, 2008).

Reimann and colleagues, (2015) shows up-regulation of S100A4 mRNA in microdissected intrapulmonary arteries from explanted end-stage COPD patients and mice exposed to 8 months cigarette smoke. High S100A4 expression was observed in pulmonary arteries and in non-muscularized vessels with a diameter of ~ 50 μ m. These findings may point to a role of S100A4 in vascular remodelling. The end stage COPD patients have frequent occurrence of hypoxemia (Weitzenblum *et al*, 2005), whereas Barbera *et al*, showed vascular remodelling in smokers with normal lung function or mild COPD patients without hypoxia indicating the other

mechanism other than hypoxemia are causative for pulmonary hypertension in COPD and in earlier stages (2013). The murine model in Reimann *et al*, (2015) study does not suffer from hypoxemia. This supports the hypothesis that S100A4 may be involved in early vascular remodelling even in non-hypoxic, mild-COPD stages.

TGF- β is one of the important cell-regulatory networks which drives EMT, particularly its isoform TGF- β 1 (Willis & Brook, 2007, Takizawa *et al*, 2001 & Zhang *et al*, 2009). TGF- β 1 induces EMT in mouse kidneys with a concomitant increase in S100A4 expression that similarly can be reversed by the BMP7 treatment via activation of Smad proteins (Iwano *et al*, 2002). This places S100A4 as a key regulator of the downstream processes in kidney fibrosis and EMT.

TGF- β 1 can induce EMT in human alveolar and bronchial epithelial cells via Smad 2 activation (Kasai *et al*, 2005). TGF- β transmit signals through its serine-threonine kinase receptors which upon activation are internalized into early endosomes where it associates with 'Smad anchor for receptor activation' which modulates the formation of complexes with Smad 2 or Smad 3 (Willis & Brook, 2007). Majority of TGF- β target genes are controlled through Smad-3 dependent transcriptional regulation (Ju *et al*, 2006). Evidence of EMT driver TGF- β 1 in asthma and chronic bronchitis in human lung mucosal biopsies shows correlation between thickness and basement membrane and increased expressions of TGF- β 1 (Vignola *et al*, 1997). The expression of TGF- β 1 mRNA in small airway epithelium from smokers and patients with COPD was significantly higher than in non-smoker controls (Takizawa *et al*, 2001). Soltani *et al*, (2012), showed an increased expression of vessel associated TGF- β in bronchial reticular basement membrane (RBM) in COPD and in normal smokers, but didn't observe rise in epithelium in large airway biopsies. Apart from TGF- β , uPAR signalling pathway may be present in small airway tissue from

COPD (Wang *et al*, 2013) or increased cyclic monophosphate activity in EMT in the airways (Milara *et al*, 2013). There is a great possibility that other signalling pathways may play significant role by participating in progression of COPD disease at different stages.

In COPD, fibrosis and inflammation are involved and these both processes are greatly depending on tissue remodelling and cell motility. Extracellular S100A4 pass signal in paracrine manner via RAGE receptor to promote cell motility (Belot *et al*, 2002) These expression and function correlates with and likely promotes EMT in several diseases (Thiery & Sleeman, 2006 & Lee *et al*, 2006).

RAGE is highly expressed during development, especially in the brain, but its expression level decreases in adult tissues. Neurons, smooth muscle cells, mesangial cells, mononuclear phagocytes, hepatocytes and cardiac myocytes possess low level but in lung tissues high levels were found (Brett *et al*, 1993). The interaction of S100A4 and RAGE was first shown in human articular chondrocytes by immunoprecipitation (Yammani *et al*, 2006). Multi-ligand receptor RAGE interacts with serotonin for S100A4 mediated proliferation and migration of human pulmonary artery smooth muscle cells (Lawrie *et al*, 2005 & Spiekerkoetter *et al*, 2005). Pulmonary S100A4 may play an important role via several mechanisms, messenger RNA (mRNA) and protein levels increase in fibroblasts during administration of bleomycin in pulmonary fibrosis (Lawson *et al*, 2005) and also in bronchial epithelial cells of transplanted lungs (Ward *et al*, 2005). The interaction of extracellular S100A4 and RAGE in colorectal cancer (CRC) cells showed hyperactivation of the MAPK/ERK and the hypoxia signalling pathway (Dahlmann *et al*, 2014).

