Glucocorticoid resistance in COPD patients and lung cancer

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List of content

List of figures

List of tables

List of abbreviations

ACTH	Adrenocorticotropic hormone
AP-1	activator protein 1
BMI	Body Mass Index
CCR	C-C chemokine receptor
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CT scan	Computed tomography
DBD	DNA-binding domain
DLCO	Carbon monoxide diffusion capacity
EGFR	Epidermal growth factor receptor
ERV	Expiratory reserve volume
FEF25-75%	Maximum mid-expiratory flow
FEV1	Forced expiratory volume at 1 second
FRC	Functional residual capacity
FVC	Forced vital capacity
GR	Glucocorticoid receptors
GRE	Glucocorticoid response element
GR-α	Glucocorticoid receptor-alpha
GR-β	Glucocorticoid receptor-beta
HDAC2	Histone deacetylace-2
HDL	High density lipoprotein
HPA	Hypothalamic–pituitary–adrenal axis
HSP	Heat shock protein
IFN-ϒ	Interferon gamma
IgG	Immunoglobulin-G
IL-10	Interleukin-10
IL-12	Interleukin-12
IL13	Interleukin 13
IL-1β	Interleukin one beta
IL-4	Interleukin 4
IL-6	Interleukin-6
IRV	Inspiratory reserve volume
JNK	c-Jun N-terminal kinases
LBD	Ligand Binding Domain
LTOT	Long term oxygen therapy
MAPKs	Mitogen activated protein kinases
MMPs	Matrix metalloproteinase
NE	Neutrophil elastase
NF-KB	Nuclear factor kappa-light-chain
NHS	National Health Service
Nrf2	Nuclear factor erythroid 2–related factor 2
NSAID	Non-steroidal anti-inflammatory
NSCLC	Non-small cell lung cancer
SCLC	Small cell lung cancer

P53	Tumour protein or phosphoprotein 53
PCO2	Partial pressure of carbon dioxide
PO2	Partial pressure of oxygen
R.V	Residual volume
RNS	Reactive Nitrogen species
ROS	Reactive Oxygen species
S211	Phosphorylated GR at Serin211
S226	Phosphorylated GR at Serin226
T.V	Tidal volume
TGF-β	Tumour growth factor beta
Th-1	T-helper cells
Thr8	T- helper cells
TLC	Total lung capacity
TNF	Tumour necrotizing factor
TNF-α	Tumour necrotizing factor alpha
TPR	Tetratricopeptide repeat
TTC5	Tetratricopeptide repeat domain 5
V.C	Vital capacity
VEGF	Vascular endothelial growth factor

Declaration

No part of this thesis has been submitted in support of an application for another degree of Salford University or other institute of learning.

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Abstract

COPD is linked to inflammatory mediators that orchestrate the disease progression, mainly in the small airways and in parenchymal tissue. Inflammatory mediators, such as cytokines, chemokines and oxygen free radicals, orchestrate the inflammatory response, causing disruption of the epithelial surface and the surrounding microenvironment in the lung tissue. Glucocorticoids are the main treatment for many inflammatory diseases, however, the majority of COPD patients are resistant to glucocorticoids, so a better understanding of the exact mechanism of glucocorticoid resistance in COPD patients and lung cancer is necessary in order to improve the effectiveness of steroids. Glucocorticoids act through glucocorticoid receptors that are located in the cytoplasm. GRs are phosphorylated, and this post-translational modification affects their binding and interaction with other proteins. In this thesis the expression of TTC-5, a stress responsive co-factor, and the total and the phosphorylated GR in the peripheral lung tissue of COPD patients were examined and compared with the expression in healthy patients. Results indicate that total GR and GR phosphorylated at S211 and S226 are expressed in A549 lung cancer cells as well as TTC5. Preliminary results also suggest that TTC5 and GR interact in these cells. Furthermore, as determined by immunohistochemistry, the total GR, GR phosphorylated on S211 and S226 as well as TTC5 are expressed in human lung tissues and in associated macrophages. Finally, we detected altered TTC5 expression that correlates with disease status. These results may contribute to our understanding of the underlying mechanisms of glucocorticoid resistance in COPD and in lung cancer.

1. **Introduction**

Chronic obstructive pulmonary disease (COPD) is a considerable health problem all over the world. It is the fourth leading cause of death and expected to be the third by 2020 (Petty, 2003). Moreover, 40-70% of patients with COPD, especially in the late stages, develop lung cancer (Metcalf *et al*, 2009).

In the United Kingdom, COPD and lung cancer are common debilitating diseases and a major public heath challenge. The NHS (National Health Service) spends about £800 million annually treating COPD. Moreover the cost of management of lung cancer is about £10,000 per patient (Health news 2014, Oxford University).

1.1 Lung physiology

1.1.1 Lung volumes and capacities

Physiologically the lung is the place where respiration takes place and the function of the lungs is determined by volumes and capacities. Lung volumes reflect the amount of air that can be inhaled during different stages of the respiratory cycle. Several important definitions can be measured to estimate lung function as listed below and shown in figure 1. Tidal volume (TV) is the amount of air entering and exhaled during resting. Total lung capacity (TLC) is the amount of gases contained inside the lung at the end of maximum inspiration and it is the vital capacity plus residual volume (the amount of air that remains after full expiration). Vital capacity (V.C) is the amount of air that is expelled after deep inspiration. Residual volume (R.V) is the amount of air inside the lung after forced complete expiration. Forced vital capacity (FVC) is the amount of air that can be exhaled following full inspiration. Forced expiratory flow (FEF 25%-75%), measures the airflow at mid-point of expiratory phase. Functional residual capacity (FRC) is the volume of air left inside the lung after normal expiratory phase. Inspiratory reserve volume (IRV) is the volume of air that can be inhaled beyond normal inspiratory breathing. Expiratory reserve volume (ERV) is the air that can be exhaled beyond the normal expiratory phase. In addition, there are some terms which they are important in the alveolar ventilation-perfusion processes, such as physiological dead space which is part of tidal volume which is not involved in the O2-CO2 exchange process. Anatomical dead space is the amount of air inside the airways which does not

participate in the ventilation process and alveolar dead space, that represents the difference between anatomical dead space and physiological dead space, and this amount of air does not participate in gas exchange. Figure 1 illustrates the important values in the lung (Becker *et al*, 2009).

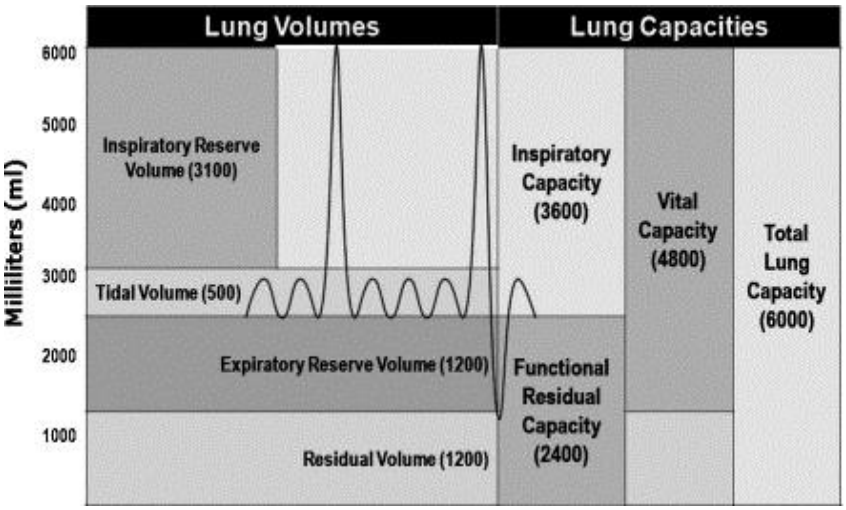


Figure1- Lung volumes and capacities. Lung volumes include (TV, IRV, ERV and Residual volume) and derived lung capacities including FRC, IC, VC and total lung capacity (Becker *et al*, 2009).

In normal individuals the peak of lung performance is achieved by age of 20 and 25 years among females and males respectively, followed by a plateau phase until the age of 35. Lung performance then gradually starts to decrease. This is shown in figure 2 (Fletcher and Petos's, 1977).

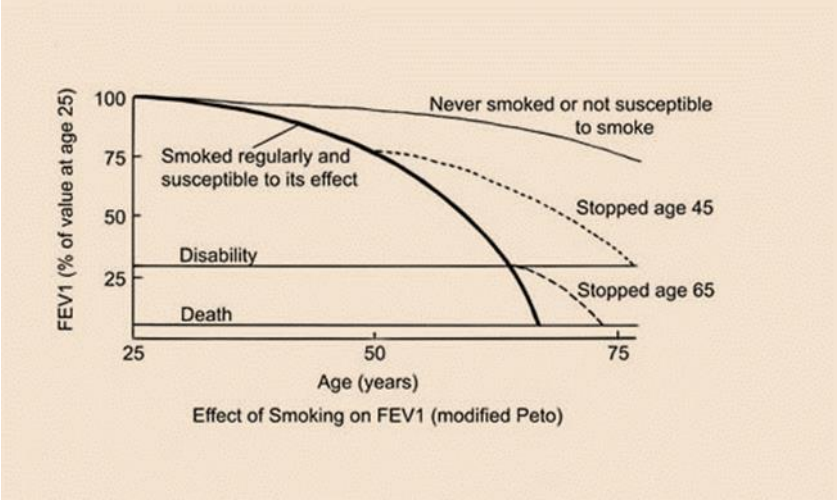


Figure 1-2. Age related decline in FEV1 in healthy individuals and COPD. FEV1 decline in normal healthy individuals (first line) is slow and usually in normal people the rate of decline is unnoticeable even after age of 75. In regular smokers (Second line), the FEV1 decreases more rapidly and the rate of decline is directly proportionate to the comorbidity and the death rate at the age of 65 and 75 years respectively (Löfdahl et al, 1998). Modified Fletcher and Petos's graph, (1977).

A part of respiration is explained by the lung function, which is subdivided into three main categories based on spirometry, lung volumes dependant flow rates, which include forced vital capacity (FVC), forced expiratory volume in one second (FEV1) and the ratio of FEV1/FVC. The second part of lung function cannot be determined by spirometry. It is known as static lung volumes comprise total lung capacity (TLC), vital capacity (VC), residual volume (RV), and functional residual capacity. Finally, the ability of blood gases to cross the alveolar blood barrier is measured by carbon monoxide diffusion capacity (CLCO).

1.1.2 Factors that may affect lung volumes

There are three factors affecting lung volumes and capacities. The first is height, because tall individuals have larger lungs, thus larger volumes and capacity. The second is body mass index. Obesity adversely affects lung volumes and capacity. The third is altitude. People who live at high altitude are more able to compensate for the low oxygen level by increased lung capacity.

1.1.3 Physiology of gas transport

In dry air oxygen accounts for approximately 21% of air, and the Partial pressure of oxygen is about 160mmHg of total air pressure (760mmgh). Physiologically, the partial pressure of oxygen and carbon monoxide is the main determinant of the oxygenation process, in which PO2 and PCO2 inside the alveoli is about 100mm and 40mm Hg respectively. The opposite is the case in capillary blood vessels (figure 1-3). In addition, the amount of oxygen across the alveolar membrane into the capillary blood vessels is directly proportionate to the alveolar surface area and reciprocal to membrane thickness and determined by carbon monoxide diffusion capacity (DLCO) (Stam*et et al,* 1994).

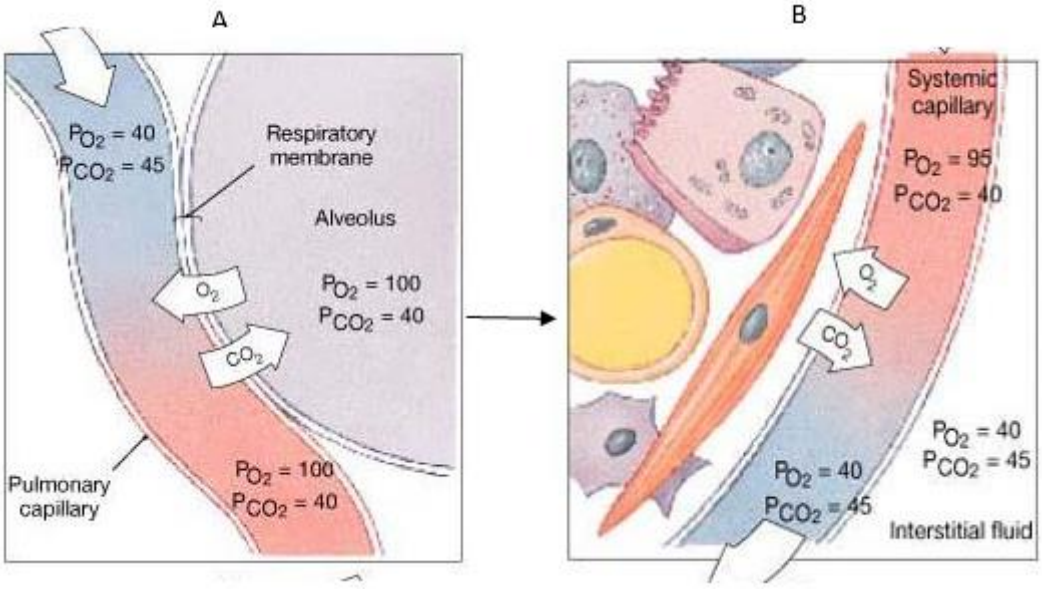


Figure 1-3 Gas exchange and partial pressure in the ventilation process. (A) O2-CO2 exchange across the respiratory barrier showing pressure difference between the alveoli and the capillary blood vessels. (B) Higher partial pressure of oxygen (PO2) in the systemic capillary blood vessels facilitates its delivery into different parts of the body (Stam*et et al,* 1994).

1.2 Defence system in the lung

There are various environmental pollutants that may have a detrimental effect on lung function, such as carbon monoxide, which has a higher affinity to haemoglobin (210 times higher than oxygen) (Ganong *et al,* 1995). However, the clearance system in the upper and lower respiratory zones are effective in eliminating the majority of deposited particles.

Two important mechanisms are well known as a first line of defence: the mucociliary system in the airways and alveolar macrophages in the air passages.

1.2.1 Mucociliary System

The mucociliary system is an important mechanism for protection from harmful organisms via ciliary movement and the mucus layer that is produced by Goblet cells (glandular simple columnar cells located in epithelial layer), and the mucus gland located in deep epidermis (Morgan *et al*, 1986).

There are a number of related congenital diseases associated with abnormality in ciliary structure and function causing an impaired elimination of noxious particles and subsequently leading to chronic respiratory disorders such as primary ciliary dyskinesia (autosomal recessive genetic disorder) also known as immotile cilia syndrome characterized by malfunctioning cilia and chronic infections. In addition, patients with cystic fibrosis and Young's syndrome may develop immotile cilia syndrome (Afzelius *et al*, 1981).

1.2.2 Lung macrophages

Lung macrophages are the most important line of defence in the lower respiratory zone, and are considered part of the mononuclear phagocyte system and one of the front line defences in the lung. They develop as a result of migration of circulatory monocytes into the lungs (Cohn *et al*, 1968). Nevertheless, the monocytes do not enter the alveoli directly, they divide and gain bio-inflammatory characteristics then migrate to the alveoli (Bowden *et al*, 1976). Macrophages play a pivotal role in both innate and acquired immunity, either by direct phagocytosis of foreign antigens or by initiation of an inflammatory cascade by releasing a number of pro-inflammatory and anti-inflammatory cytokines and chemokines. In some diseases like COPD, the phagocytic ability is disturbed by smoking, in addition it is suggested to play a role in tumour development (Mantovani *et al*, 2008). Four types of macrophages have been identified according to the site; alveolar macrophages, interstitial macrophages, intravascular and dendritic cells. Alveolar macrophages are the predominant type of this group and increase in number with the severity of COPD (Barnes *et al*, 2008). Because

macrophages are exposed to the environment, they have developed the ability to recognise and phagocyte the foreign particles, which may end by over-production of inflammatory cytokines and tissue remodelling factors triggering the inflammatory process and tissue repair. This type of cell is associated with tissue destruction in COPD through the release of inflammatory mediators including proteases such as methalloproteinases-1.The extent and severity of alveolar destruction (emphysema) is related to the number of macrophages in COPD patients (Tetley *et al*, 2005), and the macrophages are accumulated in areas where alveolar destruction exists (Barnes *et al*, 2004). Macrophages, isolated from COPD smokers, exhibit less phagocytic activity against some bacteria such as Haemophilus influenza.

Activated macrophages are also sub-divided into two groups, according to secretory mediator and their roles. The first type, classical activated macrophage or pro-inflammatory macrophages (M1), possesses an anti-inflammatory and cytotoxic character that enable them to eliminate intra-cellular pathogens especially bacteria by producing a large quantity of lymphokines. The second type, which are capable of presenting the antigens and producing anti-inflammatory

Mediators related to M2	Toxic particles to the lung
Reactive oxygen species (ROS) and Reactive nitrogen species (RNS)	Ozone, Radiation, Bleomycin and Cilica
Proteases	Bleomycin, Endotoxins and Sulphur mustard
Bioactive Lipids and Pro-inflammatory mediators(TNF-α, IL-1β and chemokines)	Ozone, silica and Sulphur mustard
Mediators related to M2	
IL-10	Ozone, Silica and Endotoxins
IL-4	Radiation, Silica, Endotoxin and hyperoxia.
IL-13	Endotoxin, hyperoxia and Asbestos.
TGFβ	Bleamycin, Radiation and Asbestos.

Table 1.1 illustrates number of mediators released by M1 and M2 macrophages in response to variable environmental and toxic particles. (Laskin *et al*, 2011).

Mediators are known as activated type (M2) and show anti-inflammatory properties, and hence are involved in tissue repair and development of T-regulatory cells. However some inflammatory mediators like IL-10 are poorly presented (Martines and Gordon, 2009). Classical type (M1) is responsible for pro-inflammatory cytokines release (IL-12 and TNF-α) and in addition enhances Th-1 immunity (Hoeve *et al*, 2005).

1.2.3 Epithelial surface

During embryogenesis the epithelial surface is derived from the endoderm tubule (Morrisey *et al*, 2010). The airways in the human lung are supported by cartilage that extends to the small airways which are lined by the ciliated pseudo-stratified columnar epithelium and surrounded by mucus-secreting glands and blood vessels. Beneath the epithelial surface, basal cells are responsible for renewal of injured epithelial and other secretory cells (Hong *et al*, 2004). Airway epithelial cells are further sub-classified into two groups, ciliated epithelial mediates dispose of the foreign particles; mucus cells, which are responsible for mucus production as well as cellular differentiation and the production of inflammatory mediators, mediate inflammatory response (Puchelle *et al*, 2006). There are many toxic particles inhaled mainly from cigarette smoking that result in alteration of epithelial cells. One of these disruptions entails epithelial cells being replaced by squamous cells, resulting in reduction of ciliated cells as well as hyperplasia of mucus secreting cells. These changes are associated with loss of surfactant producing cells (Clara cells). Additional new studies showed an inverse correlation between NF-KB expression and impaired defective mechanism in epithelial cells. Moreover, direct contact of epithelial cells with toxic materials increases airway the epithelial permeability (figure 1.4) by down regulation of the junctional barrier (Heijin *et al*, 2012).

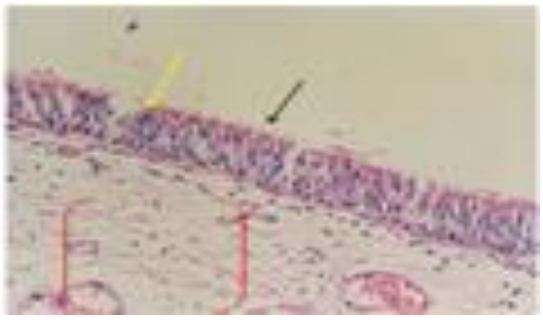


Figure 1.4. Normal epithelium histological types and significance of bronchial epithelial dysplasia. Healthy epithelial surface (black arrow) shows small tiny cilia and epithelial disruption (yellow arrow), sub-mucosal layer with small blood vessels (Heijin *et al*, 2012).

Healthy airway epithelia are an essential requirement for the integrity and proper function of the respiratory tree. Recurrent exposure to irritants may have a disruptive effect on the epithelial surface, which is participating in remodelling during its recovery (Grainage *et al*, 2013). Moreover, an intact epithelial surface is an important functional and structural barrier against inhaled particles. Frequent contact with irritants and toxic compounds such as cigarette smoke may initiate an inflammatory cascade, causing disruption in epithelial integrity, followed by a repair process via epithelial cells leading to the proliferation and regeneration of pseudo-stratified cells. Remodelling can result at any step during the repair process, including squamous metaplasia, smooth muscle hypertrophy, sub-mucosal and goblet cell hypertrophy and fibrosis.

There are many triggering factors involved in the disruption of the epithelial surface, including bacterial or viral infection, allergic reaction (asthma, cigarette smoke) and trauma. During the repair process, the reactive inflammation to antigens is the same, however, long term response to the irritants varies in different parts of the lung, according to the architecture, level of oxygen, abundance of surfactant and blood supply of the area. For example a disruption to alveolar capillary blood vessels leads to irregularity in the basement membrane and induces fibrosis by activation of fibroblasts. The role of the epithelial surface is also important in the pathogenesis of COPD and dysfunction of this barrier could cause bacterial colonization and infection in COPD patients.

According to the shape and function of epithelial cells in the alveolar region, they are classified into, type I squamous cells, which represent about 90% of the total number of

the cells, and type II cuboidal cells, which are responsible for production of surfactant, which is responsible for decreased surface tension during the respiratory cycle.

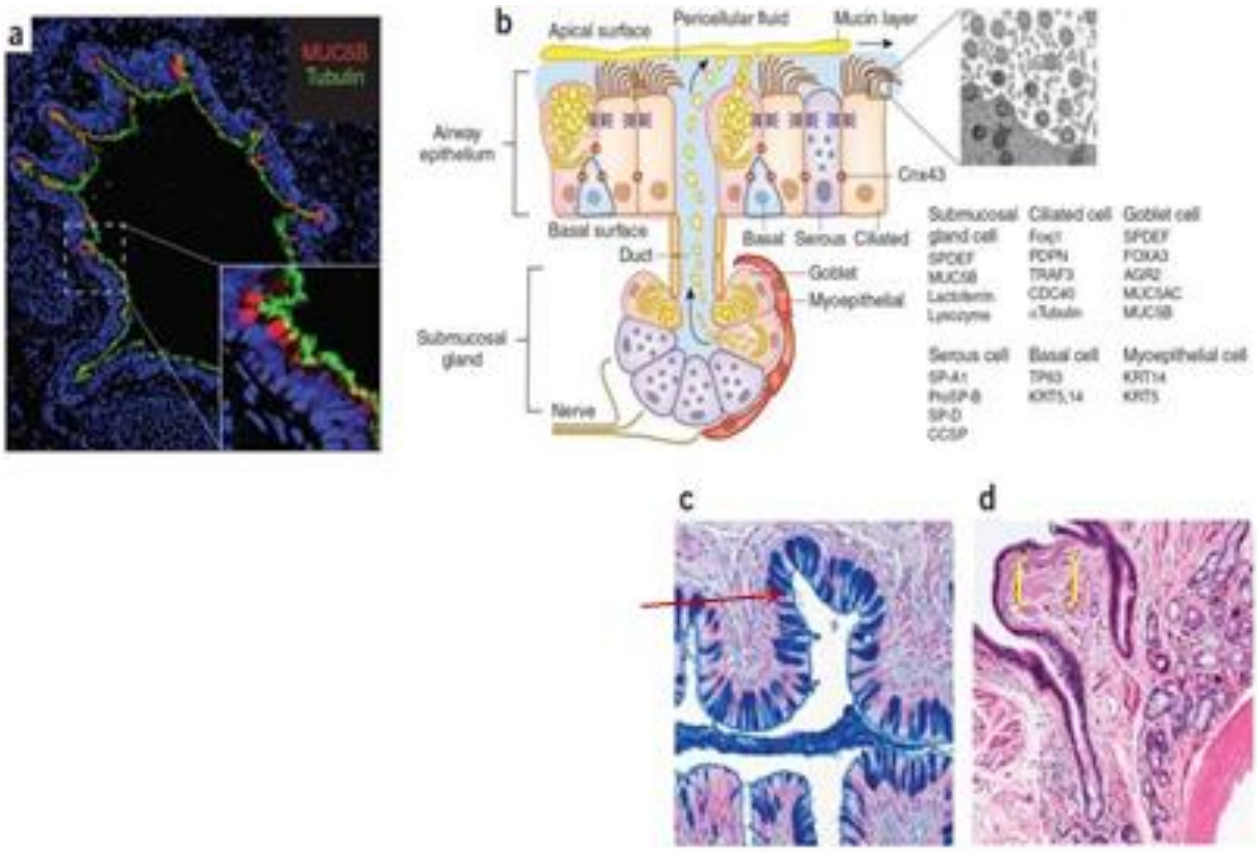


Figure 1.5 Gas exchange function of the alveolar region in the terminal respiratory unit. (a) Lining epithelial surface (b) illustrates gas exchange through alveolar lining cells and mucus production from sub-mucosal glands, in addition to the formation of the mucin layer (yellow line). (C) and (d) respectively represent the pseudo-stratified epithelial surface (red arrow) and the underlying mucosal structure (yellow bracket) http://www.nature.com/reprints/index.html

1.3.4 Dendritic cells

Dendritic cells are a group of mononuclear cells, which have the ability to identify and uptake pathogens (Henderson *et al,* 1997). They are found close to the epithelial lining surface and possess cytoplasmic branches to interact with the environment and to facilitate their interaction with PMN and to induce an acquired immune response (Iwasaki *et al,* 2007). Dendritic cells originally existed in bone marrow and circulate throughout the body in immature form. Dendritic cells present antigens to lymphocytes

in regional lymph nodes. There are 100 times fewer dendritic cells in the alveoli and small airways of COPD patients than in healthy individuals. Moreover, this type of cell returns to normal as soon as patients stop smoking (Rogers *et al*, 2008).

CD-8 T lymphocytes

CD8-T lymphocytes also have an essential role in the adaptive immune response to viral infections, and especially in respiratory syncytial viral infection and in disease progression. CD8-T- lymphocytes have been detected in both large and peripheral airways during inflammatory response (Kim *et al*, 2008). Compared with heathy smokers, the number of CD8-T lymphocytes are significantly increased, mainly in the wall of the small airways, and the number of CD8-T lymphocytes are increased with progression of the disease (Saetta *et al*, 1998). T -lymphocytes are antigen-presenting cells. They are activated by determining antigens. In COPD non-activated cells return to the circulation. In addition to the involvement in the inflammatory process in the release of inflammatory cytokines, the CD8 T-lymphocytes are related to disruption of the micro-vascular environment as a consequence of chemokines such as IL-8 and monocyte chemotactic protein-1, produced by T-cells (Abbas *et al*, 2000).

1.2.6 Natural killer cells

This type of cells is related to innate immunity. Natural killer cells act in a non-specific manner and secrete variable inflammatory mediators such as IFN-ϒ, TNF- α and IL12, which are involved in activation of immune cells to kill viruses. However, these cells are less active in smokers than healthy individuals (Ferson *et al*, 1979)

1.2.7 Neutrophils

Unlike alveolar macrophages and dendritic cells, neutrophils are part of innate immunity and account for 3 to 19% of all cells. Neutrophils flow into inflammatory or injured places to destroy pathogenic microbes in response to inflammatory signals from epithelial cells or regional macrophages. Neutrophils produce a variety of enzymes, the nature of whose effect is controversial. For example neutrophil elastase may cause

alveolar destruction if not controlled properly. Neutrophils are observed in the epithelial layer and the number of the neutrophils is directly related to restriction in the airways and to the severity of the disease (Chua *et al*, 2006). In addition, Neutrophils isolated from COPD patients respond less vigorously than normal to chemoattractants (Beeh *et al*, 2003). The speed of travel toward the presenting antigens is also affected by the disease, moreover the chemotactic property of neutrophils isolated from COPD patients is less responsive than normal to chemo-attractants. The number of neutrophils is nearly the same in both healthy smokers and smokers with COPD, however the phagocytic ability of the neutrophils is decreased in COPD patients.

1.3 Alveoli

Alveoli are where gas exchange takes place. The alveolar sacs are lined with two types of cells, type I pneumocytes represent the majority and are responsible mainly for gas exchange, while type II account for approximately 5% of the cells and are more resistant to damage and are mainly responsible for surfactant production that reduces surface tension. They also have the ability to repair injured type I pneumocytes. In addition, these cells are responsible for xenobiotic metabolism and facilitate trans-epithelial movement of water. Type II pneumocytes are implicated in pathogenesis of chronic obstructive pulmonary disease via production of inflammatory cytokines and are responsible for fibroblast inhibition during pathogenesis in some diseases (Kotton *et al*, 2014).

Surfactant is a mixture of phospholipids and proteins, surfactant synthesis and production can be affected by genetic disorders. Cigarette smoking may alter surfactant synthesis by increasing the neutrophil elastase activity, MMPs and proteolytic enzymes (Hogg *et al*, 2004).

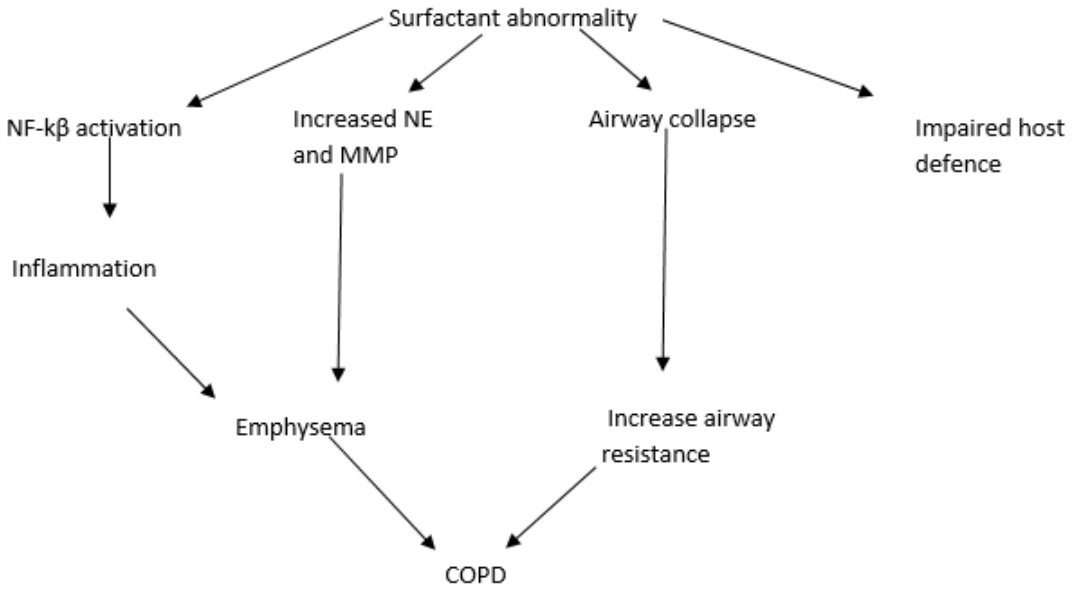


Figure 1.6. Involvement of surfactant dysfunction in pathogenesis of COPD. Impaired surfactant results in impaired immunity and airway collapse, causing more resistance in the small airways. On the other hand, surfactant causes an increase in neutrophil elastase and in metalloproteinases. Moreover, surfactant may activate NF-KB, which in turn induces inflammation. Together with increased resistance, the airway inflammation ends in COPD (Hogg, *et al* 2004).

In chronic obstructive pulmonary disease, the surfactant system is impaired due to frequent contact with noxious particles from cigarette smoking, resulting in a reduction of the total amount of the surfactant (figure 1.7) (Barnes *et al*, 2009).

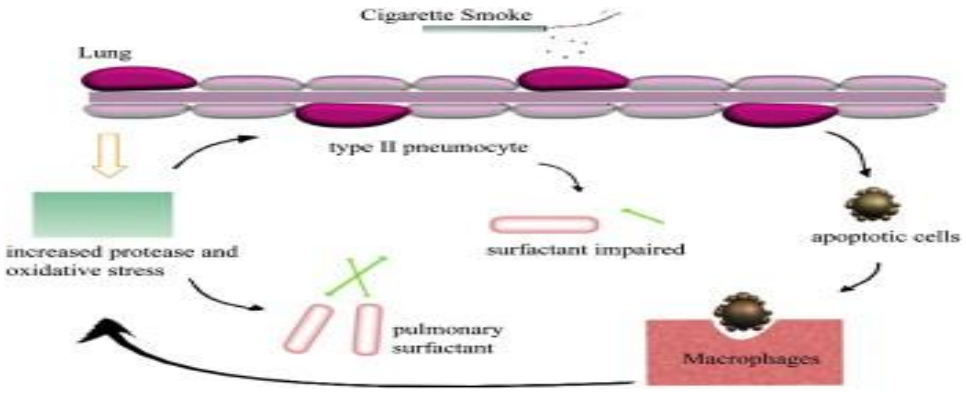


Figure 1.7 Cigarette smoke and noxious particles increase apoptosis and injury of alveolar cells type II. The direct effect of toxic materials from cigarette smoke on type II alveolar cells, leading to activation of macrophages, which subsequently enhance phagocytosis. Defective surfactant function may increase the activity of proteases and oxidative stress (Zhao *et al*, 2010).