Upregulation of S100A4 under hypoxia is induced via HIF transcription factors (Kwapiszewska *et al*, 2005). Guo *et al*, 2012 showed that nicotine, a major component of cigarette smoke induces HIF-1 α expression via mitochondrial reactive oxygen species in human small cell lung cancer cells. HIF independent but ROS dependent regulation of S100A4 has been postulated but upregulation of S100A4 in COPD lungs in absence of hypoxia via ROS and HIF have to be determined by further studies.

Reimann and colleagues (2015) highlighted vascular remodelling in COPD with increase in S100A4 expression (FSP1) in vasculature of Human COPD and murine lungs, but there is no clear information how S100A4 account for increase in vascular remodelling. So recently Sohal S, (2016), proposed the process of endothelial to mesenchymal transition (EndMT) may be the possibility in COPD lungs.

Smoking may put patients at risk of development of EMT and because levels are high in ex-smokers, it appears to be a non-reversible process. If the levels had been lower than current smokers, this would have indicated a reversibility of the process and therefore a benefit to quitting smoking. The data in this research appears that there may be no benefit to quitting smoking once the change in biomarker expression is onset. Therefore, this research supports the emphasis on continued better education and preventing of smoking habit development in the population.

GLUCOCORTICOSTEROID RECEPTORS: ROLE OF TTC5 AND S211:

We investigated the role of biomarkers of glucocorticoid steroid receptor expressing cofactor TTC5 and GC receptor phosphorylated site S211. The co-factor and phosphorylation status of GR was analyzed by IHC using the cohort of 45 patients with different categories- (Non-Smoker=5, Ex-Smoker=13, Current Smoker= 8,

COPD Current Smoker=8 and COPD Ex-Smoker= 10) in relation to lung function (FEV1% Predicted), smoking. Our findings of IHC show that both TTC5 and S211 were expressed in all patient groups- peripheral lung tissue, epithelial cell lines and macrophages. Statistically a significant difference was observed among the ex-smokers in TTC5 and S211 compared to other groups.

TOTAL GLUCOCORTICOID RECEPTORS:

Steroid receptors are major class of nuclear receptor representing ligand activated transcription factors, known to regulate many cellular functions, including inflammatory process, energy production and apoptosis (Evans *et al*, 2005). In humans, homeostasis is maintained by a glucocorticoid 'cortisol' in adrenal cortex via hypothalamic-pituitary-adrenal (HPA) axis and are effective anti-inflammatory drugs used to control both acute and chronic inflammatory responses in wide range of diseases (Chrousos, 2000). Glucocorticoids are current pharmacologic treatment for COPD. However, COPD patients responds poorly to inhaled and oral glucocorticoids, even at high doses. The inflammatory response seen in the lungs of COPD patients is glucocorticoid insensitive (Barnes & Adcock, 2009).

Glucocorticoids diffuse across cell membrane and interact with glucocorticoid receptors (GR) inside the cytoplasm and mediate hormone-induced actions. GR is a molecular protein with domain carrying distinct regions (Gigurer, 1986). The two isoforms-hGR α and hGR β are generated on splicing of hGR. GR is localized in alveolar wall, airway epithelium and vascular endothelial cells (Adcock *et al*, 1996 & Pujols *et al*, 2004). GR β resides in the nucleus of cells whereas GR α is in the cytoplasm and translocate to the nucleus upon ligand binding (Oakley *et al*, 1999). GR α is the predominant isoform of the receptor, it has steroid binding activity and

mediates either transactivation or trans repression of target genes (Reichardt, 1998), whereas GR β does not bind to glucocorticoids and is unable to trans activate glucocorticoid responsive genes (Oakley, 1996 & Hechkt *et al*, 1997). Furthermore, when hGR β was transiently transfected together with hGR α , a dominant negative effect was observed on hGR α (Oakley *et al*, 1996) and mineralocorticoid receptor (Bamberger *et al*, 1997), leading to the hypothesis that the cellular ratio of hGR α to hGR β may have profound influence on cells sensitivity to glucocorticoids. Each GR isoform originating from alternative processing of the GR gene is subject to a variety of post translational modifications that further modulate its function and expand glucocorticoid signaling.