1.4 Chronic inflammation

Inflammation is the protective mechanism against injury. Acute inflammatory response is initiated by recruitment of leukocytes from the circulatory system, starting by leakage of polymorph nuclear cells, followed by monocytes, which transform into macrophages (Majno, 1975). Chronic inflammation is generally characterized by tissue infiltration with mono-nuclear cells, lymphocytes, and plasma cells and sometimes is associated with tissue destruction as a result of the direct effect of chemicals or from inflammatory mediators that are secreted from the recruited cells. At the same time, as a part of chronic inflammation, a countervailing process of repair is usually mediated by angiogenesis and fibrosis, whatever the end-result of repair: restoration of normal architecture or permanent fibrosis (Rennard *et al*, 1999).

1.4.1 Classification of inflammatory lung diseases

Inflammatory lung diseases are classified according to the site of inflammation. Firstly, airway diseases mainly affect the passages of the air like, for example, asthma (a disease of large airways), bronchitis and bronchiectasis (permanent destruction of air spaces distal to terminal bronchioles). Secondly, parenchyma diseases usually affect the architecture of the lung, leaving either permanent destruction or fibrosis (emphysema, interstitial lung disease). Additionally, some inflammatory diseases affect blood vessels either by blood clotting or by scarring.

1.5 COPD

Term COPD is an international abbreviation for chronic obstructive pulmonary disease. It is classified as a systemic disease because of the extra-pulmonary involvement of the heart and skeletal muscles (MacNee, 2013). The most frequent leading causes of COPD are tobacco smoking, genetic inheritance and the environment. It is the 4th most common cause of mortality and morbidity. It is estimated to affect 20-30% of smokers and has a prevalence of 10% of the population as a whole (Afonso *et al*, 2011). The course of the disease is usually progressive, and many factors determine the disease severity including FEV-1, inspiratory capacity, DL-CO, hypoxaemia, impaired exercise capacity, BMI (body mass index), hypercapnia, dyspnoea and health status. Death

among patients with mild COPD is usually attributed to lung cancer and cardio-vascular diseases, however, in the advanced stages, the causes of mortality are non-malignant in origin (Berry *et al*, 2010).

1.5.1 Nature and structural feature

The prominent characteristic feature of chronic obstructive pulmonary disease is the involvement of the mucociliary system accompanied by airway structural changes, and more evidently loss of elastic recoil which is the reason behind hyperinflation and tapering of CO2 (Saett *et al*, 2001). The progressive airflow limitation in COPD is due to two major pathological processes: chronic bronchitis and emphysema.

1.5.2 Chronic bronchitis

The term chronic bronchitis refers to inflammation in the surface lining of the airways, manifested as a chronic productive cough for three subsequent months of two consecutive years. The clinical implications for chronic bronchitis are increased mucus production by goblet cells, frequent exacerbation, and remodelling of the epithelial surface (Kim *et al*, 2013). The inflammatory series starts at the small airways (less than 5 mm in diameter) and lung parenchyma causing physiological and clinical complication. The cellular changes in the airways start with mucosal infiltration of the airways by inflammatory cells (macrophages, neutrophils and CD8 T-lymphocytes) leading to epithelial disruption, smooth muscle hypertrophy and fibrosis in severe cases (Cosio *et al*, 2002). In turn, these inflammatory cells start the inflammatory process by releasing different inflammatory mediators such as TNF, interleukins, C-reactive protein and fibrinogen. Angiogenesis is another structural change resulting from activation of the hypoxia inducible factor that leads to an increase of VEGF transcription. Nevertheless, level of VEGF expression is decreased in emphysema that is induced by smoking. Emphysematous alveolar septa are avascular (low angiogenesis).

Inflammatory lung diseases are characterized by the new formation of small blood vessels as a result of the narrowing of blood vessels (Siafakas *et al*, 2007). The remodelling process may be induced by inflammatory mediators such as interleukins or

growth factors such as vascular endothelial growth factor (VEGF). Furthermore, micro-vascular remodelling in COPD may enhance the thickening of the airways, which contributes to the progression of the disease (Lieckens *et al*, 2001). Although the thickening is increased as the disease progresses, the microvasculature is reduced especially in patients with co-morbidity like lung cancer and right side heart complications (Soltani *et al*, 2009).

Structural changes are varied according to the stage of the disease. In COPD, smoking is the main cause of the structural and cellular changes. In healthy smokers respiratory bronchiolitis (inflammation of small airways) is the pathological hallmark in young smokers and the structural changes usually occur along the respiratory system in healthy smokers (not established COPD) (Piqueras *et al*, 2001). The main changes observed in many studies are infiltration of lung parenchyma with macrophages and T- lymphocytes in the absence of alveolar destruction or fibrosis. Meanwhile, a disrupted epithelium has been identified in the membranous bronchioles. In smokers with established COPD, the inflammatory cascades occur predominantly in small airways, resulting in resistance in small airways of less than 2 mm which is the hallmark in COPD patients. In (1968), Hogg *et al* discovered that small airway resistance is 4 times normal among patients with mild COPD, while total lung capacity was nearly normal. Conversely, in moderate to severe cases, because the resistance in small airways increased the total lung capacity, was higher than normal. The second effect is morphological, which dramatically affects the lung performance because of physiological abnormalities in the small airways, including mucus plugging, inflammation and increased smooth muscle and fibrosis. One report from the national lung and heart institute revealed that a 50% reduction in the number of small airways is related to a two-fold increase in peripheral resistance in COPD patients (Hogg *et al*, 2004). Moreover, a number of pathological changes has been reported in epithelial airways, including goblet cells hyperplasia in the proximal region of the airways in both ex-smokers and current smokers, as well as squamous metaplasia in the proximal and distal parts. In addition, short cilia have been reported in COPD patients (Hessel *et al*, 2011). According to the global initiative for chronic obstructive pulmonary diseases, squamous metaplasia and mucus hyper-secretion are characteristic of stages III and IV (Hogg *et al*, 2004). Additionally, squamous metaplasia is related to the number of cigarettes smoked per day. 46% of

smokers and only 23% of ex-smokers develop squamous hyperplasia. Furthermore, the extent of metaplasia is related to a decrease in the forced expiratory volume in 1 second (Araya *et al*, 2007).

Peri-bronchial fibrosis in COPD is enhanced by interleukin 1-β, secreted by squamous metaplastic cells, which in turn enhance production of the transforming growth factor and fibrosis (Kolb *et al*, 2001). Peri-bronchial inflammation and fibrosis may cause the centrilobular type of emphysema, which is usually associated with FEV25 changes in Spirometry. Disruption may occur in lung parenchyma and is considered to be a consequence of inflammatory reactions (Petty *et al*, 1985).
The smooth muscle hyperplasia in patients with COPD is prominent in the small airways, however, compared to asthmatic patients it is less marked in COPD (Hogg *et al*, 2004). At cellular level, persistent contact with tobacco smoke causes lung inflammation, characterized by invasion of white blood cells into extracellular spaces of the lung, causing accumulation of inflammatory cells in the lung tissue.

1.5.3 Emphysema

Abnormal dilation of airspaces beyond the terminal bronchioles, associated with alveolar destruction, is usually caused by an imbalance between protease/anti-protease activities in the lung microenvironment (Snider *et al*, 1985). Emphysematous changes have many effects on lung physiology, with gas transfer more affected than FEV1 (Parr *et al*, 2004). Furthermore, the alveolar destruction is the pathological hallmark in emphysematous lungs and loss of alveolar attachment and elastic fibre are related to collapse of distal airways during the expiratory phase (Vlahovic *et al*, 1999).

The inflammatory series starts at small airways and lung parenchyma, causing physiological and clinical complications. Moreover, the risk of bronchogenic carcinoma is 32 times higher in patients with bullous lung diseases (Ogawa *et al*, 1999). In practice the emphysematous lung is diagnosed by high resolution CT scan in addition to spirometry changes. Morphologically, emphysema is sub-classified into three types: centrilobular, pan-lobular and para-septal emphysema (Figure 1.8).

Centriacinar is the most frequent type. It features dilatation of central parts of lung bronchioles. The alveolar sacs are usually not affected (Leopold *et al*, 1957). The upper

lobe is the most affected part. Furthermore, this type is predominantly related to cigarettes. In the early stages of centrilobular emphysema, the border between the inner affected area and the outer region is not well identified by a high resolution CT scan. Panacinar emphysema is less frequent than the centriacinar type, and the α1- antitrypsin is the commonest cause (Figure 1.8-C). The destructive dilatation of the affected areas in this type started from respiratory bronchioles up to the alveoli and lower parts are more affected than the upper zone (Heppleston *et al*, 1961). Panacinar emphysema is localized or diffuse in form, and the affected parts tend to be hypo-echoic in contrast to the normal lung. In addition, the borders of panacinar sacs are not well defined (Stern *et al*, 1994).

Para septal emphysema is the least frequent type. The peripheral parts of acini are usually the affected site and the posterior surface of the upper parts of the lung is the predominant affected area. This type of emphysema in young age groups is usually asymptomatic, although the risk of pneumothorax is higher (Figure 1.8-C)

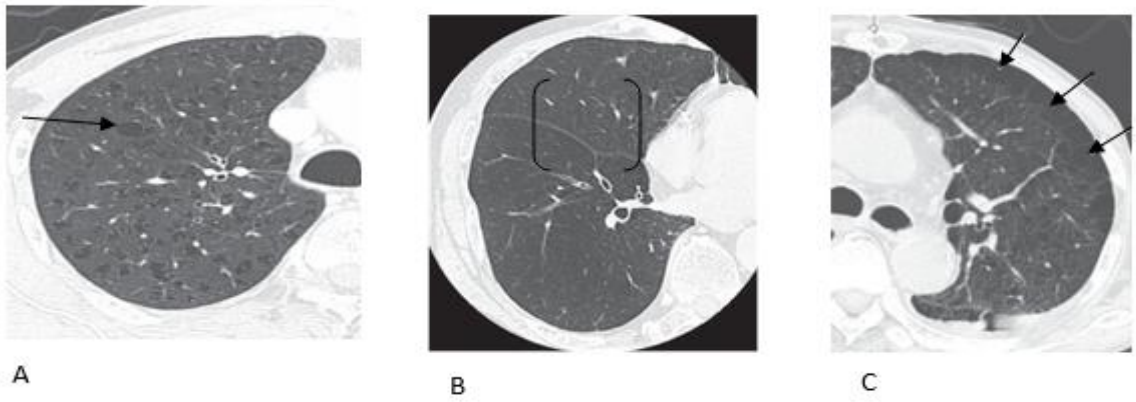


Figure 1.8 High-resolution CT scan of the chest. (A) High-resolution CT scan of the chest shows ill-identified margin of emphysematous sacs (arrow) in mild emphysematous changes (early stages). (B) CT scan shows radiological appearance of diffuse form of panacinar emphysema with ill-defined border (between the brackets). (C) CT scan chest with para septal emphysema with sub pleural air spaces (arrows). (Masashi *et al*, 2008)

1.5.3.1 Alpha1-antitrypsin deficiency

Autosomal recessive disorder affects 1 in 3000 births per annum, and manifest in the liver and lung, including emphysema and chronic bronchitis. In emphysema about 90% of anti-trypsin activity is α1- antitrypsin. Malfunctioning or deficiency in α1- antitrypsin is a leading cause of emphysema.

Alpha1-antitrypsin is an acute phase reactant secreted by hepatocytes. It is the major inhibitor of proteases such as neutrophil elastases and proteases (Anderson and Lomas, 2006). In addition the most common genetic abnormality is linked mutation in SERPIN A1 gene.

A genetic defect in the protein causes misfolding, which is referred as α1-antitrypsin Z (ATZ), and the accumulation of ATZ in the endoplasmic reticulum of hepatocytes has a proteotoxic effect on liver cells (Erikson *et al*, 1986), and causes fibrosis and cirrhosis and hence increases the risk of hepatocellular carcinoma.

1.5.3.2 Emphysema in α1-antitrypsin deficiency:

Emphysematous changes in alpha-1 antitrypsin deficiency are caused by protease-antiprotease imbalance, inflammation, and mechanical damage. In a clinical setting the patients are usually asymptomatic in early life, however, affected patients develop their symptoms at third or fourth decade. Characteristic features of emphysema related to α1-antitrypsin deficiency are panacinar and usually affect the upper lobe. The symptoms usually start at the fourth or fifth decade (Tomashefski *et al*, 2004)

1.5.3.2 Neutrophil Elastase (NE)

Neutrophil Elastase (NE), is a serine protease, whose molecular mass is 28000-31000 g/mol (Sinha et al, 1987). It is produced by variety of cells, mainly neutrophils, and is inhibited by α1-antitrypsin. A deficiency in the lung tissue and an elevated elastase in the sputum are correlated with a decline in FEV1 (Betsuyaku *et al*, 2000). NE also increases expression of IL8 in pulmonary epithelial cells in cystic fibrosis patients. (Nakamura *et al*, 1992). Moreover, disruption of elastase causes emphysema by affecting the Elastin which is the cardinal part of the architecture of lung tissue.

1.5.4 Blood vessels

In severe cases of COPD, blood vessels, especially those smaller than 500 µm in diameter are usually affected by hypoxemia, causing more resistance. Recently it has been discovered that even in early stages of COPD, smooth muscle proliferation, elastin

and collagen deposition exist in the inner surface of small blood vessels. Moreover new small blood vessels are the pathological hallmark in COPD. Angiogenesis is strongly associated with bronchiectasis and asthma. As well as COPD and lung cancer, the angiogenesis of squamous dysplasia is considered as premalignant change (Caldenhoven *et al*, 2012).

New growth of blood supply is known as angiogenesis, however, changes in morphology such as increasing the diameter without formation of new blood vessels is considered to be blood vessel remodelling. Hypoxia is responsible for new vessel formation in severe cases of COPD by upregulating VEGF (McDonald, Walsh and Pearson, 2001). Tumours in general are characterized by new vessel formation (angiogenesis) (Strieter *et al*, 2004).

Area affected	Pathological changes in COPD
Proximal airways (>2mm in diameter)	↑ .Number of macrophages and CD8-T lymphocytes . Few neutrophils and eosinophils . Enlargement of sub-mucosal gland and goblet cell metaplasia. . Infiltration of mucus glands with lymphocytes and neutrophils Smooth muscle hypertrophy and squamous metaplasia of lining epithelium.
Distal small airways(< 2mm in diameter)	↑ . Macrophages and CD8 T-lymphocytes . B lymphocytes, lymphoid follicles and Fibroblasts. . Small number of neutrophils and eosinophils . Evidence of bronchiolitis . constriction and Peri-bronchial fibrosis . squamous metaplasia of epithelial lining small airways
Parenchymal tissue	↑ . CD8 T-lymphocytes and macrophages . Destruction of alveolar wall . Microscopic and gross enlargement of airspaces (emphysematous bullae). .
Pulmonary blood vessels	↑ Total number of macrophages and T-lymphocytes . Initial changes include intimal thickening and dysfunction . delayed changes represented as hypertrophied smooth muscle and collagen deposition leading to pulmonary hypertension and cor-pulmonale

Table 1.2 cellular and structural changes in COPD. Proximal and distal airway changes as well as cellular infiltration associated with COPD (William, 2006)

1.5.5 Spirometry changes

Spirometry provides objective measures about the severity and reversibility of obstructive and restrictive disease (Figure 1.9). In addition, the quantitative data obtained by spirometry is important to confirm a diagnosis of COPD, and to measure the severity and progression estimation of the disease (Vestbo *et al*, 2012). A diagnosis of COPD is based primarily on reduced FEV1 to FVC ratio in addition to other parameters that have emerged as important predictors for small airways disease, like FEV25-75 (Marseglia *et al*, 2007).

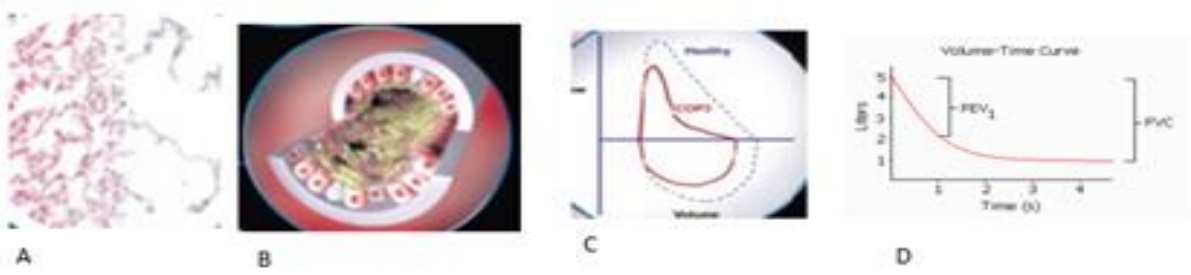


Figure 1.9 (A) Inflammatory and Spirometry changes in COPD. Parenchyma destruction in COPD patients. (B)Inner surface changes in the small airways showing disruption in the surface with mucus hyper-secretion. (C) Shape of the curve in normal heathy and COPD individuals. (D)Volume-time curve showing FEV1 in litres and expected FVC in 4 seconds (Vestbo *et al*, 2012).

1.5.6 Classification and severity of COPD

The global initiative for chronic obstructive lung disease (GOLD criteria) has launched criteria dependent on symptoms, spirometry changes and history of exacerbation in diagnosis of COPD. A bronchodilator test (percentage of change in FEV1 after bronchodilator nebulizer) is one of the most common tests to distinguish asthma from COPD in practice. A post bronchodilator measurement of FEV1 is usually negative in COPD patients. There are four categories of COPD: mild, moderate, severe and very sever. In mild COPD, symptoms are non-specific in the form of a cough, and FEV1/FVC<70%, FEV1≥80% is predicted. At this stage the patient is not aware of any symptoms. In moderate COPD FEV1/FVC<70%, FEV1 ≥ 50% and < 80% is predicted and the patient complains of progressive shortness of breath usually during exercise. Severe COPD is characterised by FEV1/FVC<70%, FEV1 ≥ 30% and <50% and the patient's symptoms and exacerbations constrain his daily activity. In very severe cases,

the lung performance is markedly affected and FEV1/FVC<70%, FEV1<30% or FEV1 less than 50%, usually accompanied by respiratory failure. In addition, the quality of life is affected at this stage.

1.5.7 Physiological changes in COPD patients

The most striking physiological abnormality in COPD patients is expiratory flow limitations, which occur as a consequence of alveolar destruction leading to air trapping and hyperinflation (Kainu *et al*, 2008). Moreover, during exercise the harmful effects of resting hyperinflation are exaggerated when the inspiratory demands are increased. Additionally, decreased expiratory timing leads to more air trapping, dynamic hyperinflation and increased mechanical restriction. Therefore inspiratory capacity, which reflects the end expiratory lung volume (EELF), is decreased as EELV (end expiratory lung volume) increase (Bikker *et al*, 2009).

Airway and parenchymal inflammation in the majority of COPD patients are associated with a decrease in FEV1 and fibrosis in the long term (American Thoracic Society, 2000). Furthermore, patients with established COPD have abundant macrophages compared with healthy smokers (Finkelstein *et al*, 1995). Tissue remodelling is a crucial pathophysiological change in COPD patients, which is characterized by abnormal repair of the epithelium and an accumulation of fibroblast cells, leading to fixed bronchial obstruction (Stockley *et al*, 2002). Moreover, frequent contact with harmful particles enhances inflammatory cells in the mucosa, sub-mucosa and glandular tissue, resulting in excessive secretion of mucus and thickening of small airways.

The major cellular components of this process are neutrophils and macrophages, which are the prominent cells in the parenchymal tissue, bronco-alveolar lavage, and sputum. In addition, these cells are involved in tissue destruction and remodelling, and defence mechanism (Stockley *et al*, 2002). Moreover, relocation and activation of these inflammatory cells are regulated by cytokines and chemokines such as TNF-α, interferon -ϒ, interleukin- 1β and IL6 (Wouters et al, 2009).

Tissue remodelling in COPD has two forms: thickening of the small airway, and alveolar destruction. It is presented, however, by mucus hyper-secretion and small airway fibrosis. Small airway remodelling in COPD patients affects the transition zone,

which lies between the airways and the alveoli. Alveoli show alveolar destruction and peri-bronchial fibrosis because of epithelial irregularity and hypertrophied smooth muscle.

1.5.8 Alveolar hypoxia and hypoxemia in chronic obstructive pulmonary disease.

The term "hypoxia" reflects a limited oxygen flux which causes oxygen deficiency to occur at tissue or cellular level. The term "hypoxemia", on the other hand, refers to a reduction of the oxygen level in the arterial blood (haemoglobin saturation <92%, arterial oxygen pressure < 70% or alveolar arterial gradient > 25 mm gH). Oxygen enters the body without any effort at atmospheric pressure of 760 mm gH and this is more or less the pressure inside the lung. However when the process of respiration starts the negativity inside the alveoli increases, as a result of stretching the visceral pleura outside, which allows the air to come inside. The process of expiration differs and depends mainly on the elastic recoil of the lung parenchyma. Loss of this property may cause air trapping and hyperinflation observed in COPD (Incalzi *et al*, 2009)

Oxygen is transported by capillary blood vessels in the lung tissue into different parts of the body through the heart and any defect in the blood vessel formation in the peripheral lung of COPD patients, for example a decrease in VEGF (vascular endothelial growth factor), may contribute to hypoxemia.

The severity of the disease in both obstructive and restrictive lung diseases is related to the degree of alveolar hypoxia and hypoxemia (Han *et al*, 2006). As well as an increase in airflow limitations, the ventilation/perfusion mismatch increases, which may be aggravated by sleep and exercise (Rodriguez *et al*, 2009). The most determinant factor in the development of hypoxemia in COPD is ventilation/perfusion mismatch, which results from emphysematous destruction of the pulmonary blood vessels and airflow limitation.

In COPD patients the ventilation/perfusion mismatch results in destruction of the capillary bed of the alveolar septa. Moreover frequent exacerbation is associated with deterioration in gas exchange and hypoxemia. Long-term disturbance in oxygen delivery may cause serious complications if the hypoxemia is not corrected, including systemic inflammation, pulmonary hypertension, muscle wasting and polycythaemia.

Long-term oxygen therapy (LTOT) can improve the quality of life, and hence is one of the options in the management of alveolar hypoxemia (Rabe *et al*, 2007).

Systemic inflammation is one of the possible complications associated with COPD. Many factors are proportionally related to systemic inflammation in COPD. These include tobacco consumption, degree of hypoxemia and a narrowing of the airways (Sin *et al*, 2006)

1.5.9 Complications

COPD is a common cause of hospitalization among elderly patients and co-morbidities are a frequent cause of hospitalization. Coronary artery disease, diabetes mellitus, infections, osteoporosis, and muscle wasting are the most frequent complications.

7-10% of COPD patients develop lung cancer (Mannino *et al*, 1993; Hansell 2003). Long-term complications are associated with cachexia (weight loss), which occurs in 25-65% of cases associated with an increase in leptin level (adipose derived hormone). Leptin is considered as pro-inflammatory factor, which is involved in the release of other inflammatory factors like IL6 (Yang *et al*, 2006).

1.5.10 Management of COPD

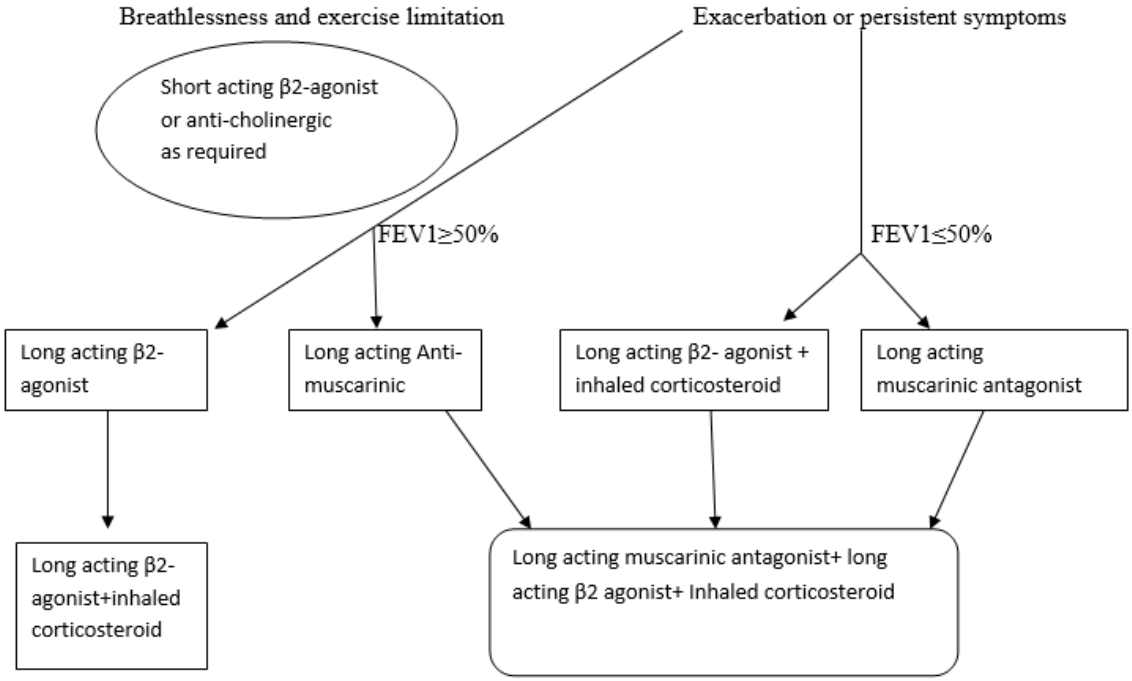
Deterioration of lung performance in COPD starts slowly at the beginning and rapidly progresses with advancement of the disease. Nonetheless, smoking cessation remains the first and the most important way to halt further progression and reduction of FEV1.

The pharmacological treatment depends on the clinical stages of the disease. In mild COPD (FEV1/FVC $\geq$70%), where breathlessness on exertion is the most common symptom, the mainstay of treatment is short acting β2-agonist which acts via stimulation of the cell surface of β2-adrenoreceptors (member of G-protein) (Jonson and Malcom, 1998), which in turn activate protein kinase-A by a sequence of reactions resulting in phosphorylation of the intracellular regulatory proteins, which then block histamine and cysteinyl-leukotrine efflux from mast cells; moreover β2-agonists possess an anti-inflammatory property.

Anti-cholinergic medication is another group that can alleviate bronchoconstriction via relaxation of smooth muscle by blocking the muscarinic receptors (Rabe et al. 2007).

Inhaled corticosteroids are important in the management of COPD as they decrease hospital stay and reduce frequency of exacerbations. Nevertheless, the long-term benefit is not clear (Lung Health Study Group, 2000).

1.5.11 Indication of steroids in management of COPD

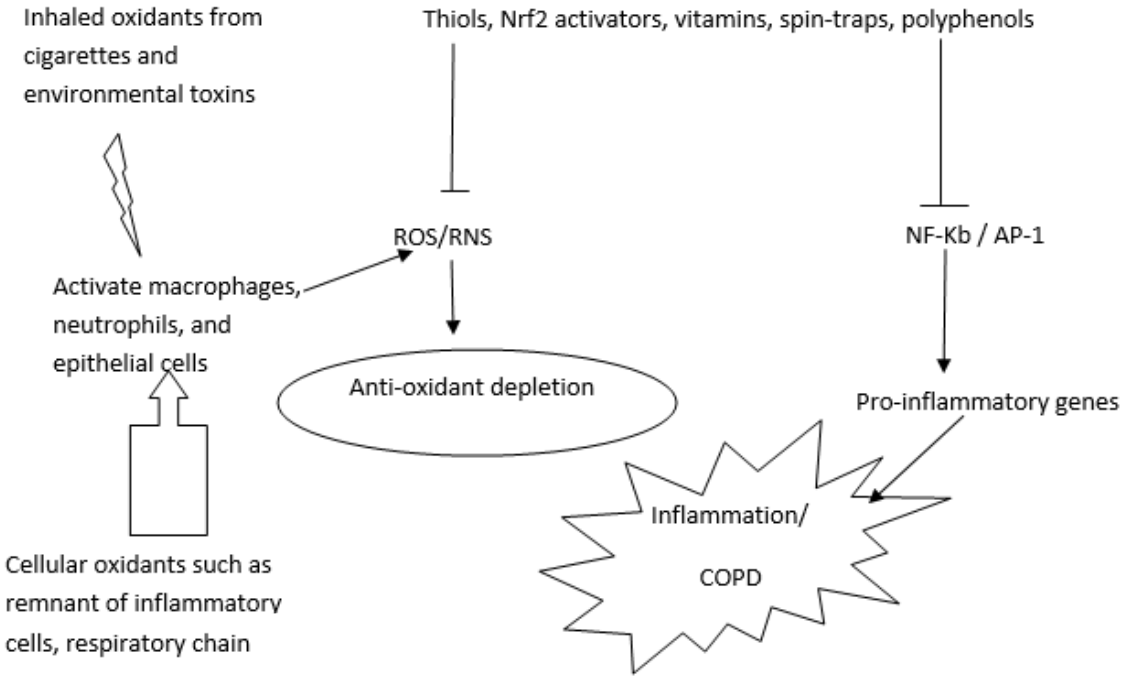


(Figure 1.12), Indication of glucocorticoids in management of COPD. Use of glucocorticoid is usually redistricted on mild cases, however during exacerbations steroids along with long acting β2 agonists are usually recommended.

1.5.12 Oxidative stress in COPD

Oxidative stress is a disturbed balance between oxidants, such as reactive oxygen species (ROS) and the anti-oxidant defence line (Betteridge, 2000). Three main sources of oxygen free radicals are smoking, inflammation and infections. Oxygen free radicals, which are responsible for alteration of oxidative stress, are released by inflammatory cells as a result of the consumption of oxygen (American Thoracic Society, 1996) (Figure 1.11). Oxidative stress is considered as the main explanation of pathogenesis of COPD.). Both oxidative and nitrates stress are increased in COPD and are profoundly

increased with frequent exacerbation (Barnes *et al*, 2009). Oxidative stress is initiated by products of neutrophils and macrophages causing tissue damage and turns on nuclear factors such as NF-KB and activating protein-1 (AP-1), leading to production of pro-inflammatory cytokines and chemokines. Meanwhile, frequent injuries or repeated infections could initiate inflammation. Antioxidants such as Nrf2 activator are inactive inside the cells until released by the effect of Nrf2 activators, then translocate into the nucleus to exert their effect by binding to anti-oxidant response elements (ARE) (Itoh et al, 2004). These anti-oxidants are markedly decreased in COPD patients because of cigarette smoking and frequent exacerbation (de Boer *at el*, 1989).



(Figure 1.11). Role of Oxidative and nitrative stress in pathogenesis of COPD. Different oxidant are involved in the activation of alveolar macrophages, neutrophils epithelial cells leading to production of ROS (reactive oxygen species) and RNS (reactive nitrogen species), as well as activation of redox sensitive transcription factors. They might have a role in inflammatory cascade responsible for COPD.

1.5.13 Role of inflammatory mediators in COPD

In heathy people the respiratory zone is sterile, however bacteria have been identified in patients with established COPD. In addition, the presence of bacteria in the lower airways is involved in the pathogenesis of the disease by releasing inflammatory

mediators that cause epithelial destruction and mucus overproduction (Grainge *et al*, 2013).

There are several inflammatory cytokines and chemokines (more than 50 different types of inflammatory mediators) involved in the inflammatory process in COPD patients (Table 1.3). Cytokines have been categorized into four main groups: 1) lymphocytes (cytokines which are released by T-cells and involved in the inflammatory process), 2) pro-inflammatory cytokines (enhance inflammation), 3) growth factors (cytokines that are responsible for structural modification which enhance the proliferation, differentiation and survival of cells), and 4) chemokines. Chemokines are inflammatory cytokines, which possess a chemotactic property for inflammatory cells and enhance the inflammatory process.

The most important pro-inflammatory cytokines involved in COPD are TNFα, IL-1β and IL6, in addition IFN-ϒ.

Cytokines	Role in COPD
Lmphokines and T-cell regulatory cytokines	
IL4	Unknown
IL5	Unknown
IL9	Unknown
IL12	Inhances the disease
IL13	Reduce the inflammation
IL17	Enhances the disease
IL25	Unknown
IFN-ϒ	Enhances the disease
Pro-inflammatory cytokines IL-1β, IL-6, TNF-α	↑ Enhances the disease.
Anti-inflammatory cytokines IL-10	Reduces the inflammation
Growth factors	
TGF-β	↑ Increase in the disease.
VEGF	↓ Increase in the disease.
Chemokines (CCR2,3,4,5 agonists)	Enhances the disease except CCR4 agonist Unknown.

Table 1.3 Cytokines netwok in Asthma and COPD (Barnes, 2008). Different inflammatory mediators are involved in the pathogenesis of COPD, involving lymphokines and T-cell regulatory cytokines, pro-inflammatory cytokines, anti-inflamatory cytokines, growth factors and chemokines

Cytokines are extra-cellular signalling proteins with relatively low molecular weight. They are produced by variety of cells and implicated in cellular interactions. In addition, inflammatory cytokines have a paracrine effect (on the neighbouring cells), an endocrine effect (at distance) and influence the original cells that secrete the cytokine. Both cytokines and chemokines act by binding to transmembrane receptors and the effect may be overlapped in a synergistic or antagonistic way by other cytokines produced by the same cells. TNF-α is one of the most important inflammatory

mediators associated with systemic manifestation, such as muscle weakness and wasting in chronic obstructive pulmonary disease (Wouters, 2002) and has been found over-expressed in patients with emphysema.

1.5.13.1 Role of Interleukin-6 in health and disease

IL6 is a multifunctional pro-inflammatory and immune-modulator cytokine produced by a variety of cells, such as epithelial cells, interstitial fibroblasts, macrophages and other inflammatory cells in response to cigarette smoking (Figure 1.10). IL6 is involved in inflammatory and immunological response, as it plays an important role in cellular proliferation and the survival of interstitial fibroblasts, thereby being involved in fibrosis. In addition, some studies show a high expression of IL6 in epithelial derived tumours (Grivennikov and Sergei, 2008). In lung tissue, it is mainly produced by alveolar macrophages and epithelial cells. Furthermore, one longitudinal study showed a relation between increased interleukin 6 in the sputum of COPD patients and deterioration in FEV1, exacerbation and pulmonary infections (Hagashimoto *et al*, 2009).

IL6 is implicated in the progression of the disease (Cosio *et al*, 2009). In addition, IL6 secretion in COPD patients is directly proportionate to C-reactive protein level during exacerbations (Kolsum *et al*, 2009). High levels of IL6 in COPD patients are related to depression and increased mortality rate (Cavaillès *et al*, 2012), and concomitant cardiovascular co-morbidities (Cosio *et al*, 2009). The cytokine IL6 has both pro-inflammatory and anti-inflammatory characteristics. The anti-inflammatory property is mediated by classic signalling, however, the majority of cells mediate the pro-inflammatory. IL6 is located on chromosome 7P 21, and it is relatively small molecular weight protein (21kDa). IL6 attaches to cell member receptors, consequently leading to activation of JAF family of tyrosine kinase, which in turn stimulates pathways such as MAPKs, PI3Ks and others (Hodge *et al*, 2005).

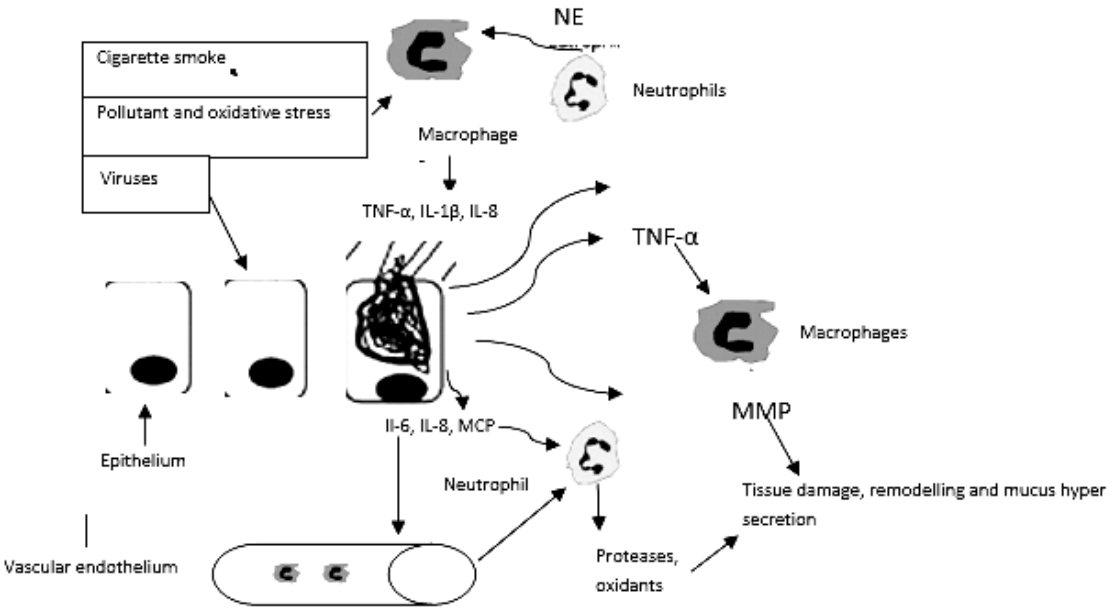


Figure 1.10 Interaction of cells and cytokines in the airway inflammation of COPD. The effect of environmental and infectious agents (viruses) on epithelial cells, as well as the effect of different inflammatory mediators on macrophages *eg* TNF-α and on neutrophils *eg*.IL6, IL-8, MCP and its involvement in tissue damage (Chung, 2001).

1.5.14 Future directions towards the new treatment of COPD

New treatment options based on the cellular and molecular mechanisms are a point of interest for many inflammatory diseases like rheumatoid arthritis and COPD. The new drugs focus on the inflammatory mediators that are responsible for the activation and recruitment of the inflammatory cells and for the ROS (reactive oxygen species) that contributes to COPD (Barnes, 2011).

TNF-α is elevated in the sputum of COPD patients. Moreover, TNF-α induces IL8 by enhancing transcription of NF-KB (Cheirakul *et al*, 2005). Anti-inflammatory drugs like phosphodiesterase inhibitors and P38 MAP kinase inhibitors are inhibitors of TNF- expression. Moreover, phosphodiasterase-4 inhibitors block expressed phosphodiesterase in the inflammatory cells suggesting a potential effect in control of the inflammation (Edelson *et al*, 2001).

Based on oxidative stress, which is increased in COPD patients (Nadeem *et al*, 2005), the anti-oxidants like N-acetyl cysteine have emerged, which show an anti-oxidant

effect both *in vitro* and *in vivo.* Recently, NAC (N-acetyl cysteine) was suggested to minimize the frequency of exacerbations (Gerrits *et al*, 2003).

1.6 Steroids in health and disease

Steroid hormones have an important role in the development, differentiation and homeostasis of the human body. Five classes of steroids are synthesized and released in organized rhythm (androgens, estrogens, progestin, mineralocorticoids and glucocorticoids), and each class exerts its effect by binding to a selected member out of 48 members of the steroid nuclear superfamily of intracellular proteins. Synthetic glucocorticoids are more potent than naturally-released glucocorticoids (Piemonti *et al*, 1999). Nuclear hormone receptors involve a superfamily of ligand dependent transcriptional factors that have role in wide cellular processes such as apoptosis, cell growth, development, differentiation and homeostasis. Many physiological processes including metabolism and haemostasis are influenced by nuclear receptors. There are two types of nuclear hormone receptors. Type I facilitate the effect of glucocorticoids (glucocorticoid receptors, GR), oestrogens (oestrogen receptors ER), mineralocorticoids (mineralocorticoid receptors, MR), progestin (progestin receptors, PR) and androgens (androgen receptors). Type II mediate the effect of thyroid hormones, 9-cis retinoic acid and vitamin-D. All nuclear receptors have same modular structure, consisting of N-terminal A/B domain, DNA binding C domain and D, E, F (LBD) Carson *et al*, (1990); Mc kenna *et al*, (1999).

There is evidence that glucocorticoid hormones are produced from various organs (Davis and Eleanor, 2003), however the adrenal cortex is the main site of synthesis and production of both glucocorticoids and mineralocorticoids. Under the control of the hypothalamic-pituitary axis various stress factors and hormones are responsible for tight regulation of glucocorticoid synthesis and production. Glucocorticoid production is enhanced by the adrenocorticotropic hormone from the pituitary gland, which is secreted under the influence of a corticotropin-releasing hormone from the hypothalamus. Mineralocorticoids are another important hormone similar to glucocorticoids and mainly responsible for reabsorption of sodium and water by the epithelial surface of collecting ducts in kidneys, salivary gland and large intestine (Schambelan and Morris, 1981).

The availability of natural glucocorticoids in tissues is regulated by corticosteroid-binding globuline in serum and by locally expressed 11β hydroxysteroid dehydrogenase enzymes (11β-HSD). Essentially, the majority of tissues are expressing glucocorticoid receptors and their effect has a crucial influence on maintaining cellular functions and mediating pathological conditions (Figure 1.13).

In the central nervous system, over-expressed glucocorticoid receptors are linked to depression and post-traumatic stress. Several experiments show a strong association between glucocorticoid signals and anxiety disorders. In addition, glucocorticoids have a critical role in maintaining immunity and suppressing inflammatory reactions following organ transplants. Furthermore, glucocorticoids increase the phagocytic ability of macrophages (Busillo *et al* 2011).

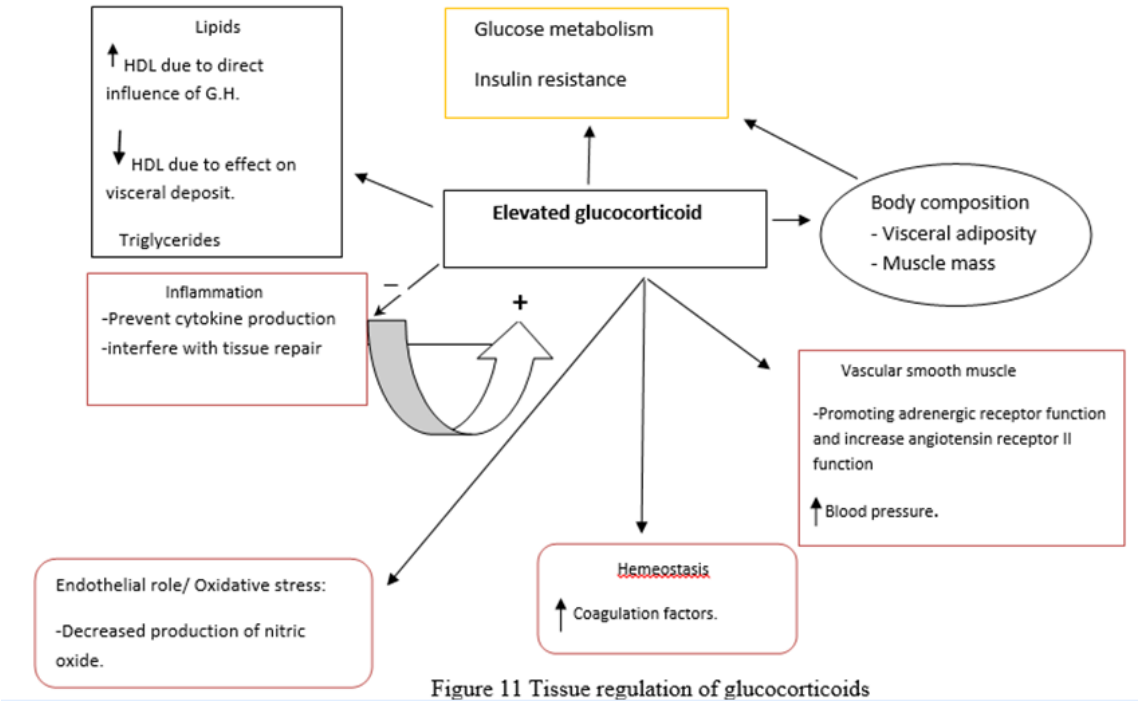


Figure 1. 13| Effect of glucocorticoids on cellular and body function. Different tissue and organs are affected by glucocorticoids, including blood glucose, endothelial tissue, lipid metabolism and homeostasis.

1.6.1 Glucocorticoids homeostasis

Cortisol is the main type of steroid, and is regulated by the hypothalamic pituitary axis under the influence of the neuroendocrine system. Furthermore, the magnitude and duration of HPA activation is controlled by glucocorticoids (Keller *et al*, 1984).

Glucocorticoids are produced in a circadian manner with diurnal variation (Rose *et al*, 1971). They have a diverse physiological effect on the majority of mammalian cells. Moreover, during some physiological conditions like stress and starvation, glucocorticoids stimulate the liver to replace the deficit by glycogenolysis and gluconeogenesis. Cushing's and Addison's diseases result from a surplus and deficiency of glucocorticoid supply respectively.

1.6.2 Response to steroids

Endogenous glucocorticoid hormones are considered to be stress related hormones released under hypothalamic control. As a response to the hypothalamic factor, corticotropin-releasing hormones enhance the release of adrenocorticotropic hormones (ACTH) from the pituitary gland, and consequently ACTH induces glucocorticoid synthesis from the zona fasciculate in the adrenal cortex. Glucocorticoids are lipophilic and circulate in the blood stream attached to corticosteroid binding protein (GBP) (Wallimann and Peter, 1997). The therapeutic efficacy of glucocorticoid is either mediated by trans-repression, which largely mediates the anti-inflammatory property, or by transactivation, which is responsible for side effects. Generally the genes that have anti-inflammatory functions are induced by GR through its transcriptional activity, for example lipocortin, IL10 and IL1 receptor antagonist. Conversely the pro-inflammatory proteins that are responsible for induction of the inflammatory process are controlled by NF-KB and AP-1, and the role of glucocorticoids in this situation is trans-repressive by blocking pro-inflammatory genes.

Glucocorticoid hormones enter the cell to interact with glucocorticoid receptors causing the release of heat shock protein (Pratt and William 1997). There are two types of glucocorticoid receptors both of which act as pro-inflammatory: GR-α which has agonistic activity and is related to glucocorticoid sensitivity, and GR-β that is related to insensitivity to the glucocorticoids. The complex then translocate into the nucleus and binds to the glucocorticoid response element (GRE) on responsive genes leading to increased or decrease transcription (Barnes *et al*, 2009). The GR gene is found on chromosome 5 (Hollenberg and Stanley, 1985). The GR function is controlled at multiple levels, by binding to hormones, nuclear translocation, interaction with cofactors and post-translational modifications such as phosphorylation and acetylation.

Four functional domains have been identified (Giguere and Vincent, 1986): N-terminal domain contains AF1 function that mediates transactivation of target genes. DNA binding domain (DBD) is involved in binding to GREs and contains two zinc fingers. The first involves AF1 which plays a role in trans-repression. The second finger has a role in dimerization of the receptor and the trans-activation that is mediated by the glucocorticoid response element. LBD (ligand binding domain) has a steroid binding function, a nuclear localization signal and ligand dependent trans-activation region which may interact with a co-activator or a co-repressor. The interaction between activated glucocorticoid receptors and GRE is considered to be classical and leads to activation of transcription, whereas binding with NF-KB is non-classical and is usually associated with transcriptional repression. The actions of NF-KB are inhibited by GR through multiple mechanisms (Auphan *et al*, 1995).

NF-KB is usually located in the cytosol in inactive state, bound with blocking proteins such as Ik B-α. Upon inflammatory signal. The NF-KB inhibitor-α is phosphorylated causing the release of NF-KB, which in turn translocate into the nucleus to interact with KB response elements and to regulate the genes coding the cytokines and chemokines to fight infection or inflammatory particles. Moreover, the imbalance or over expression in NF-KB may cause chronic inflammation (Ghosh *et al*, 1998).

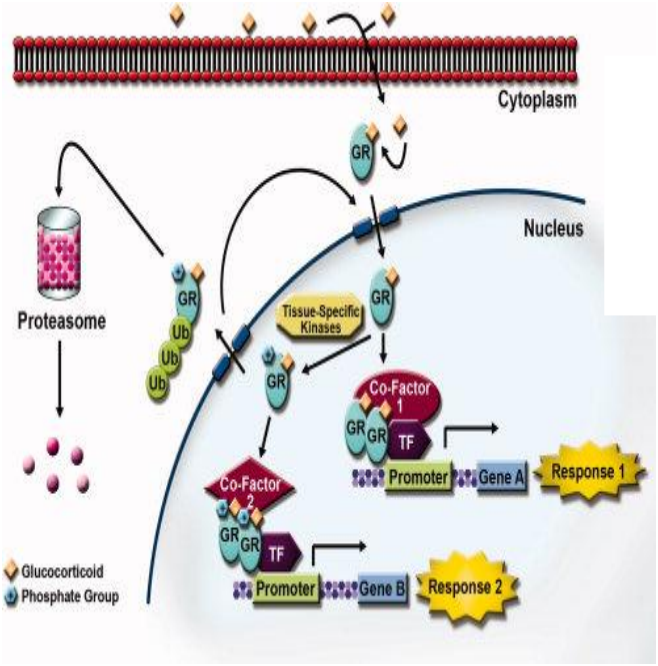
	Systemic effect	Local effect
Lung	Decreased macrophages in sputum	Decease oxidative stress
	Reduce eosinophils in sputum	Reduction in mast cells
		Down regulate synthesis and release of IL6 and IL8 from epithelial cells
Circulatory system		Decrease CRP level
Clinical	Improved FEV1, shorter hospital stay, and improve the acute exacerbations	Decrease hyper-responsiveness of the airways, reduce exacerbation and slight effect on the rate of decline in FEV1
		Improve cough and decrease sputum, Improve post Broncho-dilator test in acute exacerbation and reduce mortality rate

Table 1.4 Systemic and local effect of glucocorticoids (Barnes *et al*, 2011)

1.6.3 Glucocorticoid receptor phosphorylation

Glucocorticoid receptors are phosphoproteins that exist in most human cells. Although the glucocorticoid response is mainly determined by hormones, phosphorylation status also affects GR actions. Moreover, cellular conditions that affect phosphorylation of glucocorticoid receptors can have impact on GR transcriptional output. The most common sites for GR phosphorylation are serine 203, 211, 226 and 404. Moreover, glucocorticoid receptors have ability of phosphorylation at other sites of serine and threonine as well as tyrosine residues (Rao *et al*, 1987). Dephoure *et al* (2008) has suggested that other phosphorylation sites including threonine 8, serine 45, 134, 234 and 267 exist.

Several residues within GR, for example S211, need phosphorylation to acquire full transcriptional activity (Krstic *et al*, 1997). S211 (phosphorylated glucocorticoid receptors at serine 211) use CDK and P38 kinases pathways, which is transcriptionally active as a result of conformational changes which mediate the recruitment of GR to GRE-containing promotor. The JNK pathway is responsible for S226 (phosphorylated glucocorticoid receptor at serine 226) phosphorylation. This type of phosphorylation results in inhibition of GR activity, possibly because of increase in GR nuclear export. GR undergoes ligand-dependant self-regulation, which limits hormone responsiveness (Wallace *et al*, 2001). In addition, glucocorticoid sensitivity depends on availability of GR inside the cells. This process is also regulated by phosphorylation, in addition to transcriptional activity and nucleo-cytoplasmic localisation (Ismaili and Garabedian, 2004). In previous studies p38 MAPK was found active in alveolar macrophages in steroid-resistant asthma (Bhavsar *et al*., 2008). In response to external factors, MAPKs can affect cell behaviour and other biological processes such as migration, proliferation, and cell death. (Davis *et al*, 2003).



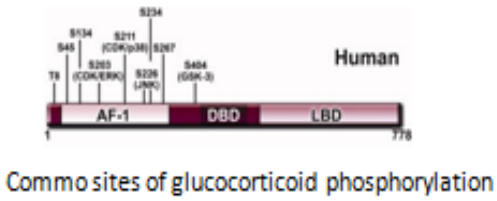


Figure 1, 14| Phosphorylation of glucocorticoid receptor. GR modulates hormone signalling. Upon binding to glucocorticoids it translocates into the nucleus and starts transcription. In addition half the life of the protein may be altered by the phosphorylation (Galliher *et al*; Amy *et al*, 2009).

1.6.4 Underlying mechanism of glucocorticoids action in COPD

A combination of long acting beta agonist and glucocorticoids has little effect on FEV1 decline rate, however long-acting beta agonist and inhaled steroids diminish the rate of exacerbations and the severity of the disease (Mahler, 1999: Dahl, 2001).

Down-regulation of inflammatory cytokines by HDAC2 in the presence of corticosteroids is a rational explanation for the therapeutic effect of glucocorticoids. Many clinical studies show that local and systemic glucocorticoids have an inhibitory effect on inflammatory cytokines (Lto *et al*, 2005). Moreover, Sin *et al*, (2006) conducted a study that showed that C-reactive protein levels are reduced in patients treated with glucocorticoids compared with placebo.

In stable COPD patients, systemic glucocorticoids have no obvious effect on FEV1 and FVC (Walter *et al*, 2005). Moreover, glucocorticoids increase the mortality rate among COPD patients above 65 (Sin *et al*, 2001). There are other side effects of glucocorticoids, such as hyperglycaemia, hypertension etc. On the other hand, during acute exacerbation of COPD, glucocorticoids improve FEV1, decrease hospital stay and reduce the frequency of exacerbation.

Inhaler glucocorticoids are involved in the management of COPD, however the exact causal mechanism of the benefit is still unclear. Pauwels *et al* (2001), carried out three years observational study on mild COPD patients who received inhaler glucocorticoids or placebo. The author concluded that there were no significant differences in FEV1 decline rate.

In COPD patients, prolonged use of glucocorticoids is responsible for systemic side effects and toxicity, including disturbance of blood sugar, increased risk of fracture, adrenal gland insufficiency (Walter *et al*, 2005), muscle weakness and depression (Decramer *et al*, 1994). However, there are some advantages including less hospital admission, fewer exacerbations and enhanced bronchodilator efficacy. (Scanlon *et al*, 2000).

1.6.5 Mitochondrial GR

GR translocation to the nucleus happen both in sensitive and resistant to glucocorticoid cells, while only in steroid-sensitive cells, glucocorticoid can translocate into the mitochondria. Mitochondrial membrane is directly affected by glucocorticoid hormones, resulting in loss of mitochondrial trans-membrane potential, hence disturbing the cellular processes mediated by function of mitochondria, for example ATP generation via oxidative phosphorylation, calcium flux and apoptosis

1.6.6 Role of co-factor TTC5 in glucocorticoid action

Nuclear hormone receptor function is controlled by numerous cofactors. GR also interacts with both coactivators, such as p300 histone acetyl transferase and corepressors such as histone deacetylases. One of the cofactors recently identified to affect GR is TTC5 (tetratricopeptide repeat domain 5). TTC5 is a cofactor protein originally identified in P53 response. TTC5 is a chaperone-like protein that mainly facilitates the assembly of multiple protein complexes.

TPR motifs are protein-protein interaction modules, identified in a variety of proteins and usually in combination with other structural motifs (Blatch and Lassle, 1999). Demonacos *et al* (2001) have identified a stress responsive activator of P300 (STRAP, also known as TTC5) as a co-factor for P300 co-activator complex. TTC5 expression was significant in lungs, brain, and kidney mouse tissues and several cell lines. TTC5 is composed of six TPR motifs and is phosphorylated and regulated by DNA damage responsive kinases (Figure 15).

TTC5 plays a pivotal role in P53 transcriptional activity in response to DNA damage and it is required to assist the interaction between JMY (transcriptional cofactor of P53) and P300 which in turn enhances apoptosis and P53 dependent transcription and prevents its degradation by MDM2 (negative regulator of p53 suppressor gene) (Coutts and La Thangue, 2006). HSP (heat shock protein) can also induce TTC5 to act as a co-factor to enhance the transcription of heat shock genes during heat shock response (Davis *et al*, 2011)

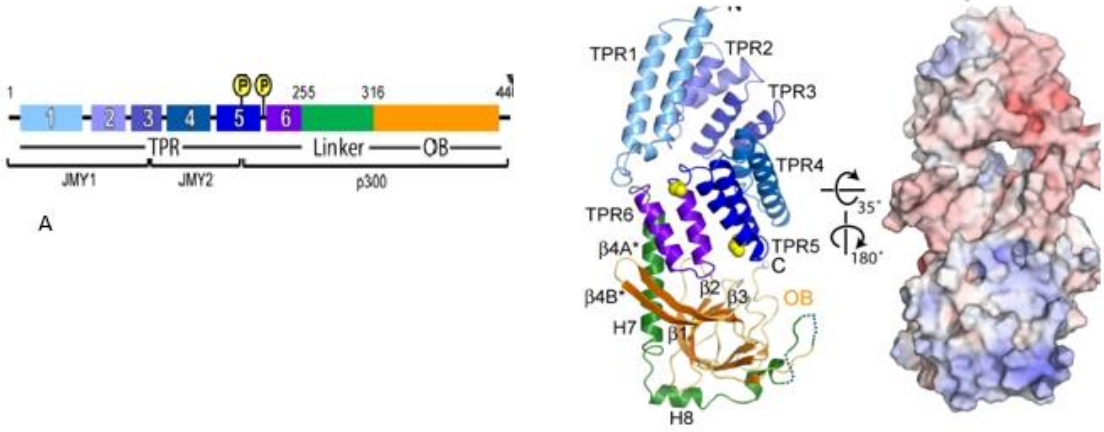


Figure-1. 15 Structure of TTC5. (A) Domain organization revealing mapped interaction for JMY, P300 phosphorylation sites (Demonacos, 2001). (B) Ribbon diagram for TTC5 with coloured structure as in A.

Stability, post-translational modifications and recruitment of co-factors are the main factors affecting glucocorticoid receptors. Post-translational modification is vital for GR transcriptional activity, stability and GR binding to other factors, suggesting that glucocorticoid hormone efficacy is highly dependent on post-translational modifications. Previous studies have shown a relation between GR and the TTC5/stress-responsive activator of P300, in which activity and ligand-dependent down-regulation of GR is affected by TTC5 (Demonacos *et al*, 2011). Phosphorylation of the glucocorticoid receptor at some sites, like serine 211, is up-regulated after exposure to dexamethasone hormone, however interaction between phosphorylated glucocorticoid receptor and TTC5 is still under investigation.

1.6.7 Complications associated with glucocorticoid treatments

Long-term treatment with glucocorticoids is associated with undesirable side effects and complications involving many parts of the body, and these complications could be immediate or long term complications, which usually result as a consequence of a cumulative dose of glucocorticoids (Figure 14). The average doses of different types of synthetic glucocorticoids are shown in table 4.

Increased weight and redistribution of fat is the most common long-term complication associated with glucocorticoids. One study, carried out for patients with rheumatoid

arthritis, has shown a 4 to 8% increase in BMI (Da silva *et al*, 2006). Another observational study concluded about 40% of patients above the age of 60 on glucocorticoids are at risk of osteoporosis and fracture, moreover, glucocorticoids can halt osteoblast activity responsible for bone formation in osteoporosis and cause pathological fracture (Canalis *et al*, 2004).

Glucocorticoids may block the pro-inflammatory cytokines, leading to decreased macrophage activity and increased risk of infection. There is a very weak correlation between glucocorticoid use and the risk of peptic ulcer, however patients on steroid treatments with NSAID (non-steroidal anti-inflammatory drugs) are at four times greater risk of peptic ulcer than patients who are taking NSAID alone (Weil *et al*, 2000). More serious complications, such as osteonecrosis, can occur. Shigemura and Tomonori (2011) revealed taking 10mg/day of prednisolone for six months can increase the risk of osteonecrosis by 4 to 5%. Other serious complications of frequent use of glucocorticoids include hyperglycaemia and secondary diabetes, ischemic heart diseases, which are the major drawback to the use of steroids in high risk patients. Moreover, hypothalamic-pituitary adrenal axis suppression may develop over a long period. In the case of small cell lung cancer there is synthesis of pro-hormone ACTH-like hormone, which is the same in character as that secreted by the pituitary gland. Negative feedback is responsible for blocking the expression and release of ACTH from the pituitary gland, nevertheless, in small cell lung cancer, glucocorticoids do not have the ability to block glucocorticoid release (Schlossmacher *et al*, 2003).

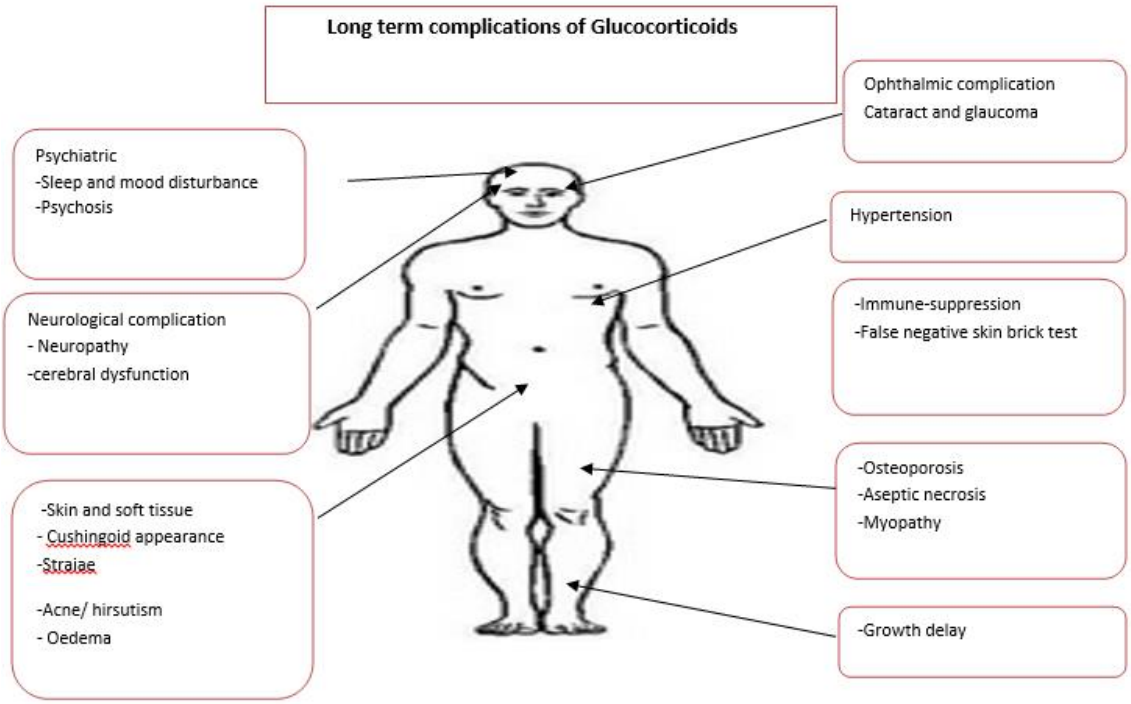


Figure 1.16| Side effect and complication from prolonged use of steroid hormones

Generic name of synthetic Glucocorticoids	Recommended dose/Kg/day
Hydrocortisone	4 mg/kg/day
Prednisolone	1 mg/kg/day
Methyl prednisolone	0.8 mg/kg/day
Dexamethasone	0.15-0.3 mg/kg/day
Betamethasone	0.12 mg/kg/day

Table 1.5 Types of synthetic glucocorticoids and recommended doses/Kg/day (Adapted from Gensler *et al*, 2013).

1.6.8 Immunohistochemistry

Immunohistochemistry is a common procedure used to identify the surface area and distribution of specific tissue antigens, and *in situ* detection by monoclonal and polyclonal antibodies. IGg is the most popular immunoglobulin used.

The idea of immunohistochemistry started when Marrack, who invented reagents acting against cholera and typhus, based on a red-stain called tetraedro. In the 1940s, antigens

against streptococcus pneumonia were identified by fleuressin stain under ultra-violet light fluorescence microscopy (Coons *et al*, 1991). In the following years, Nakane introduced the idea of enzymes as a marker antibody (Avrameas *et al*, 1966; Nakane *et al*, 1968).

1.6.7 Background of immunohistochemistry

Immunoglobulin is Y in shape and has two identical heavy and white chains. The type of antibody is determined by the weight of the chain: heavy or light. Each one has two forms: lambda and kappa (figure 1.7).

Fab portion is part of immunoglobulin that can bind to the antigen, which contains different segments of heavy and light chain and plays a role in stabilizing the antibody binding to the tissues.

The FC region (the tail of immunoglobulin) is an important region, which allows binding of antibodies by FC receptors to inflammatory cells. Moreover, this part is important for non-specific staining in the technique.

Paratop is the antigen-binding site of the antibody. The site of the antigen that binds to the antibody is known as “the epitope”.

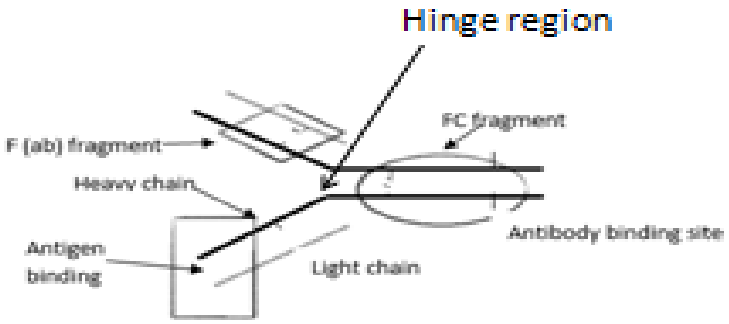


Figure 1.17|. Structure of immunoglobulin. Fab Fragment (antigen binding). Fc Fragment, crystalized. CL constant domain (Light chain). Variable domain heavy chain, and hinge region. http://www.ebioscience.com/knowledge-center/antigen/immunoglobulin/structure.htm.