Our collaborator in The Salford University Walid and group 2015, investigated effects of dexamethasone on total glucocorticoid receptors (TGR), phosphorylated expressing GR sites S211, S226 and co-factor TTC5. They show upregulated effects of TTC5 with the dexamethasone in A549 lung cell lines and it represents strong interaction with S211 and their findings were consistent to Demonacos *et al*, 2011 of TTC5 can interact with TGR at multiple sites in absence or presence of dexamethasone. Furthermore, this group conducted IHC with patient demographics ranging from non-smoker to COPD subjects and their finding represents TGR, S211 and TTC5 expressed relatively high in both macrophages and epithelial cell lines of control subjects and COPD subjects. But S226 was expressed low in macrophages of control and no expression was found in epithelial cell lines of COPD subjects. Our findings of IHC shows relatively similar results as TTC5 and S211 are both expressed in macrophages and epithelial cell lines. To gain further insight for

glucocorticoid receptors expressing components; co-factor TTC5 and phosphorylated expressing biomarker S211 were chosen to strengthen the data.

TTC5 or STRAP regulates the expression of GR target genes involved in inflammation. It acts as a cofactor for GC and estrogen receptors (Davies *et al*, 2011). TTC5 plays an important role in DNA damage and heat shock factors. It induces the interaction of JMY and p300 and increases the HAT activity of p300. This leads to enhancement of p53 acetylation, stability and transcriptional activity during DNA damage response (Demonacos *et al*, 2001). TTC5 is associated with heat shock factor 1 in a chromatin bound complex which includes p300 and resides on promoter region of HSF gene, required to prevent apoptosis (Xu *et al*, 2008). Prevention of apoptosis is linked with TTC5 in acute myeloid leukemia (AML) cells (Lynch *et al*, 2013).

Corticosteroids bind to the cytoplasmic glucocorticoid receptor (GR); this complex translocate into the nucleus where it suppresses pro-inflammatory gene transcription (trans- repression) or activates anti-inflammatory gene expression (transactivation) (Barnes, 1998). GR phosphorylation is most identified and studied modification (Galliher-Beckley *et al*, 2009 & Beck *et al*, 2009). The structure of the bound glucocorticoid can influence both the pattern and extent of GR α phosphorylation (Avenant *et al*, 2010). A study carried out by Pujols *et al*, 2004 shows that different responses to glucocorticoids in steroid sensitive and steroid insensitive interstitial lung diseases were related to changes in the pattern of expression of GR α and GR β isoforms.

Phosphorylation within the GR N-terminus occurs during GR activation, with serine (S) 211 and 226 phosphorylation associated with GR-ligand nuclear translocation and nuclear export respectively (Galliher-Beckley & Cidlowski, 2009). The ability of S211 phosphorylation to alter cofactor/corepressor activity may also account for changes in gene expression patterns in reducing inflammation. Hence, S211 phosphorylation site was preferred.

Plumb *et al*, (2013), evaluated the GC receptor function in COPD lung macrophages. GR was phosphorylated at S203, S211 in small airway epithelium and cells within the submucosa in lung tissue of COPD patients, smokers and non-smokers but S226 doesn't show any sign phosphorylation. The levels of phosphorylated GR in lung macrophages do not differ between COPD patients and controls on a group mean basis. However, GR activity may be determined by the relative expression of GR isoforms; whereas GR α is important for mediating the anti-inflammatory actions of glucocorticoids, GR β has been shown to inhibit the activity of GR α (Bamberger *et al*, 1995). The levels of GR β protein have been shown to be increased in BAL macrophages of steroid insensitive asthmatics compared to steroid sensitive asthmatics and this corresponds with reduced nuclear translocation of GR α (Goleva *et al*, 2006). It has been shown that the levels of GR β mRNA are increased in the peripheral blood neutrophils of COPD patients compared to healthy subjects (Milara *et al*, 2014).