Previous researchers hypothesized that lung macrophages in COPD are resistant to glucocorticoids. Plumb *et al* (2013) conducted research about phosphorylated GR in peripheral lung tissue, concluding that phosphorylated GR (S211 and S226) are expressed in lung macrophages in heathy and COPD patients. On the other hand, phosphorylated forms of GR (S211 and S203) were expressed in the lung epithelial surface, but the form of the GR phosphorylated at S226 was not expressed. Moreover, the phosphorylation status of glucocorticoids in lung macrophages was not affected by the disease as high degree phosphorylation in lung macrophages was detected.

The conclusion from previous research indicated that there were abundant phosphorylated forms of GR in lung macrophages, suggesting that this process was not disrupted in COPD patients (Adcock *et al*, 2005). In addition, optimum concentration of 17-BMP at small airways of COPD patients is enough to suppress pro-inflammatory mediator production by macrophages (Plumb *et al*, 2013).

1.7 Lung cancer

Lung cancer is the fourth most common cause of death in the world and about 41,000 cases are diagnosed every year in the UK. According to its histopathological features, lung cancer is classified into two types: small cell and non-small cell.

Non-small cell lung cancer accounts for approximately 85% of cases. Three types of lung cancer belong to this group: squamous cell carcinoma, large cell carcinoma and adenocarcinoma. About 40% are adenocarcinomas and usually occur in peripheral areas of the lung tissue, while squamous cell carcinoma usually occurs in central areas and more frequently in males than in females. This last detail is related to smoking patterns across sexes (Minna *et al*, 2008). Response to treatment and disease metastasis is varied from one type to another. Small cell lung cancer usually responds poorly to treatment and spreads quickly. Moreover, surgical intervention is inadvisable. However, non-small cell lung cancer responds better response to treatment. It is likewise subdivided into three main types: squamous cell carcinoma, adenocarcinoma and large cell carcinoma.

Adenocarcinoma is the most common type, and in the majority of cases the tumour is locally advanced or already metastatic at the time of diagnosis, with only 15% survival rate in 5 years.

1.7.1 Histological features of non-small cell adenocarcinoma

Non-small cell adenocarcinoma is subdivided into six groups according by histological features and clinical behaviour. Type A, known as local bronchoalveolar carcinoma, is solitary and grows by substituting the alveolar lining cells with a thickening of the alveolar septa. Usually there is no fibrotic foci with an unclear boundary. Type B, or localized bronchoalveolar type, is usually associated with foci and alveolar destruction. Its general microscopic appearance is the same as type A's, however there are some alveolar foci that develop as a consequence of alveolar collapse. Type C, or localized bronchoalveolar with foci of active fibroblastic proliferation, is the most common histological subtype. The lining epithelium is replaced with active fibroblastic proliferation and small vessels are obvious. However, there are no active fibroblasts in the foci as with type B. Type D, or poorly differentiated adenocarcinoma, is characterized by a solid tumour, which is similar to large cell carcinoma. Moreover, it has clear boundaries between the tumour and the normal parenchyma. Type E, or tubular adenocarcinoma, is comprised of tubular and acinar structures. Tumour margins are well defined. Type F, or papillary adenocarcinoma type of tumour, grows by replacing the alveolar lining cells. Additionally, it shows destruction of the septa.

1.7.2 Clinical-pathological characteristics

The clinical features depend on the stage of the tumour. In types A and B there is no lymph node metastasis and it is considered to be stage I bronchoalveolar adenoma (bronchoalveolar carcinoma *in situ*) and in type C around 30% of cases show lymph node metastasis. The male to female ratio is 1:4 and the survival rate within 5 years is 100% in patients with types A and B.

1.7.3 Smoking and lung cancer

There are more than 4,000 toxic and carcinogenic materials in each cigarette. There are approximately10^4 free radicals in each puff, which may interrupt oxidative/anti-oxidative balance, causing alveolar destruction. Moreover, apoptosis of the epithelial airways is mainly due to mitochondrial damage, and is the result of oxygen free radicals rather than nicotine (Wlkilson *et al*, 2006).

Heat shock protein 70 is a molecular chaperone important in uniting polypeptides, which importantly limit cellular injury and restore the function of damaged proteins. On the other hand, the quantity of inflammatory cytokines is directly proportionate to the development of the lung cancer in which high levels of inflammatory cytokines such as IL6 and IL8 are correlated with the occurrence of lung cancer. Moreover, IL6 and IL8 are over-expressed in pre-malignant epithelial cells and their expression is associated with a bad prognosis. Other inflammatory mediators such as C-reactive protein, a circulating inflammatory biomarker, are potential contributing factors to lung cancer

1.7.4 Inflammation in development of lung cancer

The role of inflammation was first described by Rudolf Virchows in 1863. He noted that tumours could arise from the inflammatory sites. Cancer is defined as a complex of disease diversity in the same cells, and usually occurs as a result of uncontrolled growth. Genetically normal cells are under tight control of two main classes of genes: oncogenes and tumour suppressor genes. The risk of malignancy increases whenever the activity of oncogenes exceeds that of the tumour suppressor genes. Although genetic inheritance remains an important risk factor, environmental and life style factors are considered as the main cause of genetic mutation for some diseases (Stein *et al*, 2004).

Despite inflammation's health-giving function in restoring injured tissue, it may cause malignancy in the surrounding areas. The inflammatory reaction and carcinogenesis have the same molecular aspect and signalling pathway (Yan *et al*, 2006).

Chronic inflammatory response is linked to the development of cancer. It provides active materials from cellular inflammation of tumour areas. Examples of such active materials include cytokines, chemokines and growth factors, which may maintain the

optimum environment for the cells to avoid apoptosis and enhance other beneficial processes such as the reorganization of energy metabolism (Stein *et al*, 2004).

Cytokines potentially are low molecular mass proteins (21 kDa) that have a role in cellular communication. They are synthesized by stromal cells. For example, fibroblasts regulate different metabolic processes, such as differentiation and migration.

Once the inflammatory process starts, it leads to the release of these mediators that then play an important role in the development of tumours (Hanahan *et al*, 2011). Saetta *et al*, (1998) reported that IL6 is involved in the inflammatory process in human COPD, and has shown the highest expression in lung COPD. Moreover, high levels of inflammatory cytokines such as IL6 and IL8 are correlated with a high risk of lung cancer. IL6 and IL8 are expressed in pre-malignant epithelial cells, which expression implies a bad prognosis.

1.8 Genetic susceptibility in COPD and lung cancer

Genetic and environmental factors are both responsible for COPD and lung cancer. The risk of lung cancer in patients with chronic obstructive disease is 5 times greater than in healthy smokers. Genetic backgrounds are not fully understood. It is well known that patients with α1-antitrypsin deficiency are at risk of emphysema, as it accounts for only 1 to 2 % of cases of COPD. Other genetic factors are mentioned in the development of emphysema such as TNF, which have the coding of TNF-α protein. Previous studies in mice show that excessive production of TNF-α protein is associated with inflammation and the development of emphysema (Bouma *et al*, 2006), and that TNF-α is involved in 70% of COPD-related smoking. BAL (bronchoalveolar lavage) of COPD patients shows an increase in TNF-α compared with healthy smokers. However, the reason behind the increased TNF-α in COPD patients is not clear. One possibility is that the TNF is associated with 308A allele in different disorders. It is well known that smoking increases the oxidative stress and inflammatory process by inhalation of oxidants and invasion of inflammatory cells in the lung tissue. Moreover, both oxidative stress and inflammatory cascade have an effect on the cells causing genetic instability. Furthermore, histone tails are altered by an extensive group of non-histone chromatin associated proteins known as chromatin modified enzymes which are present in cells as

a multi-component complex that is usually recruited to chromatin with DNA bound transcription factors.

Chromatin-modifying enzymes are categorized based on their function into:

1) Acetylation by histone acetyl transferase (HATs). 2) Deacetylation by histone deacetylase (HDAC). 3) Methylation by methyl transferase (HMTs). 4) Demethylation by histone demethylases (HDMs).

The level and activities of histone deacetylase, particularly HDAC2 and Sirtuin 1(SIRT1), are diminished in lungs and alveolar macrophages, resulting in steroid resistance in COPD patients as well as reduction in HDAC2 activity, which may lead to acetylation of NF-KB and glucocorticoid receptor α resulting in abnormal inflammatory response and resistance.

Alveolar and parenchymal macrophages show a reduction in the level of HDAC activity especially HDAC2 and Sirtuin. This reduction is associated with acetylation of NF-KB and glucocorticoid receptor-α, resulting in excessive inflammatory response in COPD. Since histone deacyteylases control the activity and expression of many proteins related to cancer initiation and progression and alteration in somatic genes encoding HDACs activity is related to tumour progression. It is widely believed that returning HDAC activity could have positive effects on steroid efficacy. For the lung cancer, the risk was found in carriers of mutant TP53 (Tumour protein 53). Moreover, carriers of TP53 who smoke have a 3 times higher chance of developing lung cancer than non-smokers (Hwang *et al*, 2003). Additionally, Hemminiki (2004) has reported germline epidermal growth factor receptor (EGFR) T790M sequence variation in families with NSCLC.

1.8 Apoptosis in COPD and lung cancer

Apoptosis is defined as programmed cell death, a specific program encoded in the genome responsible for regulated activation of cell death. Apoptosis is also considered as morphological aspect of cell death, which is characterized by cell shrinkage, nuclear condensation and membrane swelling. Apoptosis is an important aspect of homeostasis (the opposite of cellular division). In addition, the pathogenesis of some diseases, like ischemia, neurodegenerative diseases and viral diseases is related to apoptosis. Necrosis, on the other hand, occurs due to the effect of toxins, physical stimuli or

ischemia. Moreover, it can be differentiated from apoptosis by intense inflammatory response due to early loss of cellular content, cell swelling and nuclear lysis. (Vandenabeele *et al*, 2010).

Nonetheless, apoptosis is involved in eliminating inflammatory cells, which is considered as homeostasis in the lung, this process has a harmful effect on defence mechanism if it exceeds normal limits. Furthermore, the abnormal apoptotic process is related to the development of emphysema (figure 1.16), fibrosis, and bronchiolitis obliterans (non-reversible collapse of small airway) (Kasahara *et al*, 2001). The process of apoptosis is usually not easily detectable in pneumonia, however in severe inflammatory diseases, such as fibrosis, it could be obvious due to inefficient clearance (Vandivier and Gardai, 2005).

Phagocytosis in COPD is involved in the process of the elimination of apoptotic cells, fibroblasts, epithelial and endothelial cells. Moreover, macrophages and dendritic cells are responsible for this process. Previous studies in COPD patients showed that the inefficient removal of dead cells may be responsible for the disruption of alveolar structure (deCathelineau *et al*, 2003). Furthermore, engulfing and necrosis of dead cells could lead to the release of some inflammatory mediators, which may initiate the immunological response and attract more inflammatory cells in the surrounding tissue. Hence, the elimination of the apoptotic cells is a crucial step to restore normal tissue function.

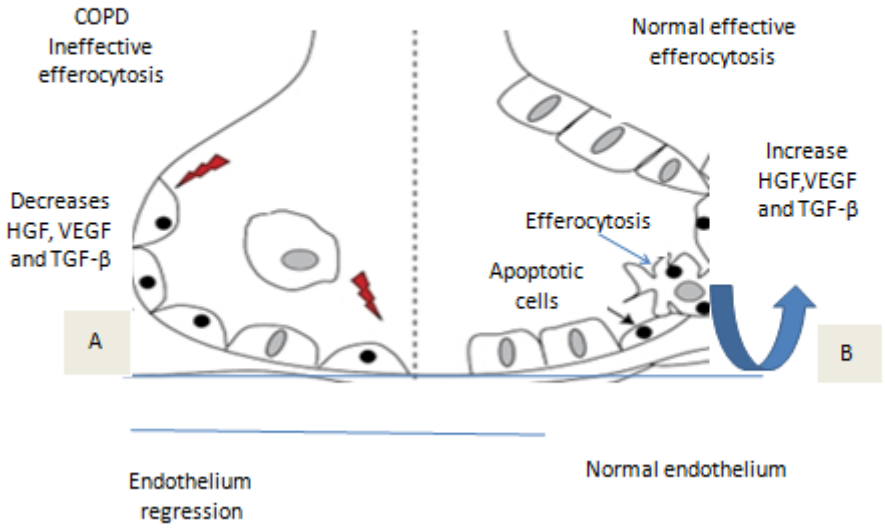


Figure 1.18| Involvement of inflammatory mediators in apoptosis. Ineffective efferocytosis (effective removal of dead cells by phagocytic cells) result in endothelial cells regression and matrix turnover. (B) Normal removal of dead cells maintains balance and prevents excessive release of inflammatory mediators that may cause erosion of the endothelium (Edward *et al*, 2009).

Programmed cell death is the principle of tumour prevention and usually occurs in normal cells as a protective mechanism. In addition, oncogenes that become active in a tumour inhibit the apoptosis of abnormal cells (Green and Evan 2002; Lowe *et al*, 2004).

The elimination of unwanted cells is regulated by apoptosis, which is a highly organized process of programmed cell death (Figure 1.16). Two different pathways, depending on the source of death stimuli, have been described in the cell cycle, intrinsic and extrinsic pathways (Figure 1.19), which they categorized according to the source of stimuli. The first pathway (intrinsic pathway) is stimulated by internal stress signals such as DNA damage, starvation and the oxidative stress (Scmitt and Lowe, 1999; Mayer and obubauer, 2003). In turn, the mitochondrial proteins leak to trigger apoptosis (Hengarner *et al*, 2000; Saelens *et al*, 2004). Upon release of mitochondrial proteins, cytochrome C initiates a dismantling of cellular components, leading to cell death (Degeterve *et al*, 2003). The second pathway (extrinsic pathway), in which the external sources such as cytokines activate the extrinsic pathway by the binding of the death

ligand of TNF superfamily and TNF-related apoptosis to cell surface death receptors (TNF receptor 1 and 2) (Walczac and Krammer, 2002; Lavrik *et al*, 2005).This in turn, activate the pro-caspase-8 after formation of death inducing signalling complex (DISC), leading to induction of mitochondrial permeability or triggering a protease cascade, which ends by death (Scaffidi *et al*, 1998; Fluda *et al*, 2002).

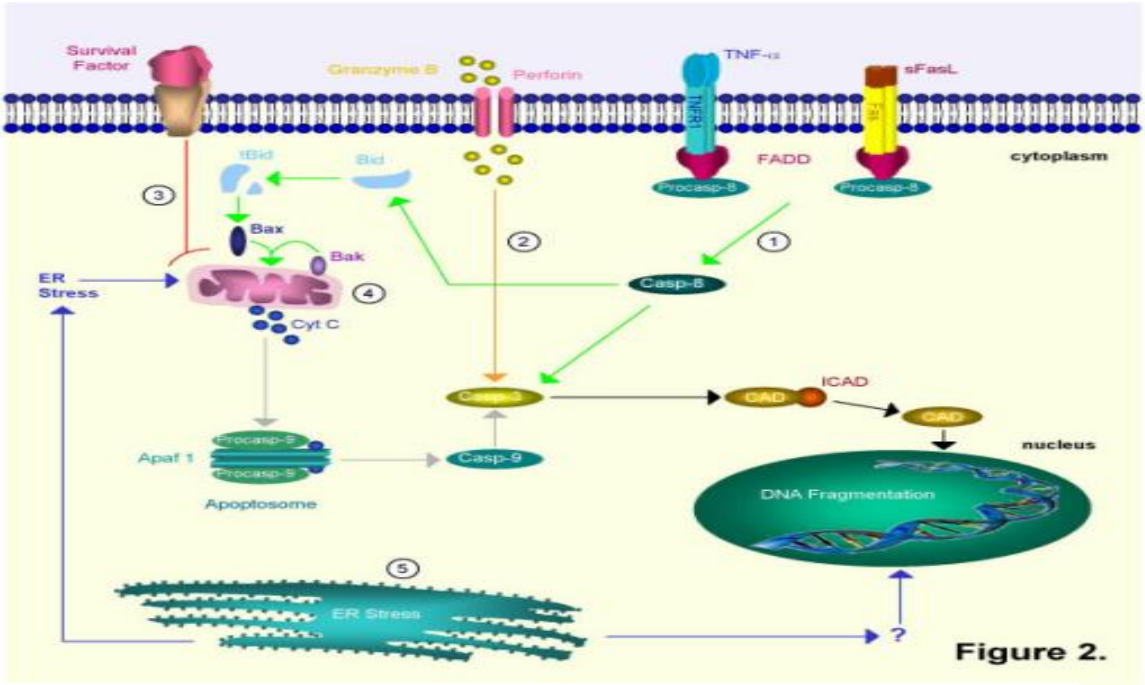


Figure 1-19| Different pathways of apoptosis in COPD and pulmonary emphysema, Intrinsic and extrinsic stimuli initiated by internal and external stimuli, which mediates two different pathways (Demedts *et al.* 2006).

Several studies carried out in COPD lung were focussing on apoptosis. Imai and colleagues (2005) pointed to decrease in the apoptosis in lining epithelial surface and endothelium in COPD patients and concluded the reduction in level of apoptosis of inflammatory cells could be responsible for chronic inflammatory process.

1.9.1 Effect of glucocorticoids on apoptosis

Glucocorticoids are used mainly as anti-inflammatories and immunosuppressant, however they are also able to induce apoptosis in many cell types and tissues (Chrouses *et al*, 2007). In glucocorticoid-sensitive cells, the GR mediates glucocorticoid apoptotic effect. The classical signalling and modulation is mediated by the high expression of

GR-α, while the role of GR-β is not well-defined and may be inversely correlated with the inhibition of GR-α.

Various isoforms of GR-α translation are related to different apoptotic rates. Apoptosis is induced by a group of proteins known as caspases. The caspase proteins are activated through the mitochondrial pathway. One of the immunomodulatory activities of glucocorticoids is to induce T-cell apoptosis. There is evidence that in the majority of white blood cells glucocorticoids use the intrinsic pathway to induce apoptosis by affecting pro-apoptotic members of the Bcl-2 family (like Bim) or blocking the anti-apoptotic members (Han *et al*. 2001, Wang *et al*. 2003, Lu *et al*. 2007). Moreover, in one study of glucocorticoid-induced apoptosis, the authors showed that there was a difference between cell lines, with the mitochondrial pathway being activated by glucocorticoids only in certain types of cell. (Sionov*et al*. 2006).

The purpose of steroids in the management of lung cancer is to enhance cell death or apoptosis, however treatment commonly fails. In addition, glucocorticoids are used as adjuvant therapy in the treatment of solid tumours to reduce oedema, pain and electrolyte disturbance. They are also used in conjunction with chemotherapy because of their pro-apoptotic properties in lymphoid cells and because they can reduce the toxic effect of chemotherapy in healthy tissue (Rutz, 2002, Rutz; Herr 2004).

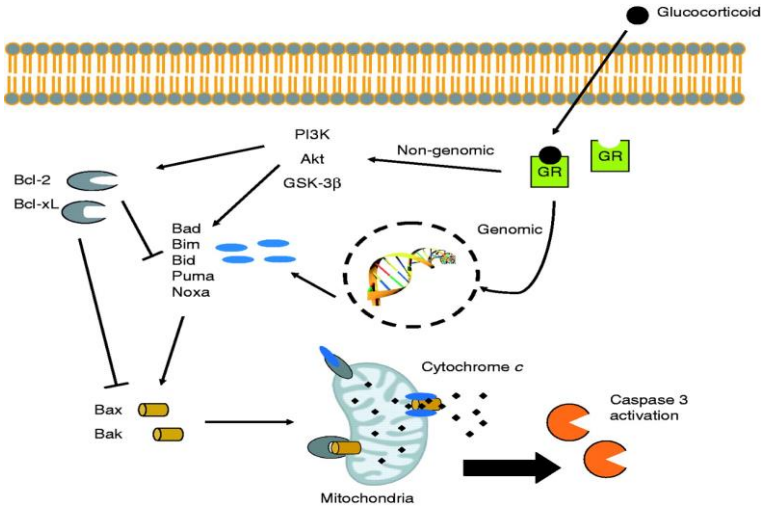


Figure-1.18|. Mechanism of glucocorticoid mediated apoptosis. Genomic and non-genomic pathway of glucocorticoid mediated apoptosis, leading to activation of caspase via mitochondrial pathway. (Elmore *et al*, 2007)

Aim and hypothesis

The risk of lung cancer among COPD patients is higher than in heathy individuals. Moreover, the degree of structural change and the variety of cells recruited in COPD patients is different from those in the case of other inflammatory diseases like asthma. Treatment failure is common in COPD, and glucocorticoid resistance in COPD and lung cancer is considered the main issue. Our study highlights the effect of glucocorticoid hormones on the transcriptional activity of glucocorticoid receptors. We hypothesize that the stress cofactor TTC5 is important for the stability and the post-translational modification of glucocorticoid receptors. In this thesis, we examine the importance of glucocorticoid receptor phosphorylation on the transcriptional activity of glucocorticoid receptors, in lung cancer cell line (A549), as well as lung tissues of healthy and COPD patients, also, we hypothesize that TTC5 interacts with phosphorylated glucocorticoid receptors at multiple sites including S211, S226, and affects the activity of glucocorticoid receptors

There is evidence suggesting that inflammatory mediators such are IL6 have an orchestrating role in the pathogenesis and the course of chronic obstructive pulmonary disease. One cross-sectional study showed that increased IL-6 in the sputum of COPD patients is directly proportionate to the disease severity. Based on these findings, our study will examine the expression of IL-6 in the peripheral lung tissue of COPD obtained from different groups of patients compared to healthy individuals. Moreover, on tissue level, we will examine the expression of glucocorticoid receptors and of the novel stress cofactor TTC5 in the macrophages and the epithelial surface, and in particular, how this expression varies with smoking status and the rate of decline in the pulmonary function. These findings could contribute to development of novel biomarkers of disease progression and drug sensitivity and improve therapy.

Chapter 2 Material and methodology

2.1.1 Methodology

Three procedures were used in this research namely maintaining the cell lines and western blot technique, co- immunoprecipitation and immunohistochemistry.

2.1.2 Maintaining the cells

Human alveolar adenocarcinoma cell line (A549 cells) were maintained in a Roswell Park Memorial Institute (RPMI) culture media. . Sterile media was with sodium bicarbonate and without L-glutamate which acts as intermediary between ammonia and ammonic acid and as nitrogen source *in vivo***.** About 90ml of media with 1% penicillin was supplemented with 10ml foetal bovine serum (FBS) and 1ml glutamate. A549 cells were inspected on regular basis under a light microscope for possible bacterial or fungal infection and contamination.

2.1.3 Feeding of the cells

Feeding of cells with nutrients and amino acids was required to maintain healthy cells. The media was replenished every 2 to 3 days, the media was prepared under as a septic technique. For safety and infection control purpose, the pipettes were changed in every step in order to avoid microbial contamination. The cells washed two times with warm sterile PBS and the existing media replaced by new one then the cells incubated in 37C (5% CO2).

2.1.4 Splitting of the cells

The aim of splitting of the cells was to maintain the cells' growth in optimum condition with enough nutrient supply. The splitting was performed whenever the confluence of the cells was 70% or more. Safety and infection control measures was followed before the procedure. The trypsin and PBS were wormed to match body temperature for enzymes activity at 37^0C, then the cells washed twice with warm PBS. In the following step about 1ml of Trypsin was added to the cells and left for about 3 minutes in 37^0C. The flasks were tapped from the sides to detach the cells from the flask and then observed under the microscope to ensure complete detachment. Once the cells were detached, they were counted using haemocytometer under the microscope. The trypsin was deactivated by adding 10 ml of new media. The following step was dividing the amount of the media between two or more flasks depending on confluence and then filled up to 10 ml of media was added to each flask.

2.1.5 Counting of cells

The cells were counted using a sterile haemocytometer. About 25µl of well mixed cells were added to 100µl of trypan blue dye and then loaded on the sides and observed under light microscope using 10x power. The vital cells which were located at four side chambers of the haemocytometer were counted and the dead cells which were blue were not counted. The total amount of cells in the four chambers are divided by four.

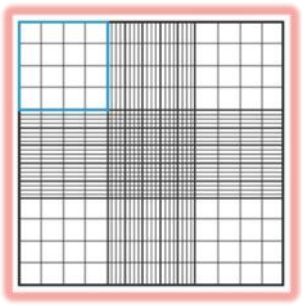


Figure 2.1 haemocytometer under the microscope.

2.1.6 Freezing of the cells

In order to freeze cells, cells were washed twice with sterile PBS followed by detaching the cells with 1ml trypsin and incubation in 37^0C for three minutes. The following step was adding 10 ml of media, centrifuge cells for 2 minutes at speed of 2000 rpm and wash them with PBS. The final step in the freezing was adding 1800 µl FBS and 200µl DMSO- (Dimethyl sulfoxide).The samples left in -20 for 24 hours before transferring into the -80 degree.

2.1.7 Cell treatments

The drug of choice was dexamethasone (1µM per 10 ml culture media for 16 to 18 hours). Dexamethasone is manufactured steroid which possesses anti-inflammatory and immuno-suppressant effect, the potency of dexamethasone is 25 times stronger than glucocorticoid, however it is weak mineral-corticoid hormone.

It is widely prescribed in management of allergic and immunological conditions such as asthma, chronic obstructive pulmonary disease, fibrosis, rheumatic diseases etc.

Before the treatment with dexamethasone, the media was supplemented with 1 ml glutamate and 10% DDC/FCS instead of FCS.

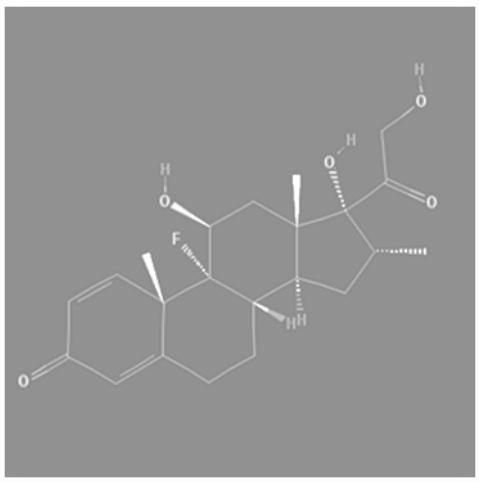


Figure 2.2| chemical structure of dexamethazone. Adopted from http/pubchem.ncbi.nlm.nih.gov/compound/5743

2.1.8 Protein extraction

Glucocorticoid receptor is a cytoplasmic protein and the aim of extraction was to analyse the protein condition and the affinity to dexamethasone hormone (treated cells with dexamethasone) relatively to control cells. The extraction of the protein was carried out using 120 µl high salt lysis buffer containing protease inhibitors per well that were previously washed with cold BPS three times. Protein extraction was done on ice to keep the proteins stable and the extracted protein was collected in Eppendorf tubes. Then the samples were rotated for 20 minutes followed by centrifuged for another 20 minutes at 12 rpm in 4C.

The samples collected and the pellets discarded. The protein concentration was measured by Spectrophotometer (protein absorbance measured at 595 nm. Jenway, Genova) aimed to have the same quantity of protein in both treated samples and the control samples. Acquiring the same amount of protein was essential to avoid false-positive or false-negative results. Moreover, the protein concentration was measured twice in order to acquire an average reading. Each sample was a mixture of 200µl of Bio-Rad reagent plus 800µl of distilled water and about 2µl of the extract were added to each sample before measuring the amount of protein (one sample was used as blank without any extract). After the measurement a simple calculation were used for quantification of protein of both control and treated cells, and then protein kept in -20^0 C with 3x DSD buffer before analysed by Western blot technique.

2.1.9 SDS- PAGE

SDS- PAGE is the way to determine protein amount and molecular weight. It is followed by the western blot analysis that uses antibodies to detect specific protein. Western blot technique is based on detection of molecular proteins on nitrocellulose membrane, thus enabling to identify specific proteins from target cells, this technique depend mainly on separation according to size of protein, and identifying target protein by using specific primary and secondary antibody.

2.1.10 Running

Before running the samples with 1x DSD buffer (made from 10x buffer that has made by dissolving 30.2g Tris base and 144g glycine), the sample was boiled at 95^0 C for 5 minutes and then the samples are subjected to 7.5 acryl amid gel (formula below), the samples run on 80 milliamp for stacking gel and 110 milliamp until the end of the resolving gel.

Acryl amide gel 7.5%

The resolving gel was prepared according to this formulation

Distilled water .....13.3 ml

Acryl amide 30% .....7 ml

1.5M Tris (Ph8.95).....7ml

0.2M EDTA 280µl

10% SDS.....280µL

10% APS.....157µl

TEMD.....17µl

The stacking gel was prepared according to this calculation

Distilled water 6.73ml

Acryl-amide 30%.....1.67ml

1M Tris (Ph6.95).....1.25 ml

0.2M EDTA.....100µl

10% SDS.....100µL

10%APS.....157µl

TEMED.....17µL

2.1.11 Transfer

Once running the samples finished, the polypeptide bands are transferred into the nitrocellulose membrane using 1x western transfer buffer, that was prepared by adding 100 ml of 10x western transfer stock to 200ml methanol and 700 ml dH2O, transfer was carried out for 2 hours, this step was crucial as the protein transferred from the gel into the membrane. Firstly the filter paper was soaked into a transfer buffer, while the transfer membrane submerged in methanol for 30 sec and then washed with the transfer buffer. A holder was prepared by assembling the following a sponge, filter paper,

transfer membrane, gel, a filter paper and a sponge from the outside, (bubbles were removed by rolling pin). The protein was transferred at 4^0 C by using ice and magnetic starrier that was placed inside.

2.1.12 Blocking

The aim of blocking was to avoid any non-specific interaction between the antibody and non-specific proteins. This step was carried out by incubating a Imobilon P membrane in blocking mixture (5% of skimmed powder milk in PBS buffer) for one hour at room temperature on a shaker.

2.1.13 Incubation with primary antibody

The membrane was incubated in each experiment with specific primary antibody to the protein of interest overnight at 4^0 C on a rotator (about 3 µl of primary antibody were added to milk/PBS/ 0.1 % tween.

2.1.14 Incubation with secondary antibody

Prior to the development which was on the second day, the membrane was washed three times with PBS tween for 10 minutes interval, and then incubated with the secondary antibody for one hour at room temperature (from 2-4 µl of secondary antibody was added to 10 ml of milk/PBS/tween

2.1.15 Development

This was the last step in western blot technique. It was done by incubating the membrane from 1-2 minutes in enhancer ECL mix (about 1000µl from each bottle of western pico), then developed by X-ray machine.

2.1.16 Stripping

It was useful to strip the membrane in order to reuse it with another antibody. The stripping was applied to the nitrocellulose membrane considering the following three steps. First step was to prepare the chemicals by adding exactly 10ml of 10% SDS to 3.2 ml of Tris (PH8.5) in a universal tube, and the completed with distilled water up to 50 ml of the universal tube. Finally under the chemical hood 350µl of 2-mercaptoethanol was added to the total amount. The second step was to place the membrane into autoclave where heated up to 50 degree for exactly 30 minutes, agitation applied sometimes aimed to remove any chemicals from the membrane. The last step was washing the membrane on a shaker twice with PBS tween for about 10 minutes, and then blocked with 5% of skimmed powder milk in 1 times TBS buffer before incubation with another antibody.

2.2 Co-immune-precipitation

It is a common purification technique usually to detect any possible interaction between two molecules of proteins, by using specific antibody to the protein of interest (target protein). The aim of the Co-immuno-precipitation in this research was to detect any possible interaction between different forms GR (H-300, phosphorylated S211 and S226) and TTC5 in physiological condition *in vitro*. The TTC5 was the protein of interest which added to cell lysate and identified each time by different antibody of GR.

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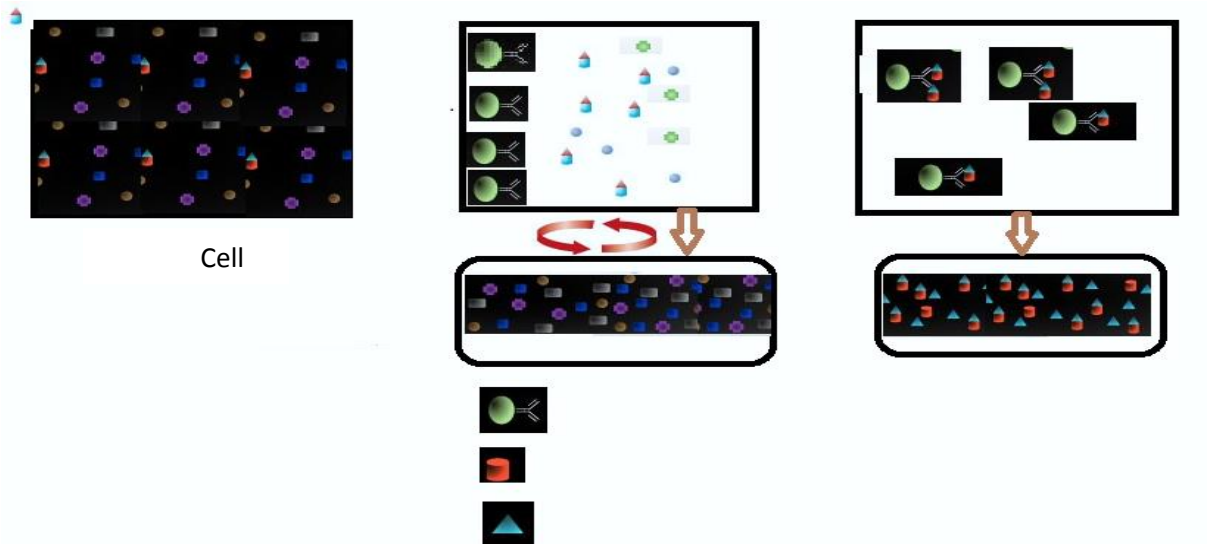

Figure 2.3| Co-immunopricipitation process using protein A sepherose. (http://www.assay-protocol.com/Immunology/Co-IP).

Co immunoprecitation was carried out as the following:

2.2.1 Cell maintenance

Obviously maintaining the cells and the splitting was the same as usual .However the buffer used for the protein extraction was 250µl of TNN buffer (50mM Tris PH7.5, 120mM Na Cl, 5mM EDTA, 0.5% Igepal, 1µg/ml PI, 1mM DTT, 1mM PMSF, Sodium orthovanadate , 5mM Sodium pyrophosphate, 20 mM β- glycerophosphate) per well plate instead of high lysis buffer .

About 20 µl of protein A sepherose beads (magnetic beads, Sigma) washed three times with 200µl of the same puffer used for cell lysate which is TNN buffer.

Some samples were saved as input in -20 and the remaining of the samples incubated with the primary antibody for the protein of interest (TTC5) overnight at 4C. In the second day the samples centrifuged at 12000 rmp, for 3 minutes and the pellet washed three times with TNN buffer at the same rate. The last step were adding 30 µl of SDS .before analysed with indicating antibody by western blot the samples boiled at 90 C for 5 minutes.

2.2.2 Immunohistochemistry

In this procedure, the monoclonal and polyclonal antibodies was used to observe in situ detection of antigens in lung tissues. The aim was to detect expression of glucocorticoid receptors and protein distribution in the lung tissues in different stages of COPD compared to clinical data for each group. Samples was obtained by bronchoscopy and lobectomy from COPD patients (permission and written consent has been taken from the patients). 39 patients were involved in this study and have been categorized according to NHS classification into 5 groups. Healthy ex-smokers, mild COPD, moderate COPD, Sever COPD and COPD with lung cancer.

The samples underwent many steps before the investigation. The first step was embedding and cutting in which, the tissue cut into small pieces and kept in cuvettes at room temperature. The cuvettes first stored overnight in 10% formalin and then processed through several different concentration of ethyl alcohol, 70% ethanol for 1 hour, 90% ethanol for 40 minutes and 100% ethanol for 40 minutes for 4 times, then transferred into Xylene1 and Xylene2 for 30 minutes respectively. The last step in the processing was transferring the tissue into a new cuvette were the wax added and the left over night in cool place.

2.2.3 Patient's demographics

39 patients from Wythenshawe hospital were investigated and analysed compared with clinical values (smoking status, FEV1, FEV1/FVC ratio) after written consent using immune-histochemistry technique. The lung biopsies in this research obtained from different regions in the lung with safety distance from the tumours, and processed into different concentration of ethyl alcohol, then cut by microtome into slides of 5µm in diameter The pathophysiological changes were similar in each group and airway obstruction was assessed by measuring FEV1/FVC ratio and predicted FEV1 (The standard deviation showed no significant difference in each group. All the clinical data and pathological characteristics are summarized in the table below.

	Healthy non-smokers	Heathy ex-smokers	Healthy smokers	COPD ex-smokers	COPD current smokers
Number of patients	3	10	9	9	8
Age	73.3 (SD 6.8)	70.3 (SD 12.2)	59.5 (SD 10.3)	68.3 (SD 8.1)	68.25 (SD 5.7)
Gender	2Female, 1Male				
FEV1	2.2 (SD 0.39)	2.19 (SD 0.45)	2 (SD 0.7)	1.38 (SD 0.36)	1.76 (SD 0.4)
FEV1 predicted	101.6 (SD2.35)	96.3 (SD 22.1)	86.5 (SD 19.3)	60.7 (SD 6.5)	72.7 (SD 10.48)
FVC	2.81 (SD 0.36)	2.8 (SD 0.67)	3 (SD 0.81)	3.5 (SD 1.04)	3.1 (SD 0.7)
FEV1/FVC ratio	78.72 (SD 3.8)	77 (SD 7.1)	68.7 (SD 9.3)	51 (SD 9.8)	57 (SD 8.4)
Height	162 cm				
Smoking history	Never	EX-smokers Past history 35.3pack/year SD (15.5)	Current smoker(42.4 P/year) SD (17.1)	Ex-smokers with COPD Stage II (33.9 Pack/ year) SD (21.7)	Current smokes with different stages of 6 patients with stage II, and two patients in stage I and III.
Medication	Non	Non	Non	Bronchodilators+ Long acting beta-agonists in combination with steroid	Bronchodilators, long beta agonists in combination with steroid

				(Seretid discus) and/or anticholinergic (Spiriva).	(Seretid discus) and/or anti cholinergic drugs (Spiriva)
Pathological findings	Normal architect-ure of lung tissue without obvious alveolar destruction seen	Variable number of macrophages with non-distinguish border and angiogenesis in the majority of the patients, however no evidence of alveolar destruction	Few to Moderate number of macrophaages with no alveolar destruction	Moderate to large number of macrophages associated with alveolar destruction in some patients moreover tar deposition.	Mild to moderate number of macrophages associated with alveolar destruction and some epithelial disruption.

Table 2.1 illustrates patient's demographics and histopathological changes in each group of patients (complete patient's demographic supplemented in table 4.1)

2.2.4 Optimization

The aim of optimization was to confirm specificity to antibodies and to achieve the best dilution for the antibodies relevant to pre-treatment condition. And it was important to test serial sections with different buffers to the same antibody.

	Antibody dilution for IHC	Antibody species (and associated vector kit)	Pre-treatment needed (y/n)	Pre-treatment buffer	Company order code + clone
IL-6	1:100	mouse	n	CB	abcam AB9324 500µg providedand diluted in 500µg to yield 1mg/1ml
Total GR H-300	1:1000	Rabbit-polyclonal	n	n/a	SantaCruz sc-8992 Provided at 200ug/ml
S226 GRphospho 226	1:100	Rabbit polyclonal	y	TEB	Abcam:ab536925 µg/ml, heat antigen retrival CB PH6 Recommended by Abcam
S211 GRphosphop horylated at 211	1:1000	Rabbit polyclonal	y	EB	Cell Signaling #4161100ul provided but no info on concentration
TTC5	1:500	Rabbit polyclonal	n	n/a	Abcamab36855U se at 10ug/ml

Table 2.2 serial dilution for different antibodies (TGR, S211, S226, TTC6 and IL-6)

2.2.5 Immunohistochemistry technique

The first step in the procedure was dewax by the tissue by histoclear reagent (National diagnostic) I and II for 5 minutes, aimed to deparaffinise the slides. Then the slides transferred into different concentration of alcohol100% for 5 minutes, 90% for 3 minutes, 75% for 2 minutes, 75% for 2 minutes and 50% for 1 minutes then the slides placed into a running water for 5 minutes before pre-treatment with different buffers before the pre-treatment with different buffers according to the optimization carried out before the incubation with the primary antibodies

The aim pre-treatment was aimed for expose epitopes retrieval(the binding part of the receptors) , which may be masked by formalin during fixation procedure . Heat applied with different solutions for epitope retrieval. Citrate buffer prepared by dissolving 2.1g of citrate in 1000ml distilled water (PH 6 with 1M HCL). 1mM EDTA buffer prepared by dissolving 0.37 g of EDTA in 1000 distilled water and PH to 8. And Tris-EDTA buffer prepared by adding 1.21g Tris base and 0.37g EDTA to 1000ml distilled water (PH to 9 where 0.5 ml tween 20 was added).

Blocking serum was important step in order to prevent non-specific hydrophilic binding between non-specific protein and primary antibody other than those attractive to receptors. Blocking the normal serum carried out by incubation of 100 µl (15µl of normal serum in 1000 µl TBS.

The last step in the first part of the immunohistochemistry was incubating the samples in the primary antibody for about 2 hours up to 18 hours the primary antibody was added to the normal blocking serum and 100µl was applied.

In the second part and before the incubation with secondary antibody, the samples washed three times with PBS tween, then the samples incubated in Vector biotinylated secondary antibody which was prepared by adding 15µl of normal blocking serum and 5µl of secondary anti (mouse/rabbit) IGg to 1ml TBS. The incubation with the secondary antibody was 1 hour, and then washed again with TBS three times.

After the incubation with the secondary antibody and to avoid inconvenient staining it was necessary to block endogenous peroxidase by incubating the samples in 3% H_2O_2 with methanol for half an hour, (the mixture was prepared by adding 48.5 ml of methanol to 1.5 hydrogen peroxide3%).

Before washing with TBS and running water for 5 minutes the ABC (Avidin-biotinylated complex) prepared and left for 30 minutes in room temperature. Any biotinylated molecule can be detected by ABC because of high affinity property of avidin to biotin.

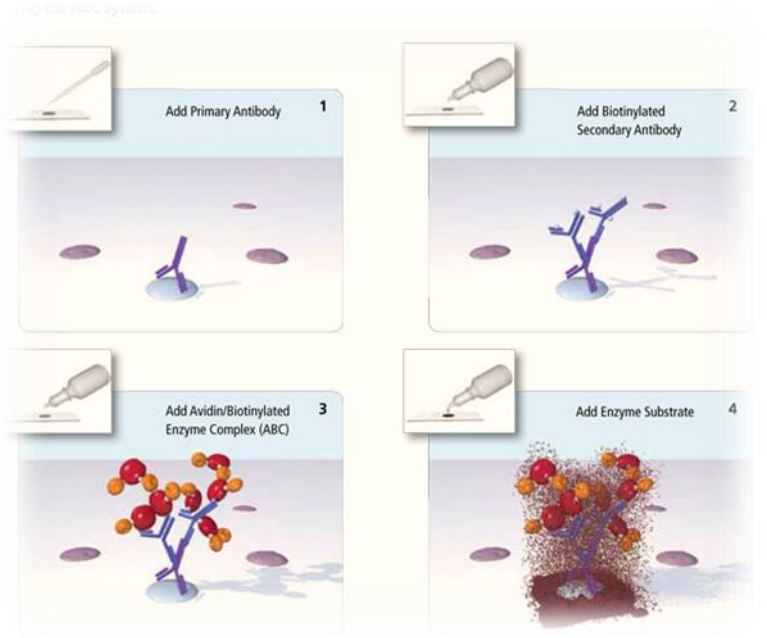

Figure2.4| using ABC system. (https://www.vectorlabs.com/uk/catalog.aspx?catID=42)

The samples washed three times by PBS tween, then incubated with DAB substrate (3, 3' diaminobenzidine), dark brown reaction was the end result under the microscope and this reaction stopped by distilled water. And the haematoxylin stain was applied from 20 to 40 seconds and rainsaned with excess of water (Haematoxylin is diagnostic dye in histology field and counter stain to nuclei in immunohistochemistry. the nuclei are stained blue as oxidized haematoxylin bound to aluminium ions. And before the last step of dehydration, the slides put under running water. The dehydration was by different concentration of ethyl alcohol as following. 50% ethyl alcohol for 1minute, 75% ethyl-alcohol for 2 minutes,
75% ethyl-alcohol for 2 minutes, 90% ethyl-alcohol for 3 minutes, 100% ethyl-alcohol for 5 minutes lastly clearance was through xylene II and xylene I for 5 minutes each

respectively

then the slides covered with slip s were DPX had applied

2.2.6 Image-J

Image-j is analytical program, its idea adopted from NIH image which was developed first time by research service branch (RSB) of the natural institute of mental health. It can be downloaded for free from **http://rsbweb.nih.gov/ij/download.html.**

Multiple tasks can be performed by image j including quantification of protein amount in western blot, also it is important tool in the pathology field which can be used in counting the number of cells like Macrophages, measurement of surface area in tissues and density histogram, the steps of cell counting are explained in the following diagram

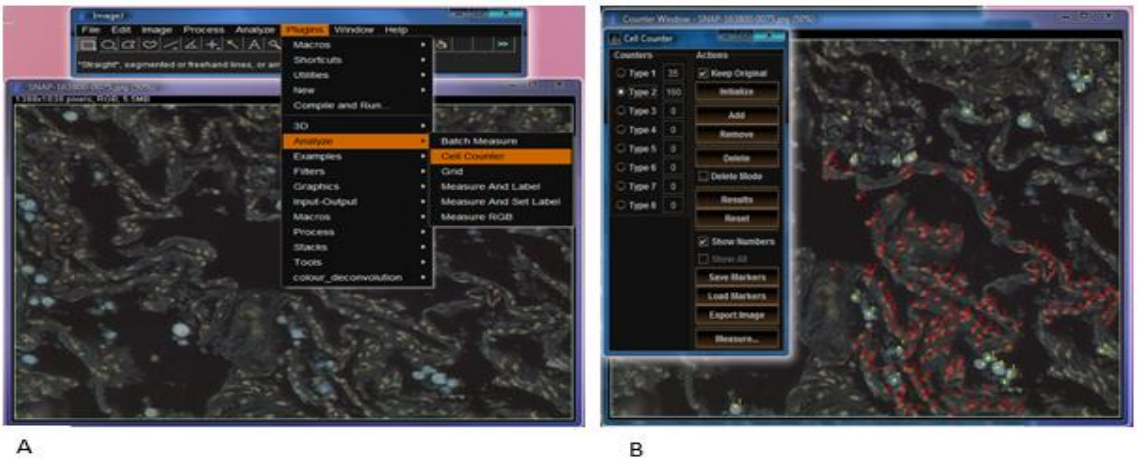

Figure 2.5 Plugins-Analyse-Cell counting process by image-j. A shows multiple windows available on the front, starting by file image option, and plugins window which is characterized by analysing the slides through cell counting icon. In picture B illustrates analysis process which involving many characters like counting each type separately and total calculation at the end of counting.

2.3 Material

Different materials were used in processing the lung tissues and different antibodies as well as chemical reagents used for western blot and Co-immune-precipitation. All the materials are mentioned in the table below as well as the supplier and the product code.

2.3.1 Substances

Different chemicals were used in this research including buffers, steroid drugs and different antibodies

2.3.2 List of chemical reagents

Name of the chemical	Supplier		Product code
Dexamethazone	Sigma		81K1100
Penicillin	Labtech		LM-A4118/100
Formalin			
Hydrogen peroxide			
Histoclear I (d-limonene)	National diagnostic		
Histoclear II	National diagnostic		
DPX			
Citrate			
EDTA	Sigma-Aldrich, UK		E6760
Tris EDTA			
Ethyl alcohol	Fisher, UK		E/0600/05
FCS/FBS(Dextran coated charcoal treated FBS)	Supplier, UK		

Vericol	Fisher, UK		005414182
Trypsin (500ml)	Labtech		LM-T1705/500
(Dimethylsulfoxide) hyprid-max-sterile filtered (DMSO)	(SLS) Scientific Laboratory Supplies, UK		D2650-5X5ML
Biorad protein essay	Bio-Rad		500-0006
SDS	Fisher UK		S/P530/48
High Lysis Buffer	1		
TTN buffer	2		
30% Acryl	National diagnostic		Ec-890
0.2M EDTA			
Page Ruler Prestained protein ladder	Fermentas, UK		SM0672
TEMD (N.N.N.N) Tetramethylethylenedaimine	Sigma		BCBH1254V
Tris base	Fisher, UK		BP152-1
PMSF (Phenylmethanesulfonyl Fluoride)	Sigma		044K0157
BGP (Disodium Pentahydrate)			
DTT	Sigma UK		D0632
Na OV	Sigma UK		S6508

APS 10% (Ammonium per sulphate)	Flowgen, UK		H17423
Glycine	Fisher		1211411
Sodium dodecyl Sulphate	Fisher		114840
Na PPI			
TBS buffer	Fisher		10214733
(PBS) Phosphate buffer saline 10X	Fisher, UK		
Tween 20	Sigma		029Ko1855
Western Pico enhancer	Thermo		N178744
Western Femto super-signal	Thermo		NH173984
2-Mercaptoethanol	Sigma-Aldrich, UK		M3148
Sodium pyrophosphate			
Methanol	Fisher UK		BPE1105-1
Haematoxylin			
ABC reagent	Vectastain		PK-6100
DAB substrate			PK-6100
Goat serum	Vectastain		
Total GR (H300)	Santa Cruz Biotechnology		SC-8992
Phospho GR (S211)	Cell Signaling		4161
Phospho GR (S226)	Abcam		Ab 53692

TTC5 (Tetratricopeptide repeat domain 5)	Abcam		Ab36855
Interleukin 6	abcam		Ab 9324
B-actin	abcam		ab8227
Glycerol	Fisher chemical		1291109
Secondary anti-mouse	GE-Healthcare		5356526
Secondary anti-rabbit IgG	GE-Healthcare		5272514
Actin	Abcam		Ab8227
X-ray	Fuji film UK		Super Rx

III. Results

3.1 Determination of GR and TTC5 protein levels in lung cancer cell line

The main purpose of western blot in adenocarcinoma cell line (A549 cells), was to investigate the difference in protein expression after treatment with long acting corticosteroid (Dexamethasone). Three main forms of glucocorticoid protein (total GR, S211, S226) were investigated in addition to TTC5 (tetratricopeptide repeat domain 5) and Interleukin-6. The procedure was performed by making a protein extract from control and hormone treated cells, and by loading the whole cell lysate on 7.5% polycrylamide gel. Actin protein was used as a loading control. The quantity of target protein was measured by image-J comparative to the control (Figure 3.1).

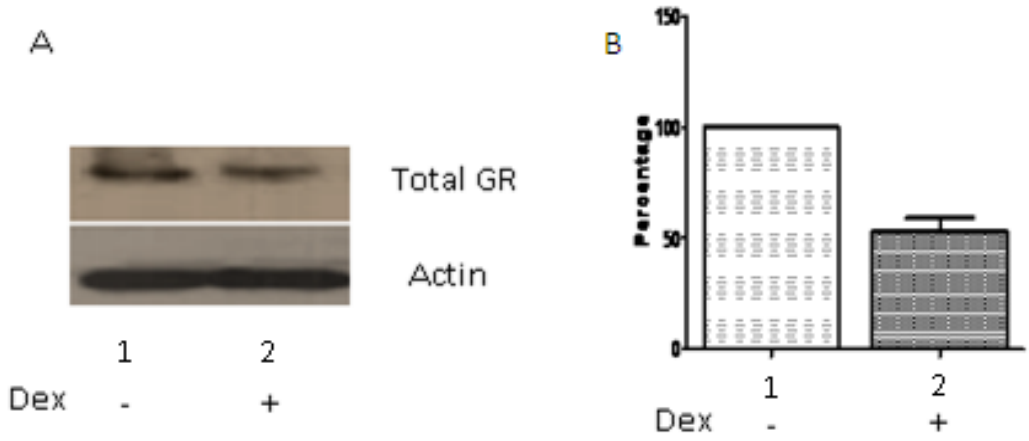

Figure 3.1| Measurement of protein expression (total GR) in A549 cells**.** (A) The level of expression in total GR relative to actin gene in A549 cells treated with 1µM dexamethasone. The whole cell lysate was analysed by SDS-PAGE using 7.5% gel, this was followed by western blot transfer and antibody analysis with total GR and actin. (B) Protein expression of TGR. The percentage of expression was obtained by normalization of non-affected gene (Actin). The results represents the averages of five different experiments (supportive figures 5.1), and the error bars represent SD (13.71) (figure 3.1 B). P value < 0.01 (shown in supplementary figure, table 5.2).

In this series of experiments the protein level of total GR was measured in A549 lung cancer cell line (Figure 3.1 and supplementary figure 5.1 in the appendix).). Protein expression of TGR shown in lane 2 compared to lane 1, was down-regulated by 53% in A549 cells treated with dexamethasone as compared to un-treated cells (figure 3.1 A and B, compare lane 2 and lane 1). Results show that GR is down-regulated (figure 3.1 A, lane 2) in A459 cells treated with dexamethasone.

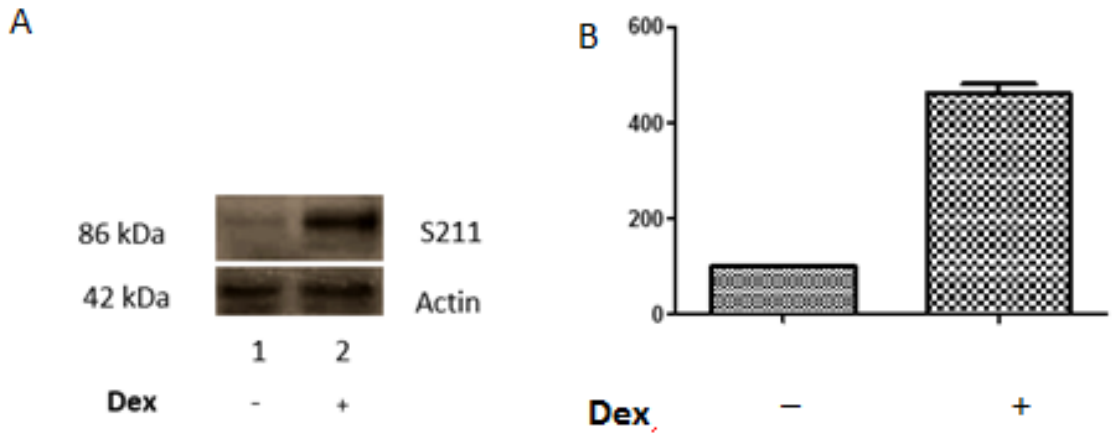

Figure3.2| Measurement of protein expression (S211) in A549 cells. (A) The level of expression in phosphorylated GR at S211 in treated A549 cells with 1µmol dexamethasone. The whole cell lysate was analysed by SDS Page using 7.5% gel, this was followed by western blot transfer and antibody analyst with S211 and actin. (B) Relative protein expression of S211. The percentage of expression was obtained by normalization of non-affected gene (actin). The result represent the averages of three different experiments (supportive figures 4.2), and the error bars represent SD (28.28), (figure 3.2 B). P value< 0.01 (shown in supplementary figure, table 5.2).

In order to analyse GR phosphorylation status in lung cancer, GR phosphorylated on serine 211 was followed by SDS PAGE (Figure 3.2 and supplementary figure 5.1 in appendix). Protein expression of S211 shown in lane 2 compared to lane 1, was massively up-regulated in A549 cells treated with dexamethasone as compared to un-treated cells (figure 3.2 A and B, compare lane 2 and lane 1). Results show that S211 is up-regulated (figure 3.2 A, lane 2) in A549 cells treated with dexamethasone.

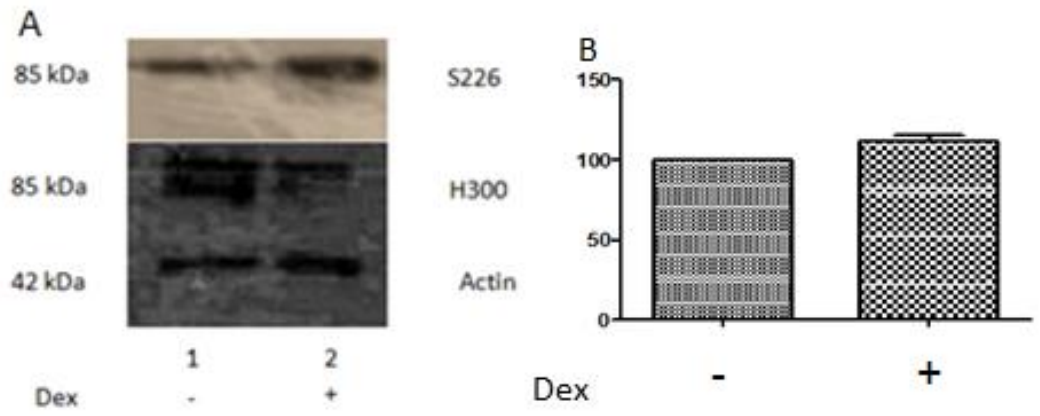

Figure3.3 Measurnement of protein expression (S226) in A549 cells. (A) The level of expression in phosphorylated GR (226) related to actin gene in A549 cells treated by dexamethasone. The whole cell lysate was analysed by SDS Page using 7.5% gel, this was followed by western transfer and antibody analysis with S226 and Actin. (B) Quantification of protein expression. The percentage of expression was obtained by normalization of non-affected gene (actin). The results represents the averages of five different experiments (supportive figures 4.2), and the error bars represent SD (5.85) (figure 3.3 B). P value < 0.05 (shown in supplementary figure, table 5.2).

In this series of experiments the protein level of S226 was measured in A549 lung cancer cell line (figure 3.3 and supplementary figure 5.1 in the appendix). Protein xpression of S226 shown in lane 2 compared to lane 1, was up-regulated by 11% (figure 3.3A, lane 2) in A549 cells treated with dexmethasone.

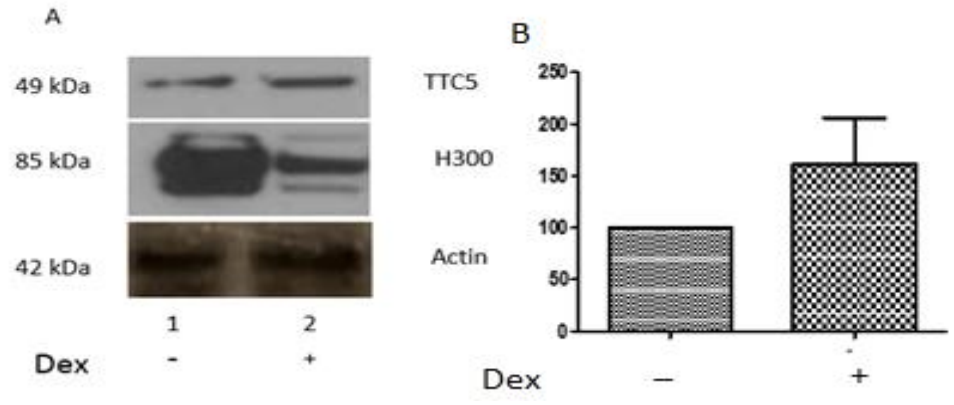

Figure 3.4| Measurement of protein expression (TTC5) in A549 cells. (A) The level of expression in TTC5 (Strap) related to actin in A549 cells treated with 1µM dexamethasone. The whole cell lysate was analysed by SDS Page using 7.5% gel followed by western blot transfer and antibody analysis with TTC5 and actin. (B) Quantification of protein expression using image J. The percentage of expression was obtained by normalization of non-affected gene (actin). The results represent the

averages of three independent experiments (supportive figures 5.1), and the error bars represent SD (62.93) (figure 4.1 B). P value < 0.05 (shown in supplementary figure, table 5.2).

The protein level of TTC5 was measured in A549 lung cancer cell line (Figure 3.4 and supplementary figure 5.1 in the appendix). Protein expression of TTC5 shown in lane 2 compared to lane 1, was up-regulated by 80% in A549 cells treated with dexamethasone as compared to un-treated cells (figure 3.4 A, lane 2) in A549 cells treated with dexamethasone.

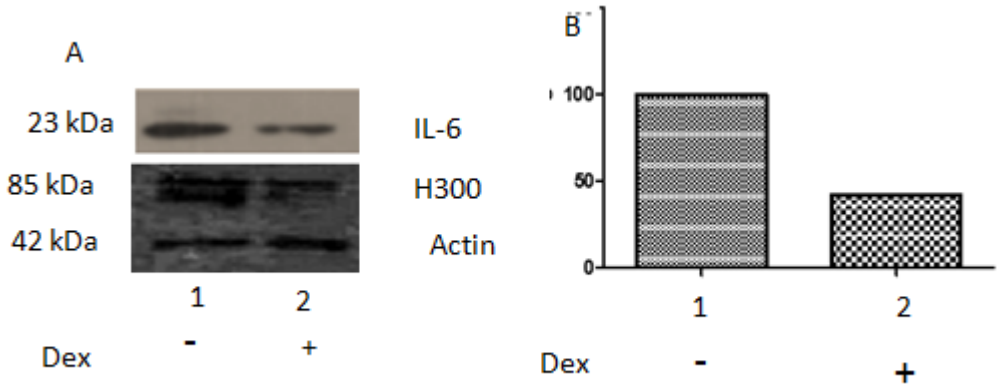

Figure 3.5| Measurement of protein expression (IL6) in A549 cells**.** (A) The level of expression in IL6 related to actin gene in A549 cells treated with 1µM dexamethasone. The whole cell lysate was analysed by SDS Page using 12% gel, followed by western transfer and antibody analyst with IL-6 and actin. (B) Protein expression of IL-6 in lane 2 compared to lane 1(figure 5A and B, compare lane 2 lane 1). The percentage of expression was obtained by normalization of non-affected gene (actin). The percentage of expression represent only one reading.

In this experiment the protein level of IL6 was measured in A549 lung cancer cell line (figure 3.5A). Protein expression of IL6 shown in lane 2 compared to lane 1, was down-regulated by 52% in A549 cells treated with dexamethasone as compared to un-treated cells (figure 3.5A, lane 2) in A549 cells treated with dexamethasone.

3.2 Conclusion of western blot experiments

In summary, in lung cancer cells treated with synthetic glucocorticoid dexamethasone, protein levels showed down regulation of total GR, slight increase in expression of the GR phosphorylated on S226, and substantial up-regulation of the GR phosphorylated on S211. On the other hand the expression of TTC5 was variable in three different

experiments. Preliminary results suggest that dexamethasone causes marked down regulation of IL6.

3.3 Analysis of protein-protein interaction using Co-immune precipitation

Glucocorticoid receptors mediate varied physiological processes. They interact with a number of proteins which are considered as co-regulators because they control gene transcription by repression or activation. TTC5 has been identified as co-regulator of p53 tumour suppressor that controls cell cycle progression and stress response (Demonacos et al, 2001). In this experiment we analysed possible interaction between glucocorticoid receptors and TTC5.

The aim of co-immunoprecipitation was to detect interaction between total glucocorticoid receptor protein, phosphorylated glucocorticoid receptor at S211 Serine 226 and TTC5. The procedure was carried out by incubation of the cell lysate with protein A-sepharose and antibody against the protein of interest (TTC5) were affixed to the complex and left in a rotator overnight at 4^0C. About 5% of cell lysate was saved as an input sample as shown in figure 3.6A (lane 1 non-treated cells and lane 2 treated cells upper panels). Co-immunoprecipitation was continued after overnight incubation, the samples were washed by TNN buffer then loaded on the same 7.5% acrylamide gel (figure 3.6A, lower panels). The immune-precipitates brought down using TTC5 antibody were analysed with H300 total GR specific antibody and non-specific secondary anti-mouse IGg. Extracts were incubated with separate cell lysate at 4^0C and loaded on the same gel to confirm there is no non-specific interactions.

The results showed down regulation of total GR protein levels isolated from hormone treated cells in the input sample in lane 1 and 2. At molecular weight 49 TTC5 levels increase with hormone treatment as shown in lane 1 and 2. On the other hand, in the

third panel co-immunoprecipitation showed that GR interacts with TTC5 in lung cancer cells and this, interaction increases in presence of dexamethasone (compare lanes 1 and in the 2) in the third panel. Panel four showed no interaction when nonspecific antibody was used. Quantification showed interaction between TTC5 and GR that was slightly higher in treated cells (figure 3.6B).

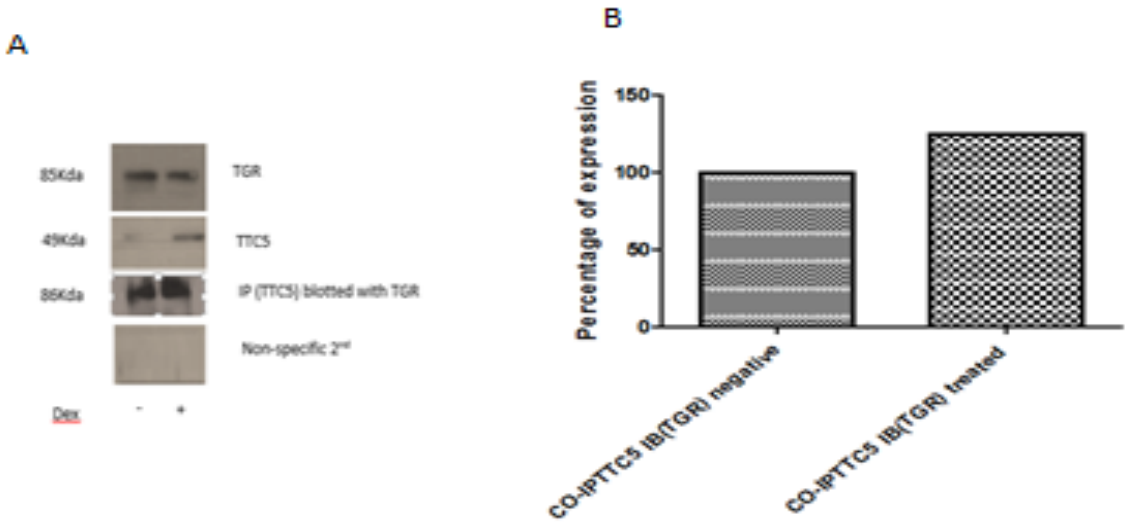

Figure 3.6 Interaction of TTC5 and total GR (A) Co-IP of TTC5 with total GR 40 µl of cell lysate (input) of non-treated cells and treated cells are loaded in lane l and 2 respectively (two upper panels). The immune-precipitant of treated and non-treated cells were shown in the third panel. Fourth panel shows non-specific anti-mouse IGg to ensure there was no non-specific interaction. (B) The results of quantification using Image J are shown (figure 3.6, B).