One of the major effects of GR α phosphorylation is that it changes the transcriptional activity of the receptors. Reports showed that phosphorylation at S211 correlated with increased transcriptional activity of GR α , whereas phosphorylation at S226

decreased the signaling capacity of the receptor (Wang *et al*, 2002 & Chen *et al*, 2008). A deficiency in S211 phosphorylation might contribute to the resistance to glucocorticoid induced apoptosis that develops in malignant lymphoid cells (Miller *et al*, 2005).

The relative lack of response to corticosteroids has been linked to oxidative stress. Cigarette smoking is the major culprit in causing COPD. Cigarette smoke contains more than 2000 xenobiotic compounds and 10^{15} free radicals (ROS) per puff causing damage to airways and lung epithelial cells (Ya & Rahman, 2011). This suggests that oxidative stress may be an important factor in inducing corticosteroid resistance in COPD. Corticosteroids showed less effectiveness in reducing inflammatory cells in BAL & sputum from smoking asthmatic patients compared to non-smokers (Chalmers *et al*, 2002 & Chaudhari *et al*, 2003). The resistance to corticosteroid action is maintained even in subjects who are no longer smoking. This implies that either the oxidant stress is persistent or that the initial chronic insult permanently alters the expression of a component involved in corticosteroid action. Okamoto *et al*, (1999) suggested that oxidative stress may influence corticosteroid function by inhibiting GR nuclear translocation or cigarette smoke can suppress GR function without affecting nuclear translocation (Adcock *et al*, 2005).

In our findings, the expression of TTC5 and S211 didn't alter during the evaluation of all patient groups in relation with forced expiratory volume in 1 second FEV1% predicted. On contrary, S211 shows raised expression level and positive correlation with smoking pack year history, indicating smoking alone is responsible for increased level despite of lung obstruction in COPD disease.

Other proposed mechanisms for reduced corticosteroid function involve nuclear events. Oxidant-mediated enhancement of inflammatory responses and development of relative glucocorticoid insensitivity may be facilitated by alteration of acetylation-deacetylation balance of core histones (Rahman *et al*, 2004). Gene transcription is controlled by histone acetyltransferases (HAT) and histone deacetylases (HDACs) are important for corticosteroid mediated suppression of NF- κ B activity. Oxidative stress leads to reduction of HDAC2 expressions in *in vivo* and *in vitro* (Ito *et al*, 2001 & Marwick *et al*, 2004). The expression and activity of HDAC2 is decreased in BAL macrophages and biopsy specimens from smokers and correlates with COPD (Barnes *et al*, 2004). These decrease in HDAC2 activity correlates with increased inflammatory gene expression and reduced responsiveness to corticosteroids (Ito *et al*, 2001).

The activation of signaling kinases is a fundamental part of physiologically controlled inflammation. COPD patients display altered kinase signaling with an elevation in the activity of p38 MAPK and JNK compared to healthy smokers (Renda *et al* 2008 & Rumara *et al*, 2008). The inflammatory gene expression in patients with reduced corticosteroid sensitivity may be associated with increased phosphorylation of GR at serine 226 by p38 MAPK, which results in reduced nuclear translocation and failure of GR to inhibit gene transcription (Mercado *et al*, 2012).