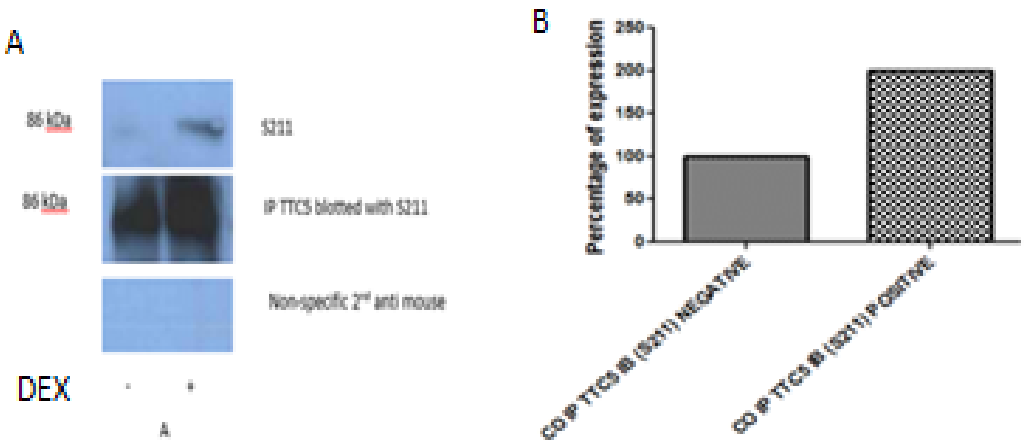

Figure3.7| TTC5 interaction with GR phosphorylated at S211. Above described experiment (Figure 3.6) was used to analyse western blot membrane with antibodies against GR phosphorylated at S211The experiment was done by loading 40µl of cell lysate as input (lane1 and 2) (upper panel) and incubation of the rest of the sample with 5µl of TTC5 antibody (both treated and non-treated cells) and developing the blot by incubating with antibody against phosphorylated GR at S211 (middle panel). (B) Quantification of results using Image J

In order to determine if GR phosphorylated on S211 interacts with TTC5, membranes were reprobed with antibody that specifically detects this GR phosphorylated form. Results show there is a strong interaction between GR phosphorylated at S211 and TTC5. However, this experiment needs to be repeated multiple times to confirm the findings.

3.4 Conclusion of the Co-immune precipitation

In summary, GR and TTC5 have been shown to interact in A549 cells according to one preliminary result (Figures 3.6 and 3.7, Supplementary figure 4. 2). This interaction increases in hormone treated cells and both total and GR phosphorylated at S211 interact with TTC5. However, given that there was more TTC5 in hormone treated cells in the input sample, more experiments are needed to confirm what the role of hormone is in controlling GR and TTC5 interaction.

3.5 Determination of protein expression in human lung tissues using immunohistochemistry

The main purpose of immunohistochemistry analysis was to investigate the extent of protein expression in healthy non-smokers, healthy ex-smokers, healthy smokers, ex-smokers with COPD and current smokers with COPD in relation to FEV1 and clinical relevance in each group. Three main forms of glucocorticoids were detected in addition to TTC5 and IL6. The total glucocorticoid receptor (detected using H300 antibody), phosphorylated glucocorticoid receptor (phosphorylated on serine 226 and serine 211) and cofactor (TTC5) in addition to IL6, were detected in COPD patients using H300, S226, S211, TTC5 and IL6 specific antibodies.

3.6 Optimization of experimental conditions

The aim of optimization was to confirm specificity to antibodies and to achieve the best dilution for the antibodies relevant to pre-treatment condition. It was important to test serial sections with different buffers and the same antibody. The antibodies directed against phospho-serine groups of glucocorticoid receptor were better detected in buffers containing EDTA and Tris-EDTA.

Conclusion from antibody optimisation protocols (figure 3.8) was that antibody against total GR (H300), and TTC5 antibodies do not require any pre-treatment (column 1) whereas the antibody against IL6 is more efficient if citrate buffer (column 2) was used.GR antibody that detects GR phosphorylated at serine 211 required pre-treatment with EB buffer (column 3) while S226 phospho GR antibody required pre-treatment in TEB buffer (column 4).

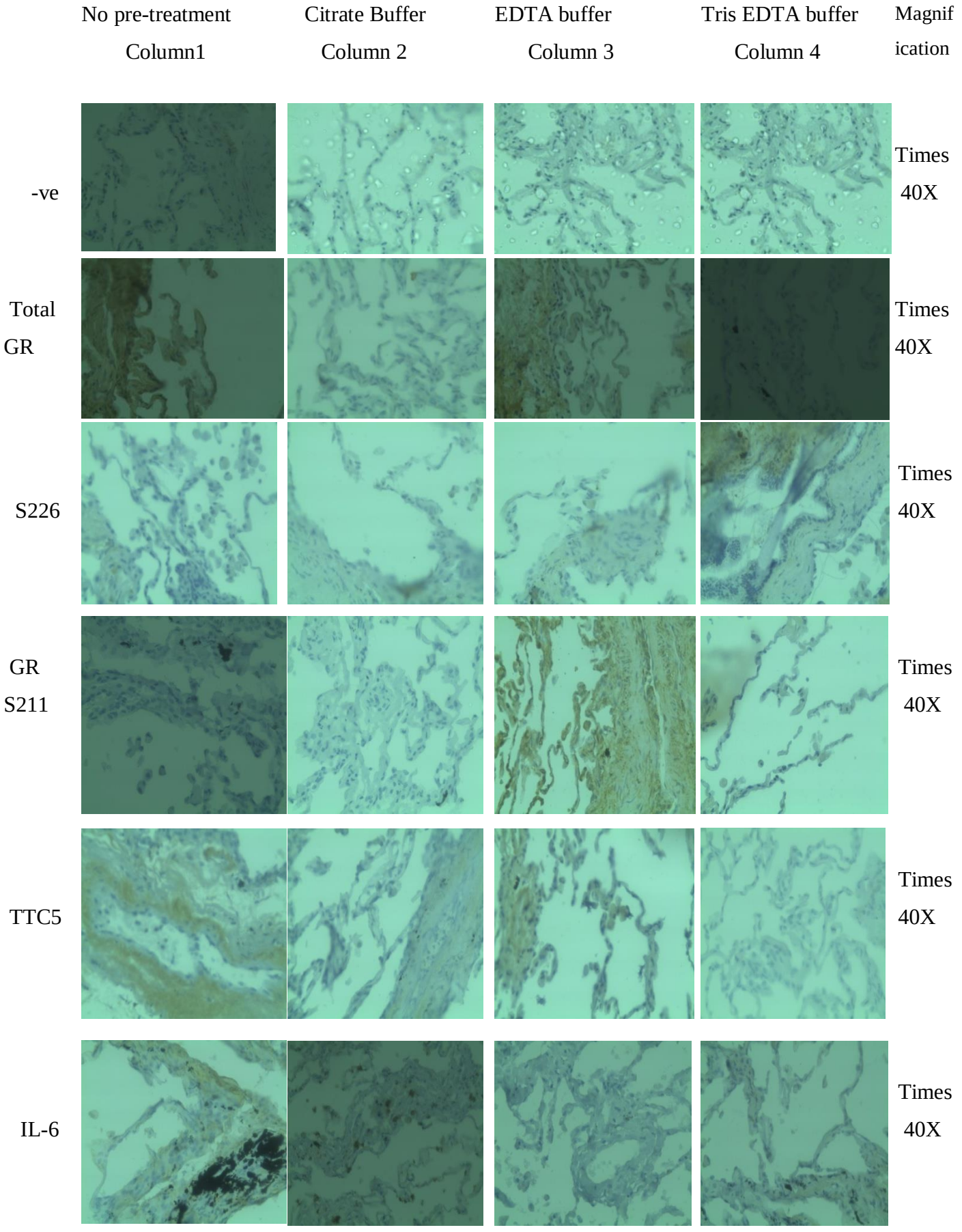

Figure 3.8 antibody optimisation

3.7 Pathological changes

40 Times magnification (A) 400 Times magnification (B)

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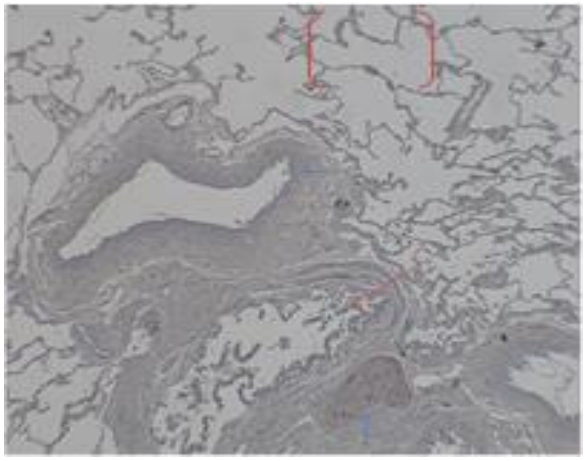
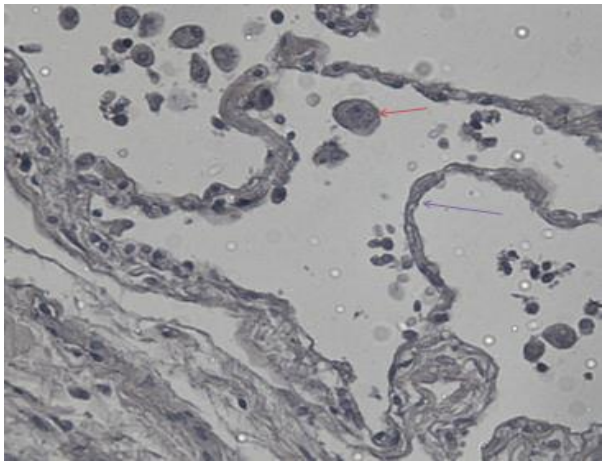
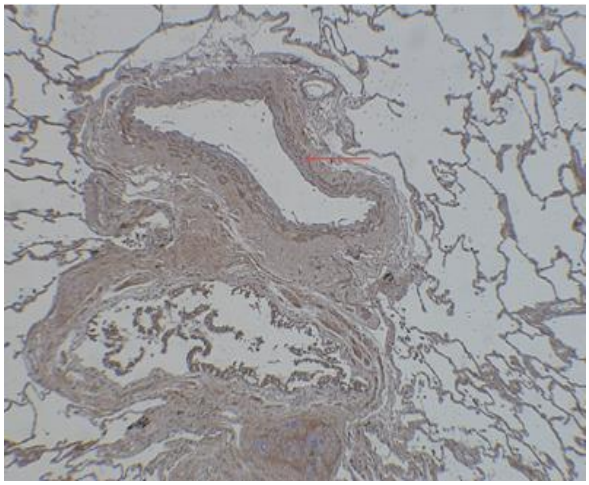
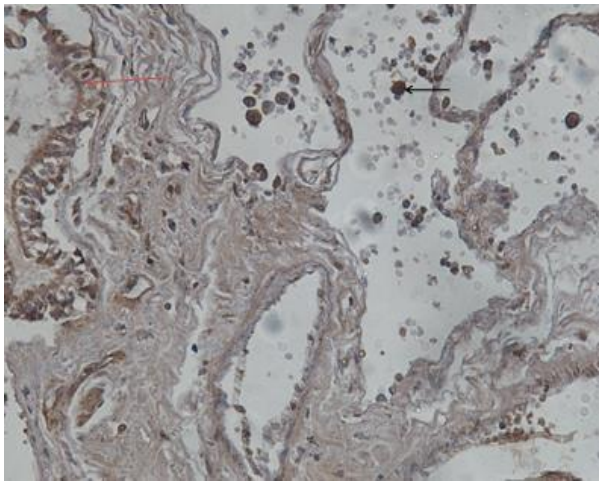


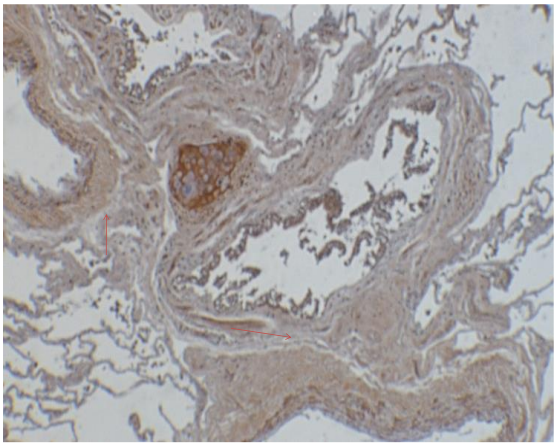


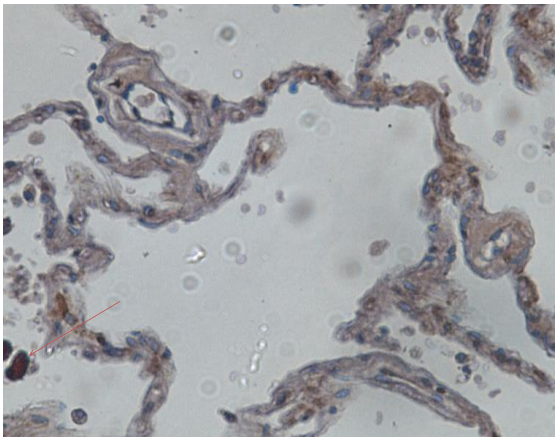
TGR



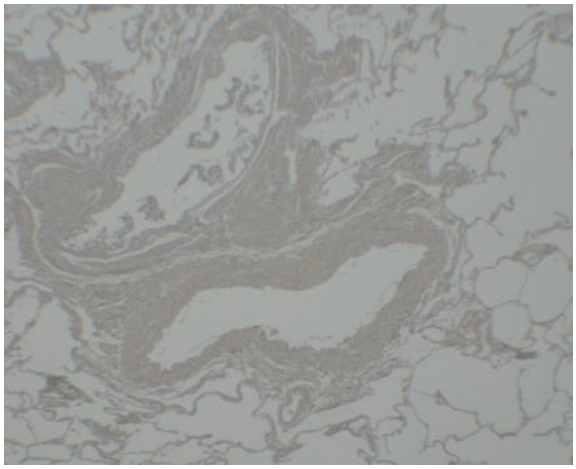


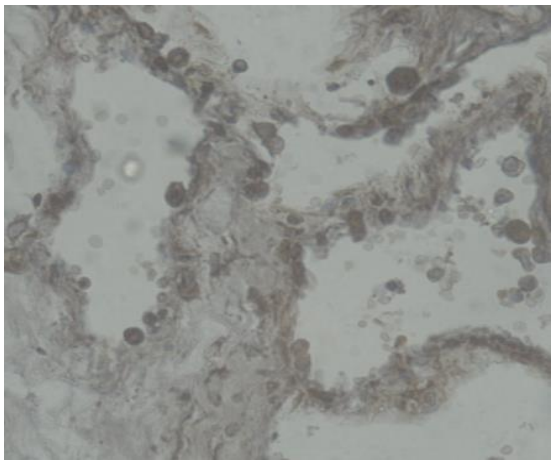
S226



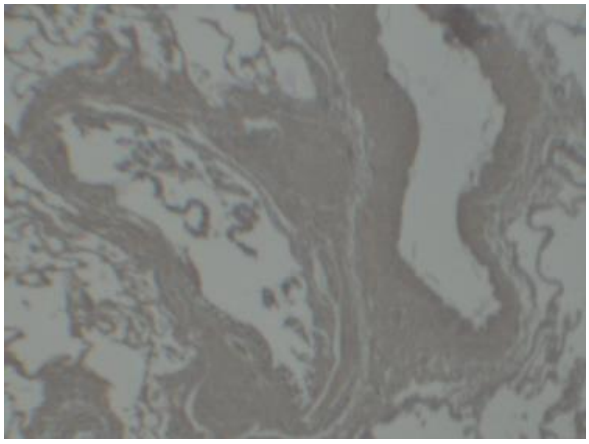


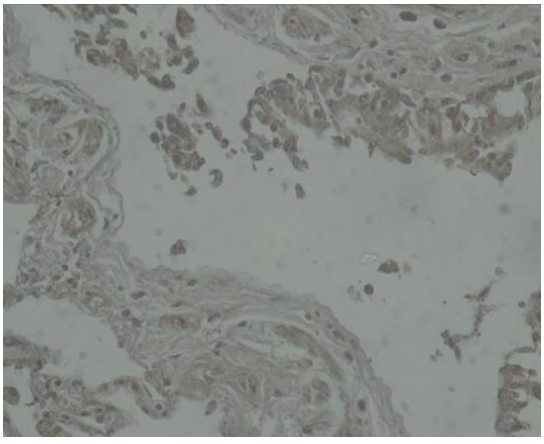
S211





TTC5





IL-6

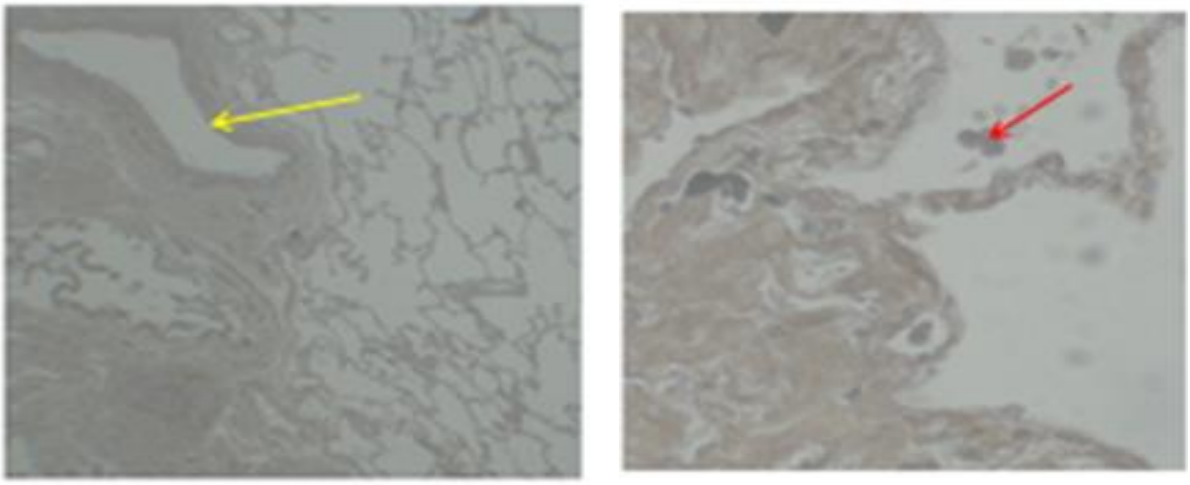


Figure 3.9| Glucocorticoid receptors, phosphorylated glucocorticoid receptors, interleukin-6 and TTC5 were investigated *in situ* using immunohistochemistry in healthy non-smokers. 40 times magnification (column-A) of peripheral lung samples from healthy non-smokers, detecting the glucocorticoid receptors (H300 antibody), phosphorylated glucocorticoid receptors (antibodies against phosphorylated GR at S211 and at S226) in addition to IL-6 and TTC5. 400 times magnification (column B) of the peripheral lung tissue analysed as described above.

In order to analyse pathological changes in the lung tissue of healthy non-smokers, immune histochemical analysis was carried out using relevant antibodies (Figure 3.9 and supplementary figure 5.4 in the appendix). The lung parenchymal tissue was intact (between the brackets, figure 3.9 column A, first panel from the top) in the majority of the involved group. The intima of blood vessels was intact (red arrow) and normal epithelial surface and submucosa (blue arrow, figure 3.9 column A, first panel from the top) with sub-mucosal gland (blue inferior arrow). In the candidate group the inner alveolar septum was intact (Figure 3.9 column B purple arrow). The expression of TGR, S211 and S226 was high in epithelial surface. However, TTC5 showed weak expression in epithelial surface. Moreover, IL6 was not expressed in epithelial surface of healthy non-smokers (Figure 3.9 column A, yellow arrow). TGR was highly expressed in macrophages (column-B, black arrow) and epithelial surface (Column B-red arrow). S211 and S226 was highly expressed in macrophages (red arrow, column B) and epithelial surface of healthy individuals. TTC5 showed higher expression in macrophages among healthy non-smokers, however expression in epithelial surface was relatively week. The expression of IL6 in healthy non-smokers was not detected in

epithelial surface, however there was some expression of IL-6 in macrophages in healthy normal individuals (red arrow).

40 times magnification A

400 times magnification B

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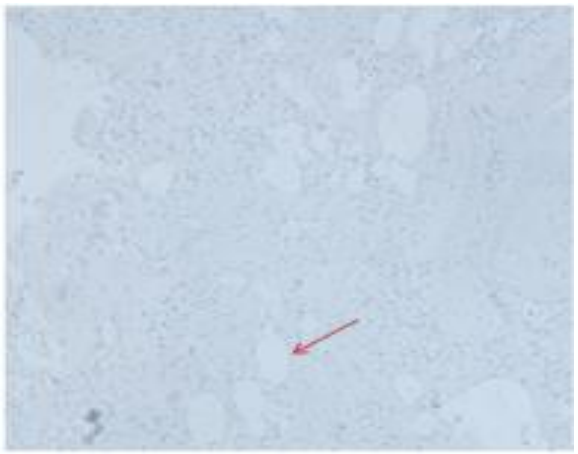
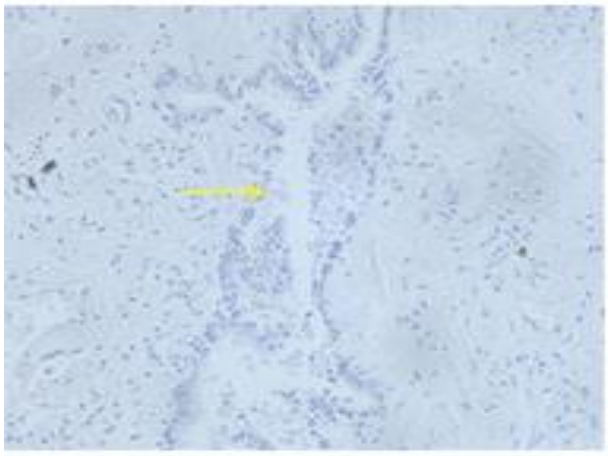
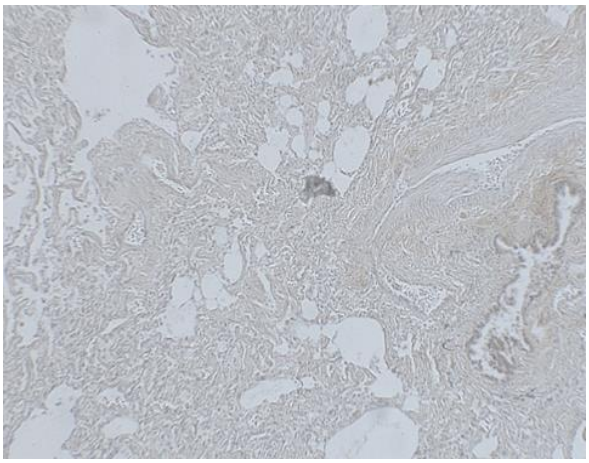
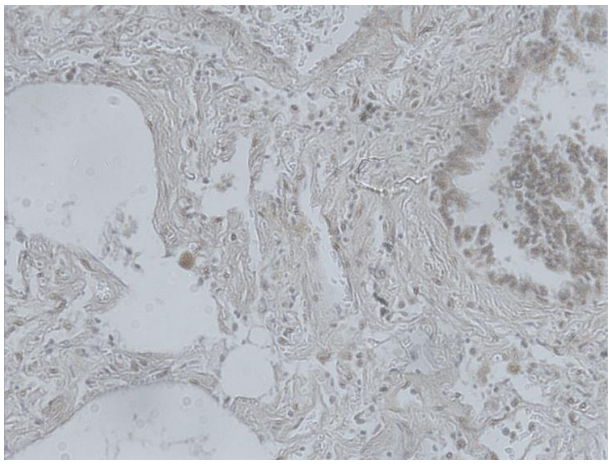


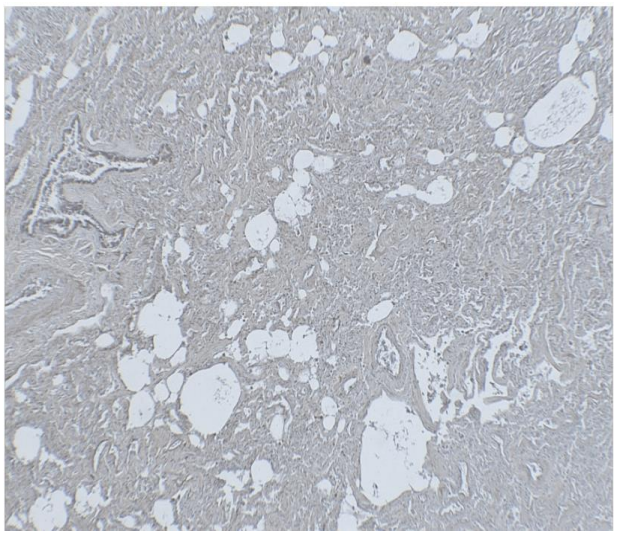


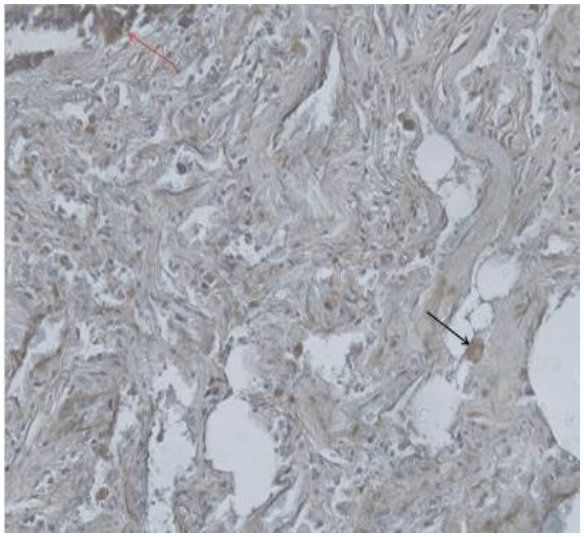
TGR



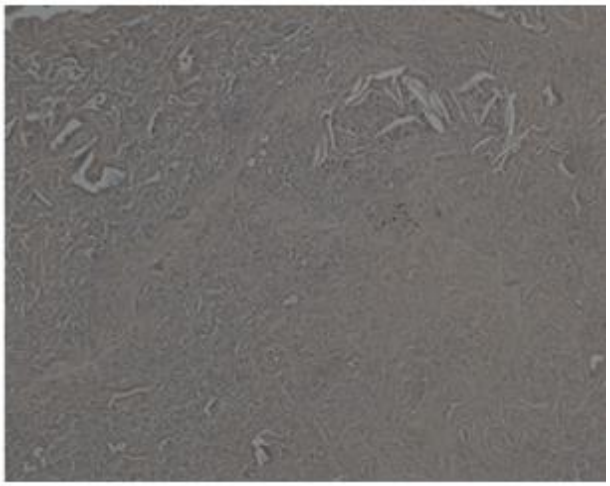


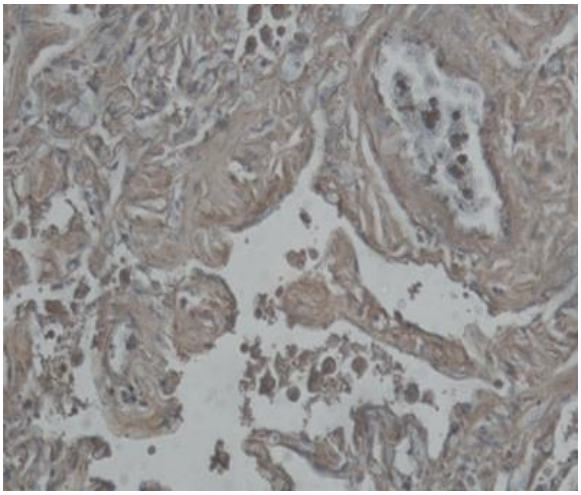
S226



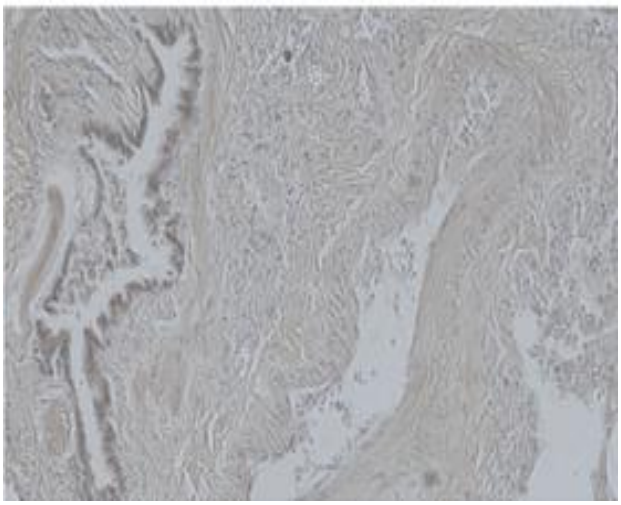


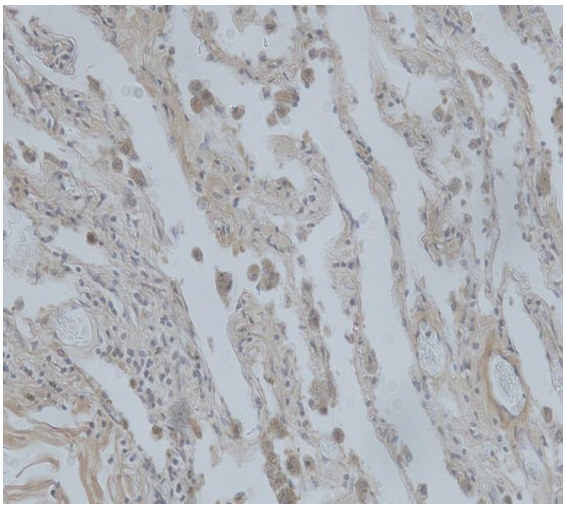
S211





TTC5





Il6

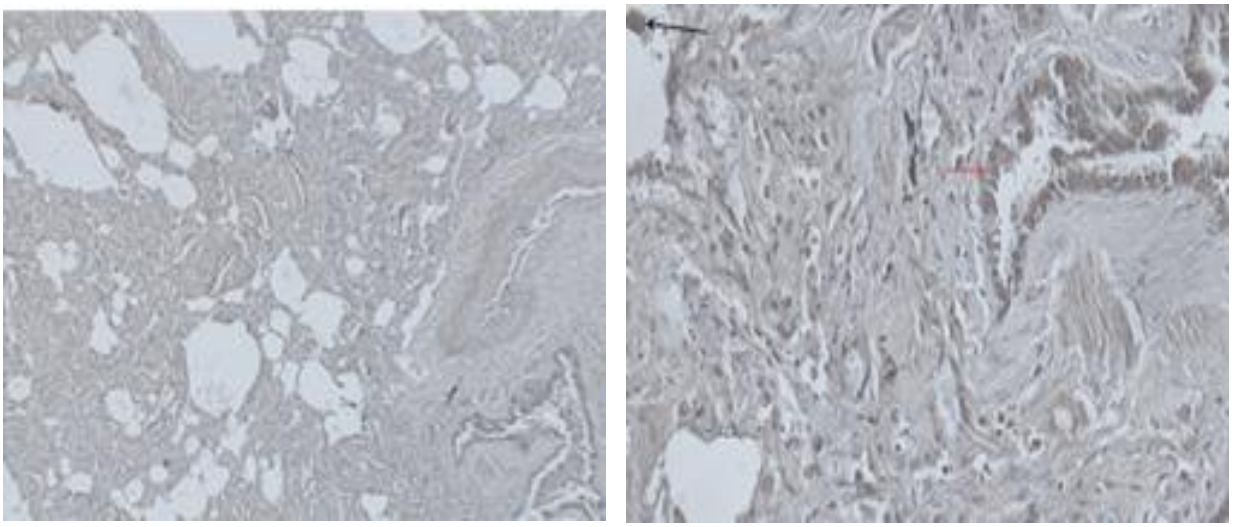
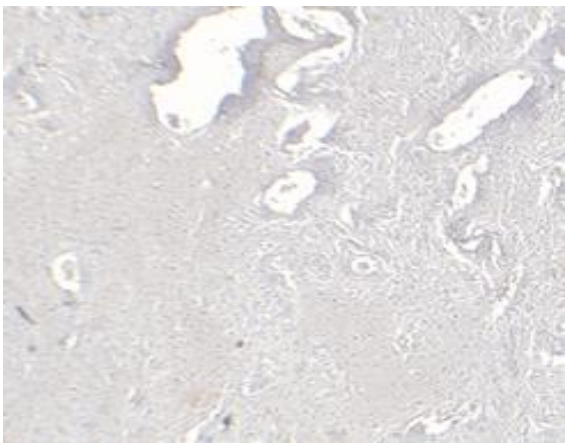
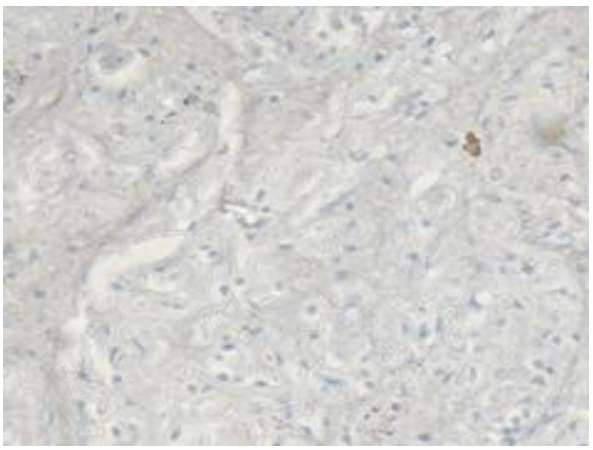
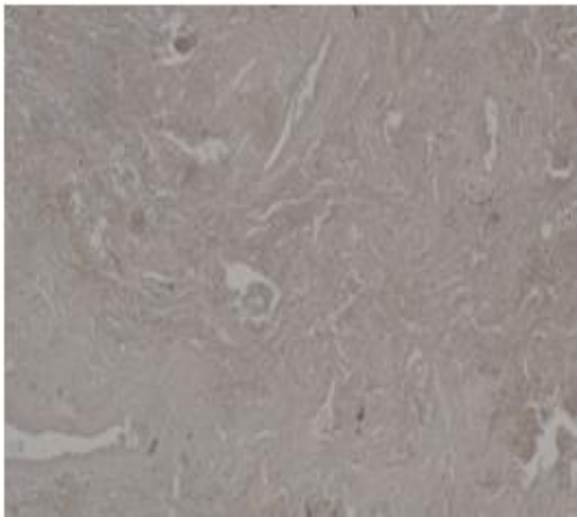
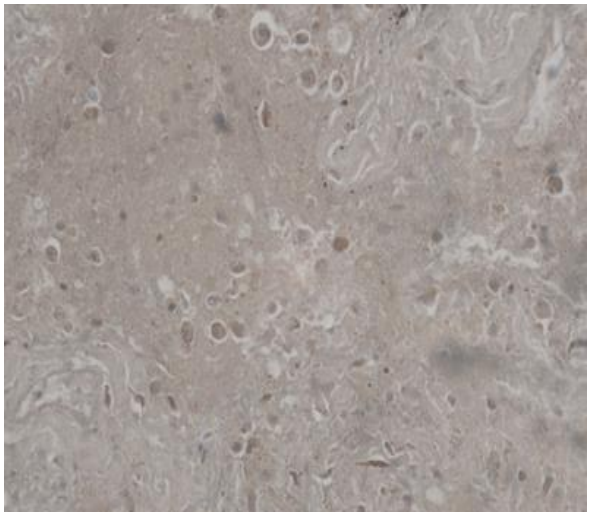
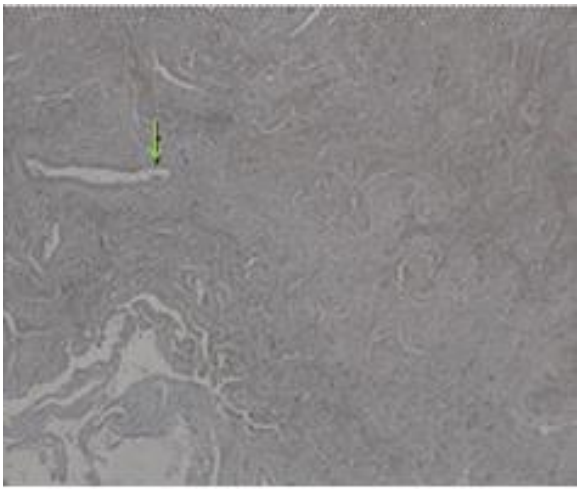
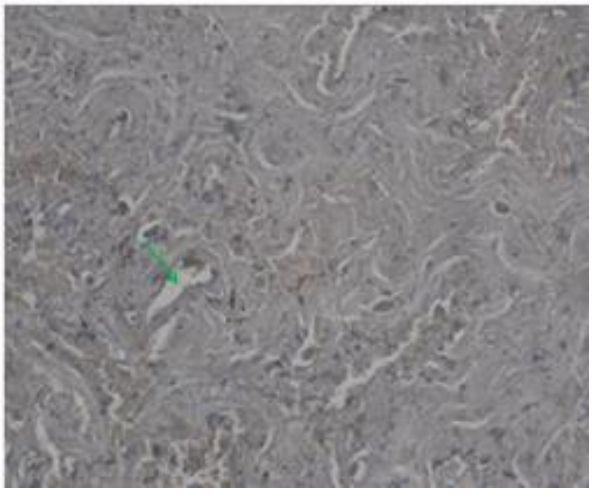
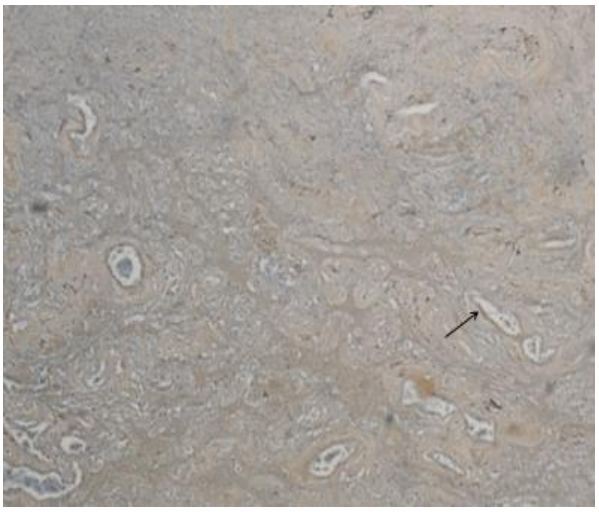


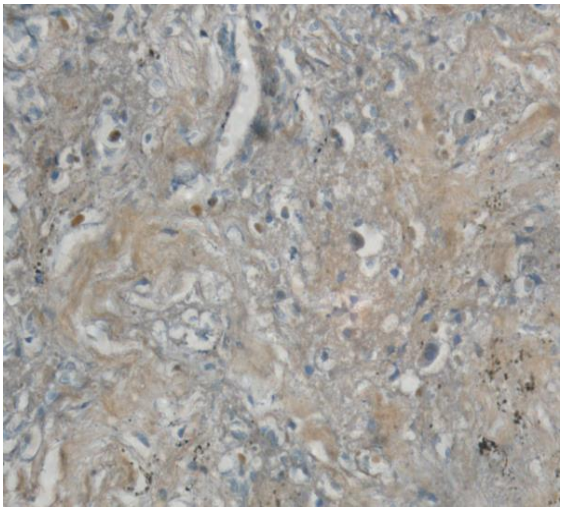
Figure 3.10. Glucocorticoid receptors, phosphorylated glucocorticoid receptors, interleukin-6 and TTC5 were investigated *in situ* using immunohistochemistry in healthy ex-smokers. 40 times magnification (column-A) of peripheral lung samples from healthy ex-smokers, detecting the glucocorticoid receptors (H300 antibody), phosphorylated glucocorticoid receptors (antibodies against phosphorylated GR at S211 and at S226) in addition to IL-6 and TTC5. 400 times magnification (column B) of the peripheral lung tissue analysed as described above.

In order to analyse pathological changes in the lung tissue of healthy ex-smokers, immune-histochemical analysis was carried out using relevant antibodies (figure 3.10 and supplementary figure 5.4 in the appendix). The lung parenchyma was intact in the majority of the patients, infiltrated with small number of macrophages (figure 3.10, column B) and the epithelial surface was intact (figure 3.10, yellow arrow, fist panel). TGR, S211, S226, and TTC5 were highly expressed in both epithelial and macrophages, moreover, IL6 was expressed in the epithelial surface in this group (red arrow, the last panel, column B). The number of macrophages infiltrating the lung parenchyma was low and expressing TGR, S211, S226, TTC5 and IL6 (black arrow, column B).

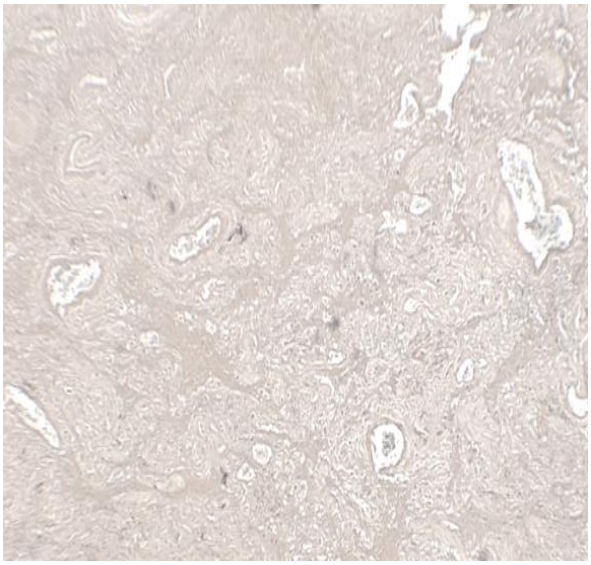
	40 times magnification A	400 times magnification B
-ve		
TGR		
S226		

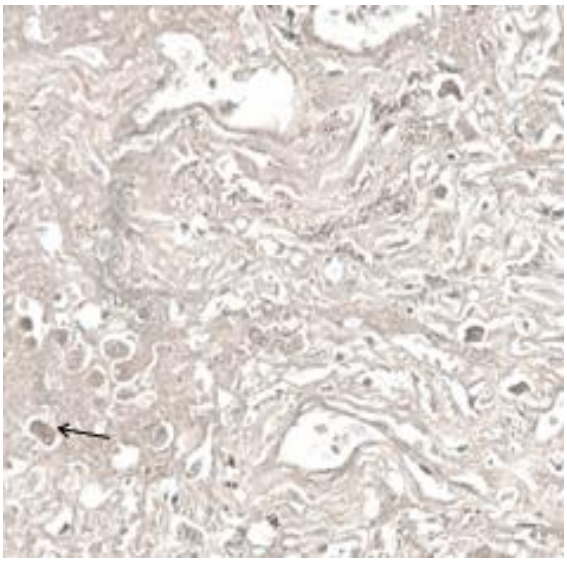
S211





TTC5





IL-6

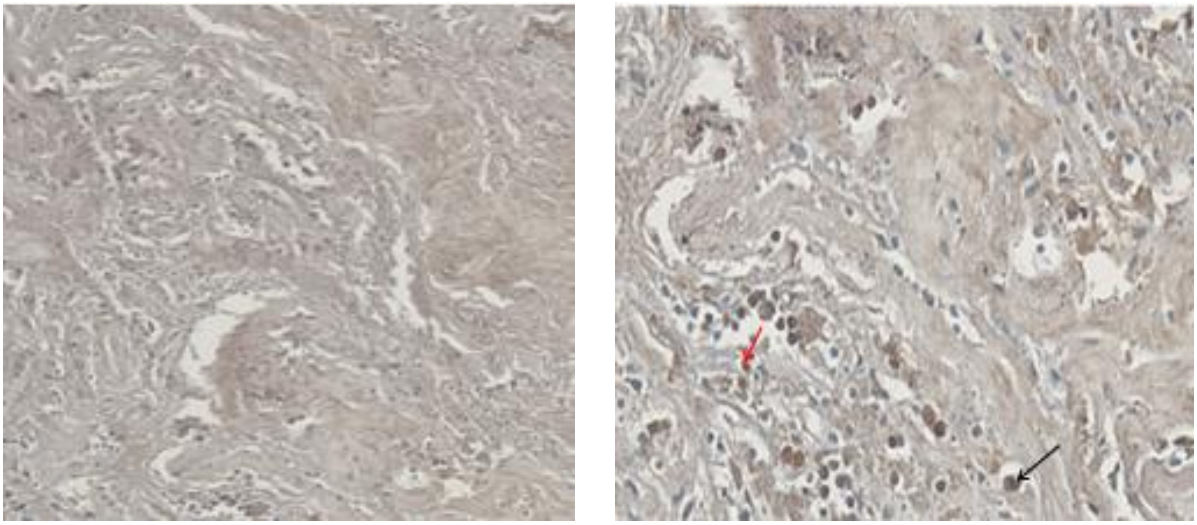


Figure 3.11| Glucocorticoid receptors, phosphorylated glucocorticoid receptors, interleukin-6 and TTC5 were investigated *in situ* using immunohistochemistry in healthy chronic smokers. 40 times magnification (column-A) of peripheral lung samples from healthy chronic smokers, detecting the glucocorticoid receptors (H300 antibody), phosphorylated glucocorticoid receptors (antibodies against phosphorylated GR at S211 and at S226) in addition to IL-6 and TTC5. 400 times magnification (column B) of the peripheral lung tissue analysed as described above.

In order to analyse pathological changes in lung tissue of healthy chronic smokers, immune-histochemical analysis was carried out using relevant antibodies (figure 3.11 and supplementary figure 5.4 in the appendix). The lung parenchyma was intact, infiltrated with large number of macrophages. The expression of TGR, S211, TTC5 and IL6 was expressed in both macrophages and epithelial surface, additionally other leukocytes express IL6 in this group of patients (red arrow, figure 3.12, column B, the last panel). S226 was only expressed in macrophages and absent in epithelial surface (green arrow, figure 3.11, column B, third panel).

40 times magnification (A) 400 times magnification (B)

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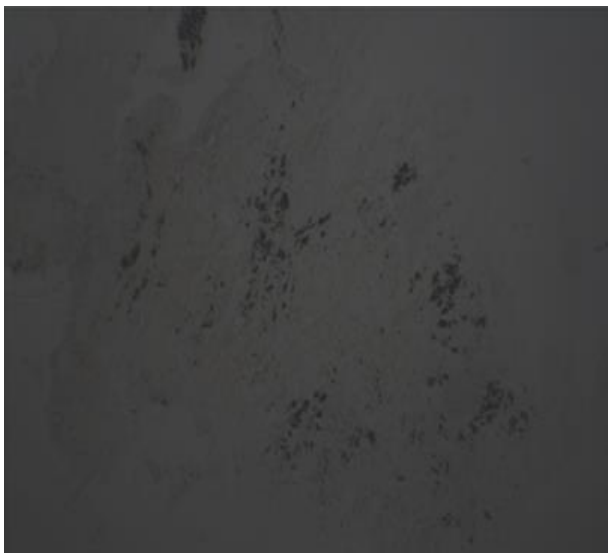
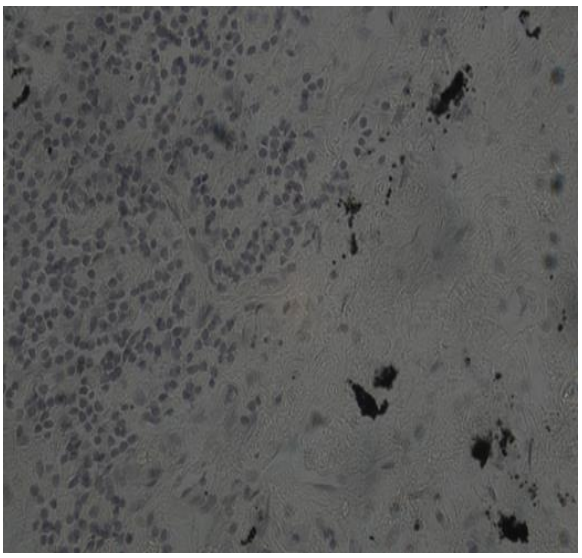
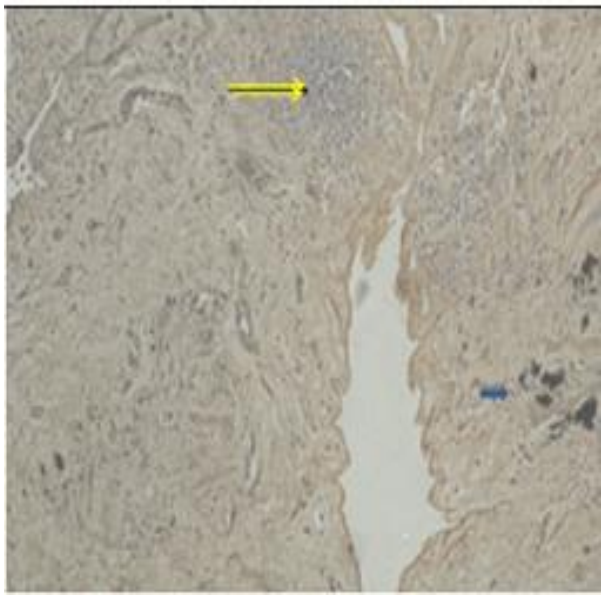
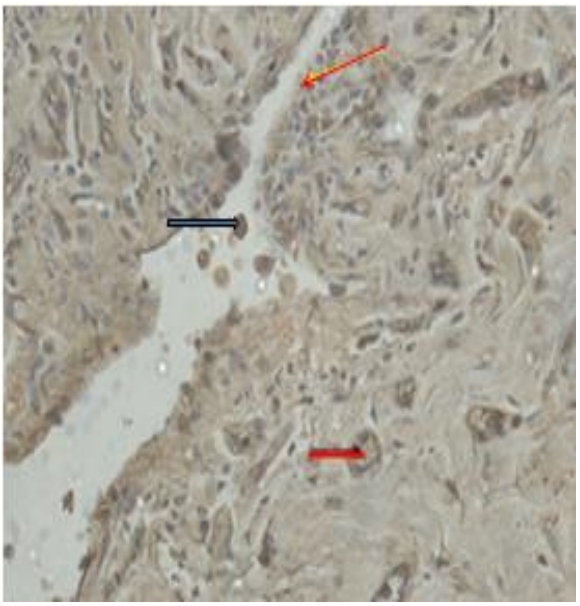


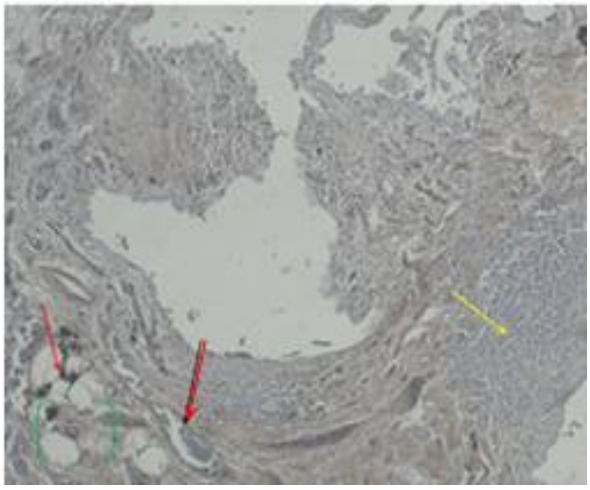


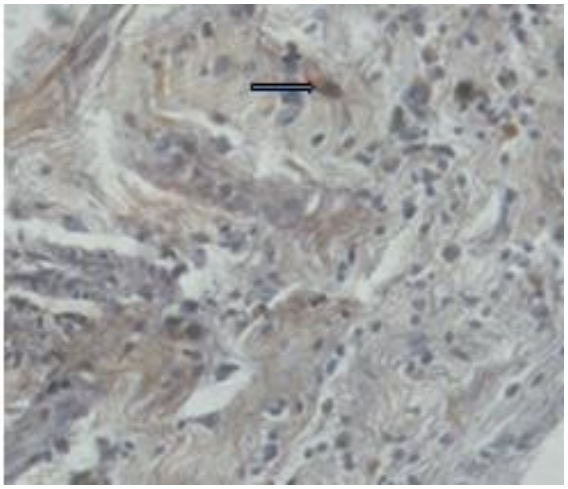
TGR



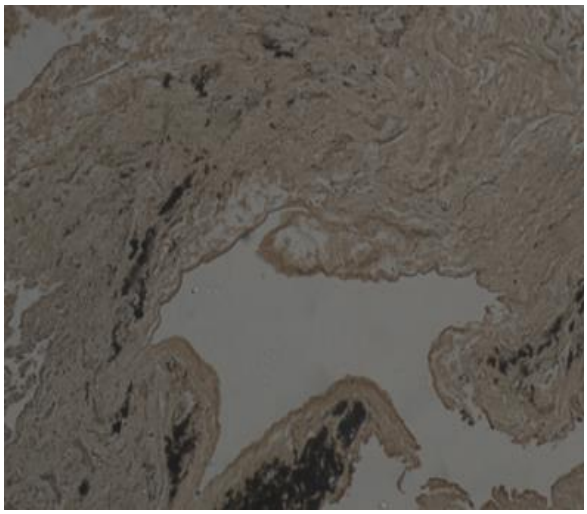


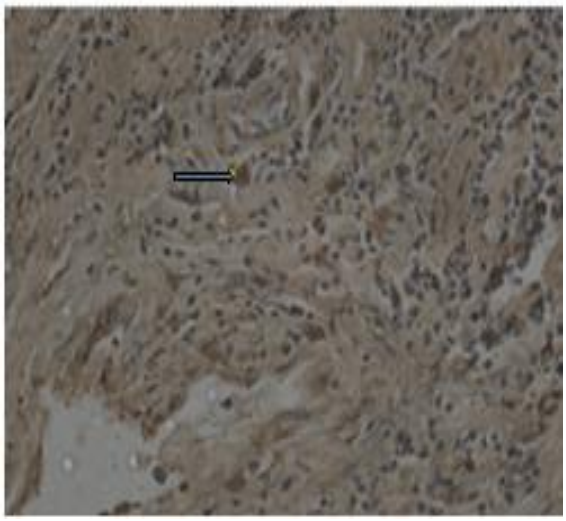
S226



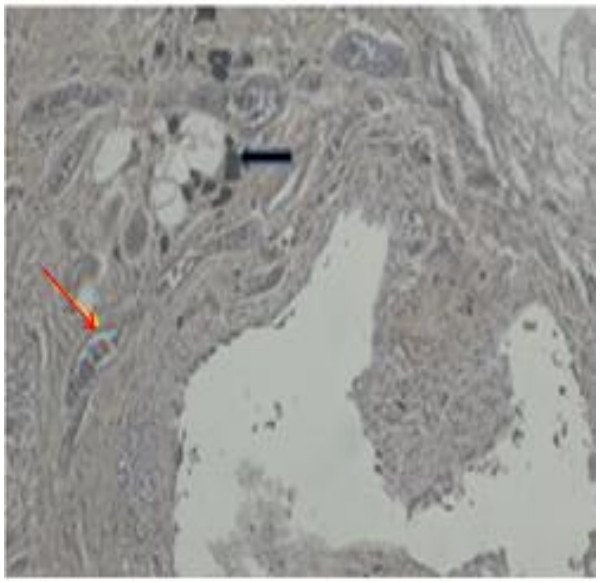


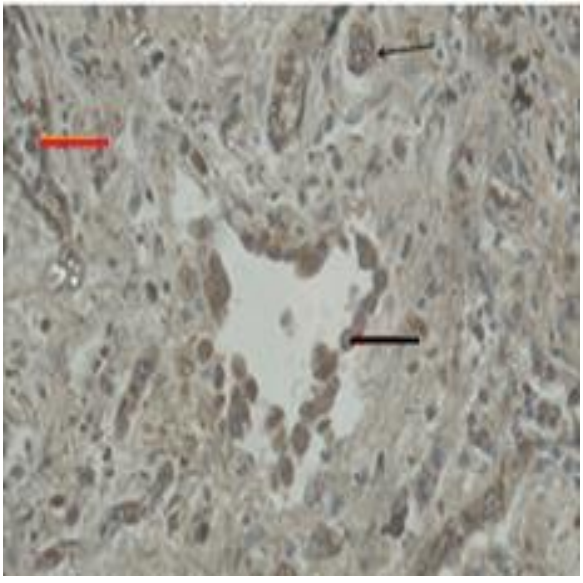
S211





TTC5





IL6

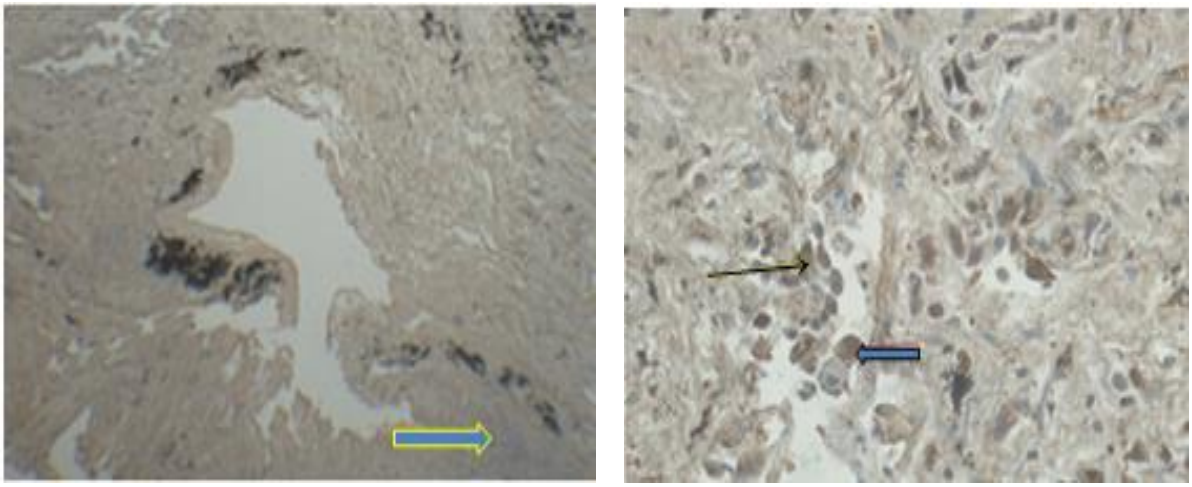
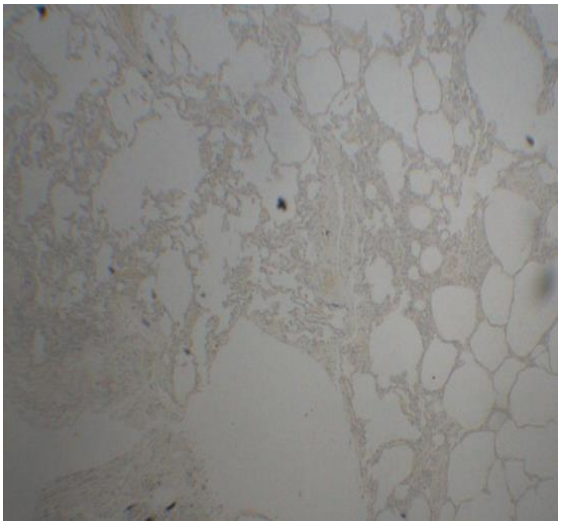
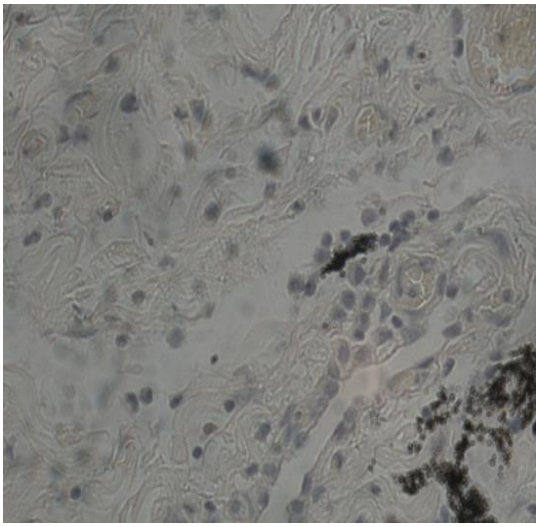
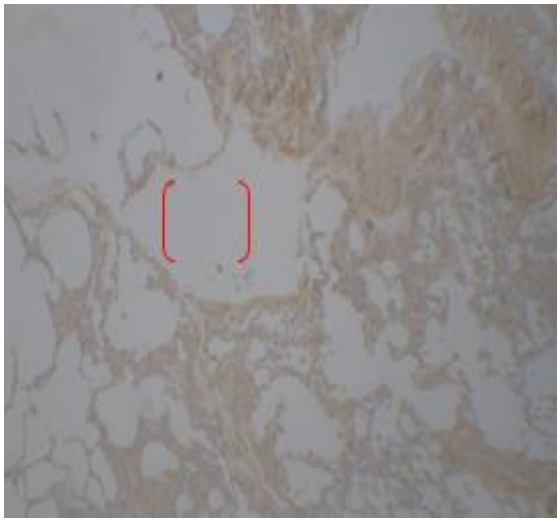
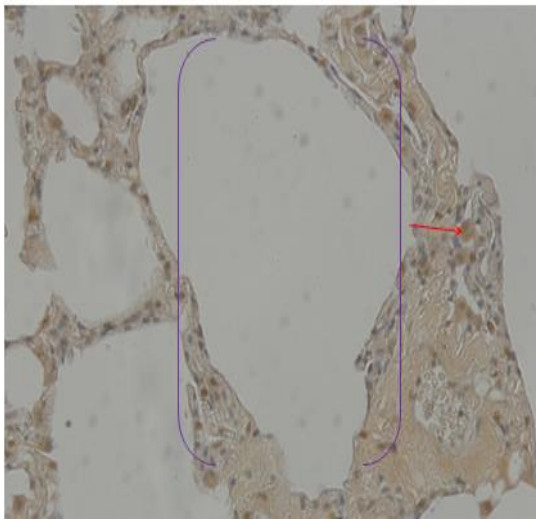
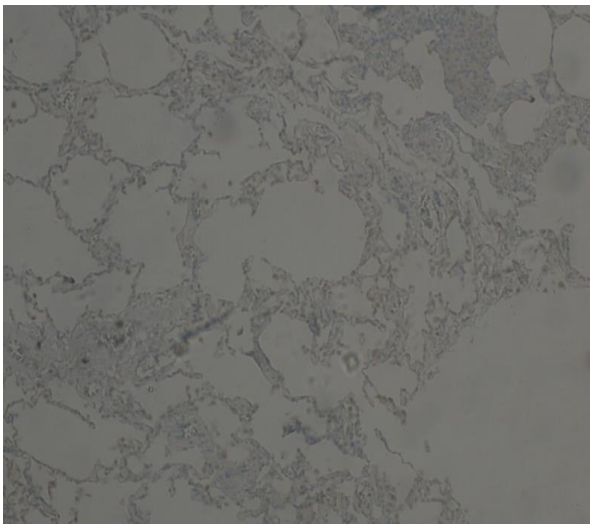
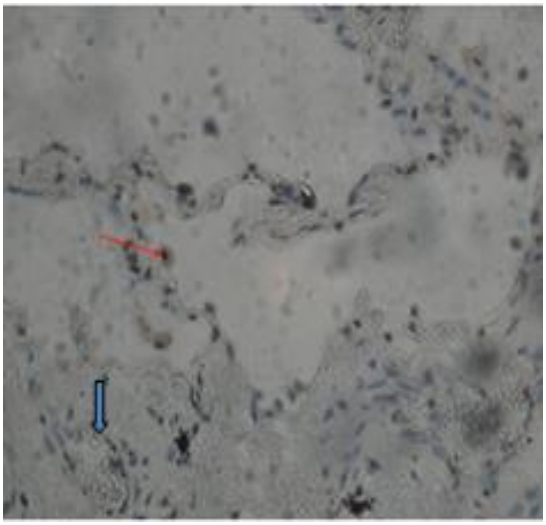
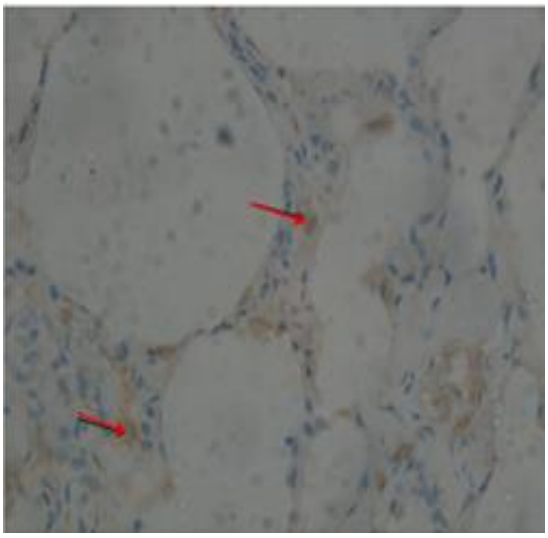


Figure 3. 12| Glucocorticoid receptors, phosphorylated glucocorticoid receptors, interleukin-6 and TTC5 were investigated *in situ* using immunohistochemistry in COPD ex-smokers. 40 times magnification (column-A) of peripheral lung samples from COPD ex-smokers, detecting the glucocorticoid receptors (H300 antibody), phosphorylated glucocorticoid receptors (antibodies against phosphorylated GR at S211 and at S226) in addition to IL-6 and TTC5. 400 times magnification (column B) of the peripheral lung tissue analysed as described above.