5. CONCLUSION AND FUTURE PERSPECTIVES

Chronic obstructive pulmonary disease is characterized by airflow obstruction that is progressive and not fully reversible and associated with chronic airway inflammation. Smoking is considered to be the crucial player in COPD. It contributes to the underlying structural changes and airway remodeling. To our knowledge the process applicable for remodeling of airways involves the differentiation of epithelial into mesenchymal cells (EMT). During injury or wound healing, myofibroblast cells are activated via inflammation. This can cause fibrosis and destruction of alveoli. The tissue remodeling lead to airflow obstruction and thickening of small airways with infiltration of inflammatory cells. However, the source of fibroblasts is unknown. It is assumed that they arise from trans-differentiation of residential pulmonary fibroblasts. They can be an effector cell in fibrotic respiratory disease like COPD.

I have contributed to the FIR group at The Salford University and shown the EMT biomarkers are linked to COPD. They observed S100A4 was highly expressed in COPD tissues of active smokers and COPD subjects and linked to smoking in disease progression. Our study is of course limited by the fact that it was carried out in living human subjects underwent surgery. Further study with larger group may get require to broaden our results of EMT in COPD. However, my research presented in this thesis strengthens the previous data from the FIR group and will contribute to a publication to be submitted to CHEST journal soon.

Our findings have shown the raised expression of S100A4 in active smokers. Smoking can put patient at a risk of development of EMT, which indicates, EMT

process may contribute a potential role in fibrosis of COPD by being partly reversible. Researches have shown a quite strong evidence that airway epithelium can undergo EMT of human airways in smokers and would explain the underlying pathological processes involved in COPD and its accompanying risks. The functional effect of S100A4 in cell motility plays a general role in disease progression by tissue remodeling of both tumor and non-tumor related tissues of normal cells in kidney, liver and heart. However, it is not clear whether the processes that S100A4 induces are similar in lung fibrosis. Several pieces of data have prompted speculations on whether S100A4 could be used as a prognostic marker for fibrotic diseases like it is used for cancer. As some evidence show S100A4 is involved in both tissue fibrosis and metastasis. Though S100A4 plays a major role in EMT, additional research is required to understand the precise molecular interactions that lead specifically to EMT in the airways in response to cigarette smoke. To understand this molecular mechanism, EMT can be observed in lung biopsies via profound histochemical analysis, laser capture dissection microscopy or by bronchoscopy.

Glucocorticoids act through the GR to regulate numerous physiologic processes, and synthetic derivatives of these hormones are widely prescribed for treating inflammatory diseases. The discovery of multiple isoforms of GR from single gene has advanced our understanding in molecular mechanism in glucocorticoid signaling. Though, Glucocorticoid resistance remains a major barrier to effective treatment of variety of inflammatory diseases including COPD.

A collaborative group of The Salford University has been working on different glucocorticoid receptors and GC receptor variants expressing in lungs. The research

conducted by this group on COPD patients and control subjects observed the effect of dexamethasone (dex) on TGR, phosphorylated GR at S211 and S226 and on stress co-factor TTC5 suggesting that protein levels of both phosphorylated forms of GC receptors is upregulated in the presence of dex hormone and observed interaction between TTC5 and S211 suggesting transcriptional activity may be present. Hypothesis being made that differential expression of GR may exist in COPD and control subjects. We also conducted a study on glucocorticoid receptor expressing two different variants- Cofactor TTC5 and phosphorylated GC receptor S211 in COPD patients Vs control subjects. Our results showed TTC5 and S211 expression were raised in Ex-Smokers and current smoker but low-level expressions were observed in non-smokers. This irreversible change implicates the mechanism of steroid resistance in COPD patients. Not all patients with COPD who smoke are insensitive to GC for some reason, so some drug will show effectiveness in some groups. Therefore rapid test to differentiate the mechanism of defective cells from this patient can be useful as a measure for selective therapy.

Recent investigation on GR provides a novel insight to understand mechanisms of GC resistance and increasing knowledge of molecular mechanism by which patient loses responsiveness to GCs opens up the possibility of defined patient specific therapy. Anti-inflammatory therapies, restoration of GC function by theophylline are important therapeutic targets for respiratory diseases like COPD. The isoforms of GC receptors may be involved differentially in signaling mechanisms. There can be tremendous potential for development of synthetic ligands that activate anti-inflammatory mechanisms but do not affect other pathways.

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