In order to analyse pathological changes in lung tissue of COPD chronic smokers, immune-histochemical analysis was carried out using relevant antibodies (figure 3.12 and supplementary figure 5.4 in the appendix). The lung parenchyma was intact in the majority of the patients, with some epithelial disruption. Moreover, the parenchyma was infiltrated with variable number of inflammatory cells including macrophages (figure 3.12, black arrows, column B), groups of lymphocytes (figure 3.12, yellow arrows, column A, first panel). The expression of TGR, S211 and IL6 in macrophages and epithelial cells was relatively high, however, S226 was not expressed in the epithelial cells.

	40 times magnification (A)	400 times magnification (B)
Negative		
TGR		
S226		

S211



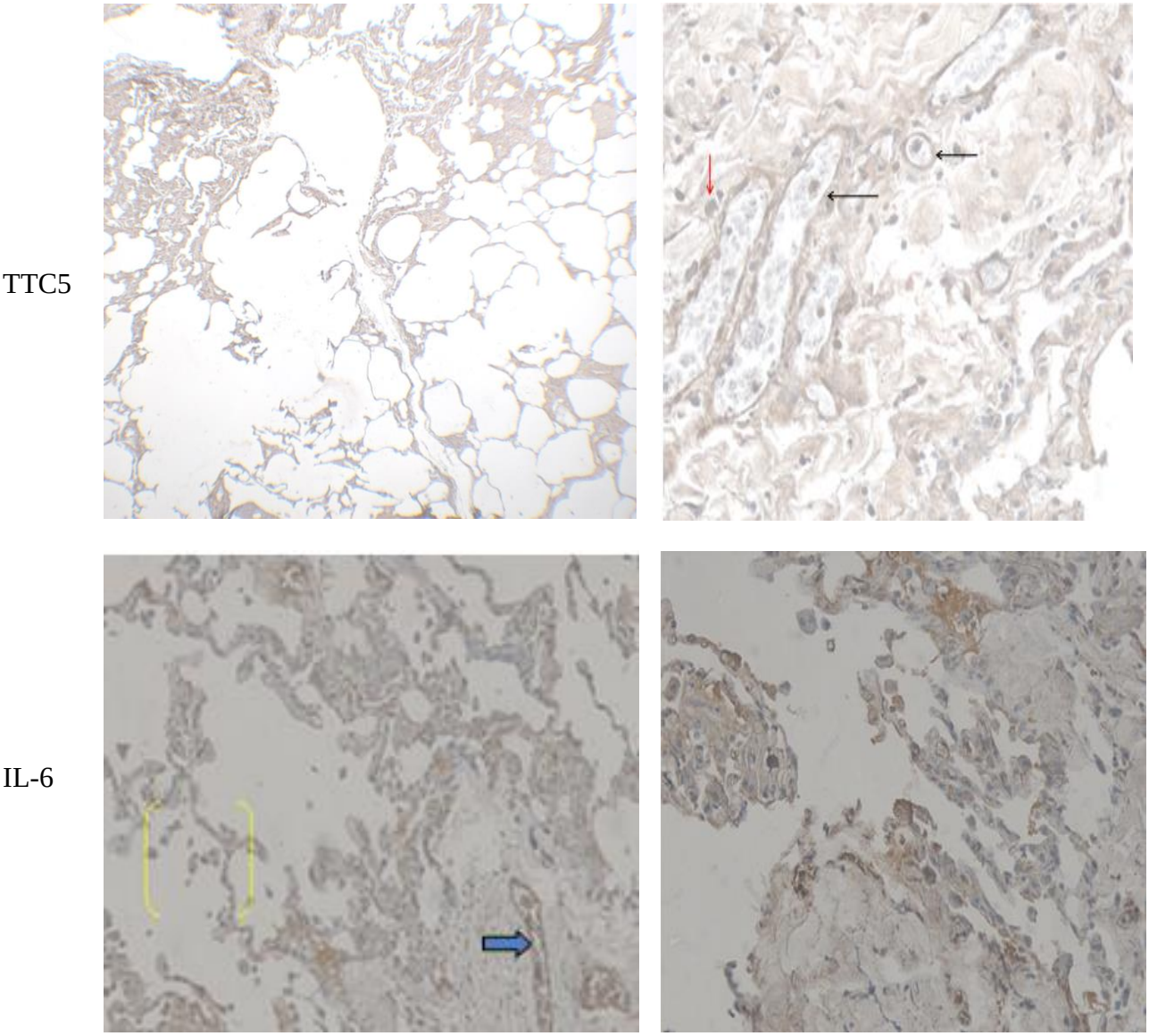


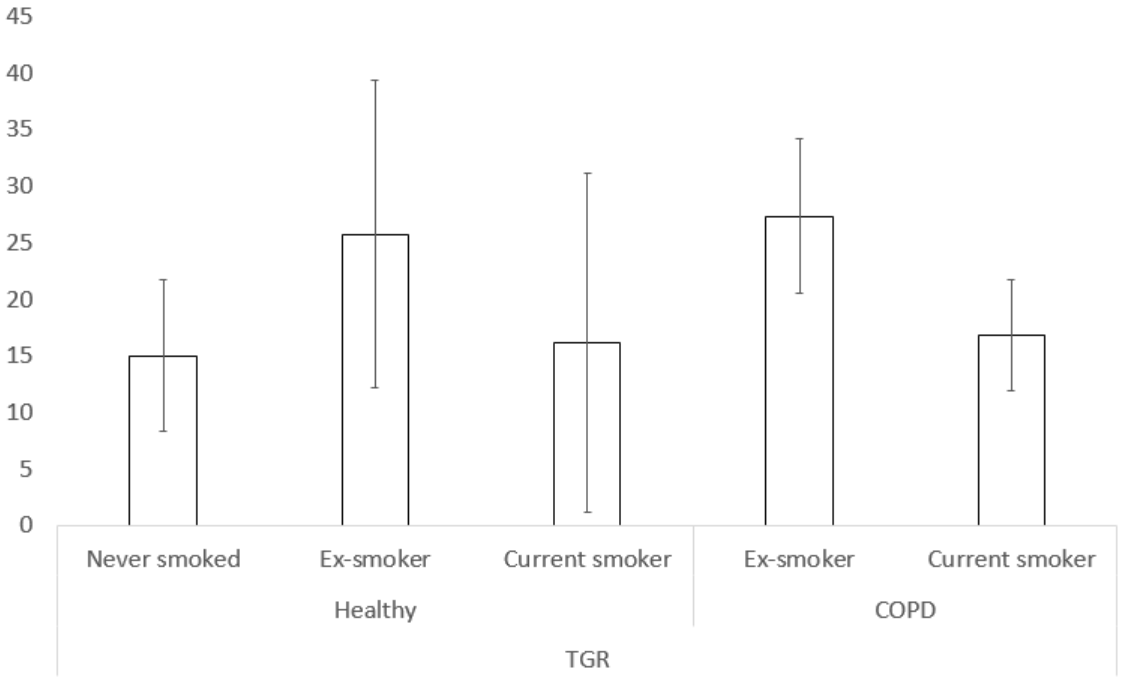
Figure 3.13| Glucocorticoid receptors, phosphorylated glucocorticoid receptors, interleukin 6 and TTC5 were investigated *in situ* using immunohistochemistry in COPD current smokers. 40 times magnification (column-A) of peripheral lung samples from COPD current smokers, detecting the glucocorticoid receptors (H300 antibody), phosphorylated glucocorticoid receptors (antibodies against phosphorylated GR at S211 and at S226) in addition to IL-6 and TTC5. 400 times magnification (column B) of the peripheral lung tissue analysed as described above.

In order to analyse pathological changes in lung tissue of COPD chronic smokers, immune-histochemical analysis was carried out using relevant antibodies (figure 3.12 and supplementary figure 5.4 in the appendix). The lung parenchyma was destructed in the majority of cases (brackets, figure 3.13, column A and B, second panel). The expression of TGR, S211, S226 and IL6 was relatively high in macrophages, however,

TTC5 was weak expression to TTC5 in macrophages (Figure 3.13, red arrow, column B, and fifth panel). S226 was not expressed in epithelial surface of this group.

3.8 Statistical analysis

Non-parametric repeated measures ANOVA, were used to examine the difference of gene expression between different groups, and multiple linear regression was performed for the complete data to test expression of TGR, S211, S226, TTC5 and IL-6 in relation to FEV1, FEV1/FVC and pack years of smoking.



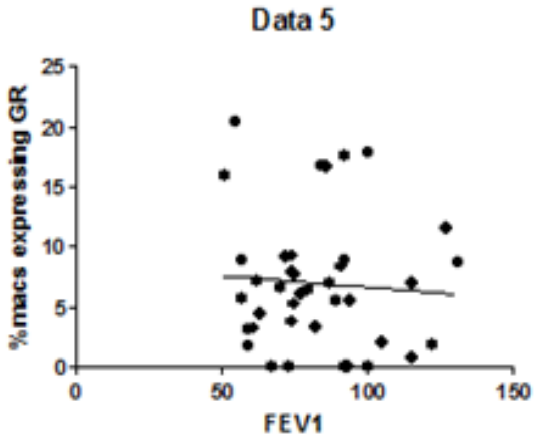
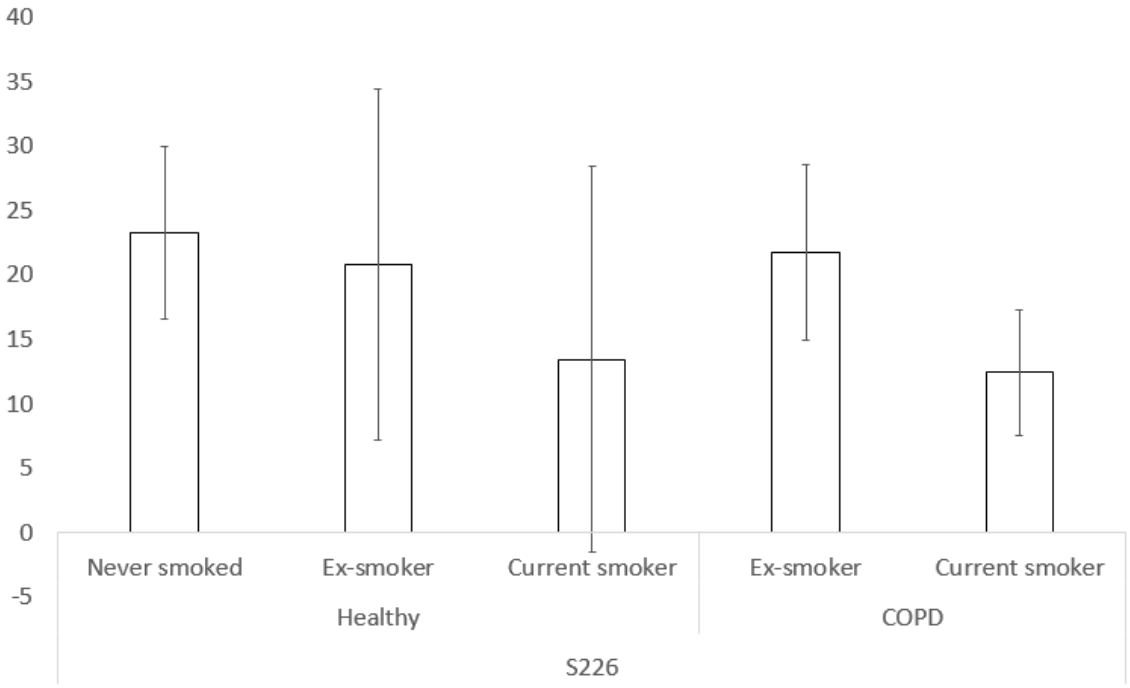


Figure 3. 14| Expression of TGR in macrophages in healthy non-smokers, healthy ex-smokers, healthy current smokers, ex-smokers and current smokers with COPD. (A) Percentage of macrophages expressing TGR as demonstrated by immunohistochemistry in 39 patients. (B) Linear regression analysis was employed to analyse the relation between TGR and FEV1, FEV1/FVC and the smoking history. Error bars represent SD and the P vale was > 0.05.

Expression of TGR was analysed in macrophages in the lung tissue of healthy non-smokers, healthy ex-smokers, healthy current smokers, ex-smokers and current smokers with COPD using immunohistochemistry (Figure 3.14). Expression of TGR was almost the same in all candidate group without any significant difference (figure 3.14A, compare healthy non-smokers with other group). Linear regression analysis was performed to determine the relation between expression of TGR and FEV1, FEV1/FVC, and smoking history of all candidate groups using one way anova. The analysis showed no significant relation between TGR and FEV1, FEV1/FVC or smoking history (Complete supplementary figures shown in appendix 5.5).



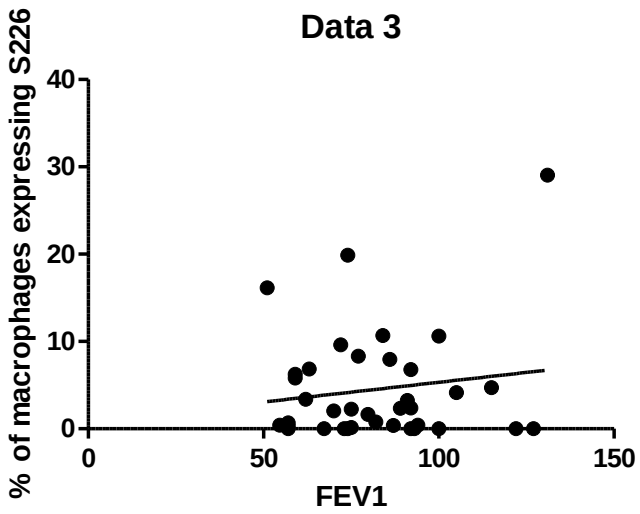
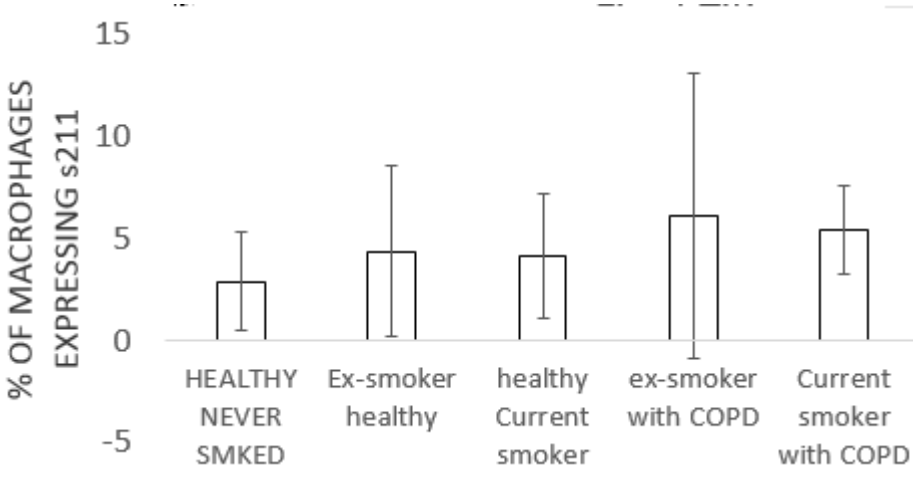


Figure 3.15| expression of S226 in healthy non-smokers, healthy ex-smokers, Healthy current smokers and ex-smokers and current smokers with COPD. (A) Percentage of macrophages expressing S226 as demonstrated by immunohistochemistry in 39 patients. (B) Linear regression analysis was employed to analyse the relation between S226 and FEV1. Error bars represent SD and the P value was > 0.05.

Expression of S226 was analysed in macrophages in the lung tissue of healthy non-smokers, healthy ex-smokers, healthy current smokers and ex-smokers and current smokers with COPD using immunohistochemistry (figure 3.15). There was non-significant difference in expression of S226 between the candidate groups (figure 3.15A, compare different groups). Linear regression analysis was performed to determine the relation between expression of S226 and FEV1, FEV1/FVC and smoking history using one way anova. This analysis showed non-significant relation between lung parameters and S226 (complete supplementary figure shown in appendix 5.5).



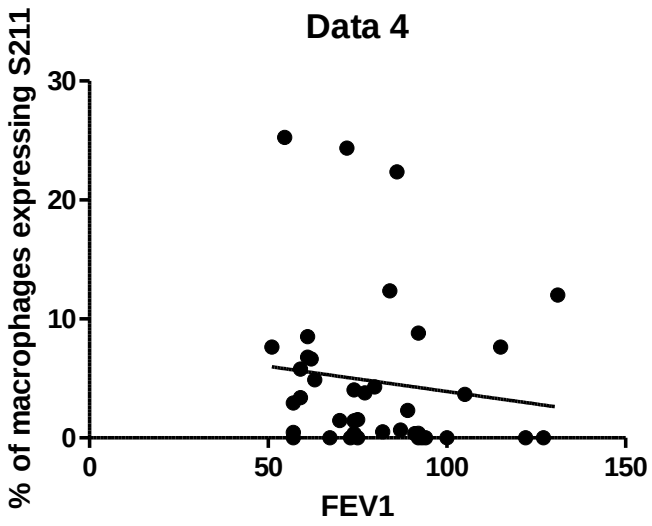


Figure 3.16| Expression of S211 in macrophages in lung tissue in healthy non-smokers, healthy ex-smokers, healthy current smokers, ex-smokers and current smokers with COPD. (A) Percentage of macrophages expressing S211 as demonstrated by immunohistochemistry in 39 patients. (B) Linear regression analysis was employed to analyse correlation between S211 and lung parameters. Error bars represent SD and the P value was > 0.05.

Expression of S211 was analysed in macrophages in the lung tissue of healthy non-smokers, healthy ex-smokers, healthy current smokers, ex-smokers and current smokers with COPD using immunohistochemistry (figure 3.17). S211 was exist in all candidate group without significant difference (figure 3.16A, compare different groups). Linear regression analysis was performed to determine the relation between the expression of S211 and FEV1, FEV1/FVC, and smoking history using one way anova. This showed non-significant relation between the expression of S211 and lung parameters or smoking history (complete supplementary figure shown in appendix 5.5)

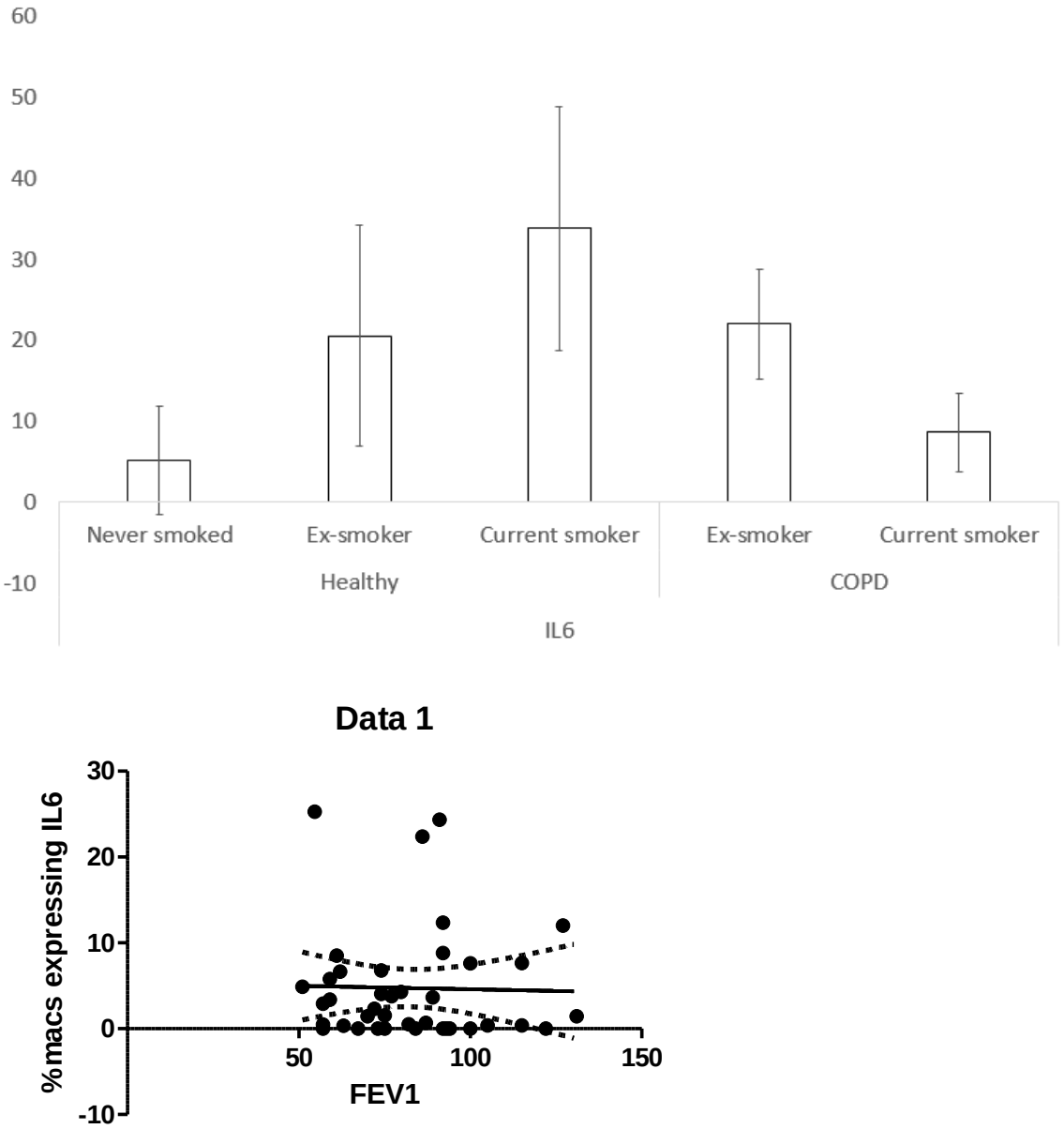


Figure 3.17| Expression of IL6 in Macrophages in Healthy non-smokers, healthy ex-smokers, healthy current smokers, ex-smokers and current smokers with COPD. (A) Percentage of macrophages expressing IL6 as demonstrated by immunohistochemistry in 39 patients. (B) Linear regression analysis was employed to analyse correlation between IL6 and FEV1. Error bars represent S.D and the P value was > 0.05.

The expression of IL-6 was analysed in macrophages in the lung tissue of healthy non-smokers, healthy ex-smokers, healthy current smokers, ex-smokers and current smokers with COPD using immunohistochemistry (figure 3.17). The statistical analysis showed no significant difference in the expression of IL-6 among the candidate group (figure 3.17A, compare different groups). Linear regression analysis was performed to determine the relation between IL-6 expression and FEV1/FVC, FEV1 and smoking status of all candidate group using one way anova. This analysis showed non-significant

relation between the expressions of IL-6 and lung parameters or smoking status (complete supplementary figure shown in appendix 5.5).

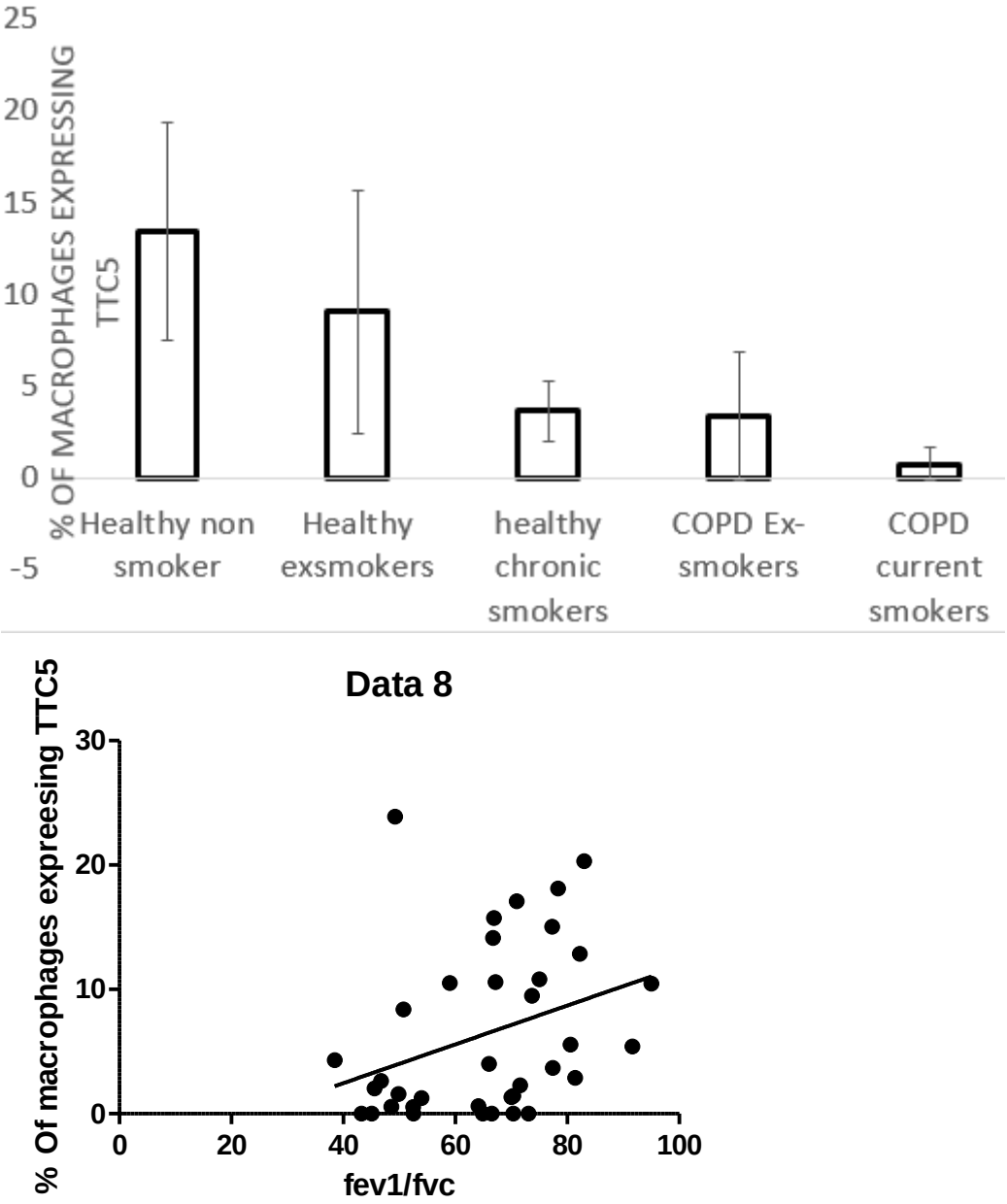


Figure 3.18 Expression of TTC5 in macrophages in healthy non-smokers, healthy ex-smokers, healthy current smokers, ex-smokers and current smokers with COPD. (A) Number of macrophages expressing TTC5 as demonstrated by immunohiustochemistry.in 39 patients. (B) Linear regression analysis was employed to analyse correlation between TTC5 and FEV1/FVC. Error bars represent SD. P value was < 0.05.

Expression of TTC5 was analysed in macrophages in the lung tissue of healthy non-smokers, healthy ex-smokers, healthy current smokers, ex-smokers and current smokers with COPD using immunohistochemistry (Figure 3.18). Expression of TTC5 was at

highest level in healthy non-smokers and its expression showed gradual decrease as disease progressed. Moreover there was significant decrease between healthy non-smokers and current smokers with COPD (figure 3.18A, compare healthy non-smokers group to current smokers with COPD). Linear regression analysis was performed to determine the relation between expression of TTC5 and FEV1/FVC, FEV1 and smoking history of all candidate groups using one way anova. This analysis showed significant relation between FEV1/FVC and TTC5, in which the percentage of macrophages expressing TTC5 are declining with the course of the disease P value <0.05. (Complete supplementary figure shown in appendix 5.5)

The expression in epithelial cells among healthy non-smokers, current healthy smokers, and current smokers with COPD, showed significant difference between healthy individuals and current smokers with COPD. The expression of IL6 was at the highest level among COPD patients who currently smoke, while healthy normal individuals showed no expression to IL-6. The expression of S226 in epithelial cells among healthy non-smokers, current smokers and COPD current smokers showed significant difference, in which the expression of S226 was higher among healthy non-smokers and very weak expression among current smokers and COPD patients. TGR, S211 and TTC5 were expressed among all candidate group (healthy non-smokers, current healthy smokers, and COPD patients).

Conclusion

TGR, S226, S211 showed no significant difference in expression among healthy non-smokers, healthy current smokers and current or ex-smokers with COPD, however the expression of IL-6 in both macrophages and epithelial cells was significantly different and increase by disease course. The percentage of average positive macrophages to TTC5 among healthy non-smokers was the highest and decrease gradually over the course of the disease.

Expression of S226 and IL-6 were opposite to each other in epithelial cells, in which healthy non-smokers showed no expression to IL6 and high expression to S226. On the other hand in COPD patients the IL6 was highly expressed and S226 was negative. (Complete supplementary table for clinical features, and statistical analysis including the averages supplemented in complete tables 4.4 and 4.5).

Discussion

The disease course in COPD varies from one patient to another, and treatment failure is common. Glucocorticoids are among the most widely prescribed drugs for the management of COPD, however, glucocorticoid resistance is the main obstacle in the management of COPD (Sin *et al*, 2003). Glucocorticoids in humans are secreted in a circadian rhythm in the form of cortisol under the control of the HPA (hypothalamic pituitary axis). The receptors mediating the glucocorticoid effect can be altered and are considered to be inflammatory markers in some diseases, such as asthma and COPD (Vilasco *et al*, 2011). The aim of this research is to follow the molecular pathways of the glucocorticoid hormone and of the stress co-factor TTC5. A further aim is to measure the expression of IL-6 and of the glucocorticoid receptors in peripheral parts of the lungs across different groups: COPD current smokers, COPD ex-smokers, healthy smokers, healthy ex-smokers and healthy non-smokers.

4.1 Disease progression and glucocorticoid resistance

Glucocorticoid receptors control many physiological functions, such as cellular differentiation and metabolism, by modulating transcription in cells in a gene specific way (Mushtaq *et al*, 2002). There are two forms of glucocorticoid receptors, hGR-α and hGR-β. The GR-α is the most abundant form. Furthermore, the level in the cells of GR-α mRNA is higher than that of GR-β mRNA (Pujols *et al*, 2002). We have analysed the expression of the hGR-α form in A549 cells (respiratory epithelial lining cells) under the effect of dexamethasone. Wang *et al*, (2002) have identified three main phosphorylation points at N-terminal transcriptional regulatory domains (S203, S211 and S226).

To gain insight into the effect of dexamethasone on the transcriptional activity of GR, we have investigated the effect of dexamethasone on TGR, on phosphorylated GR at S211 and S226, and on the stress co-factor TTC5. Our results show that the protein level of both phosphorylated forms of glucocorticoid receptors is up-regulated significantly in the presence of the dexamethasone hormone.

TTC5 (stress responsive activator of P53) or strap is a p300 interacting protein, which is necessary for P53 response (Demonacos *et al*, 2001). We have investigated the effect of

dexamethasone on TTC5 in A549 cells and the result shows varying degrees of up-regulation. Previously it has been proposed that glucocorticoid activity is linked to the TTC5 cofactor that affects its stability and transcriptional activity (Demonacos *et al*, 2001). Demonacos *et al* (2011) have stated that TTC5 interacts with TGR in the presence and absence of dexamethasone at multiple sites. Our data are consistent with this result. Our preliminary data show that there is an interaction between TTC5 and phosphorylated GR at S211.

4.2 Evaluation of glucocorticoid receptors, TTC5 and IL-6 in COPD patients

Glucocorticoids usually halt the inflammatory process by reversing the histone acetylation of activated genes (Barnes, 2010). As a result of oxidative and nutritive stress, histone deacetylase activity decreases in COPD patients, leading to glucocorticoid resistance (Barnes, 2010). Chronic obstructive pulmonary disease (COPD) is a progressive, non-reversible decline in FEV1 due to airflow limitation and parenchymal destruction (Rabe *et al*, 2007; MacNee *et al*, 2007). Many types of cell are involved in the pathogenesis of this disease. One type, epithelial cells, is activated to produce inflammatory mediators, including TNF-α, IL-1b and IL-6. In addition, epithelial cells are an important source of TGF, which mediates fibrosis.

In this study, we have evaluated a cohort of 39 patients of different categories and examined the expression of TGR, S211, S226, TTC5 and IL-6 in relation to spirometry changes FEV1, the FEV1/FVC ratio, smoking status, and the stage of the disease. In addition, a linear regression curve was used to detect the relationship between smoking history, FEV1 and the FEV1/FVC ratio.

Jan *et al* (2011) found that TGR was expressed in all groups in both macrophages and epithelial cells, showing that there is no significant difference between healthy non-smokers and COPD patients in TGR expression.

Our results show that total glucocorticoid receptor (TGR) is expressed in both macrophages and epithelial cells of all candidate involving current smokers with COPD without any significant changes in the expression. The linear regression curve shows that the alteration of FEV1 is not related to glucocorticoid receptor expression. To gain insight into the expression of phosphorylated GR at S211 in the peripheral lung tissue,

we have analysed the same candidate groups of patients: healthy non-smokers, heathy ex-smokers, heathy smokers and both ex-smokers and current smokers with COPD. Our results show that the GR phosphorylated at S211 was expressed in all the groups and there was no significant difference in the expression between the candidate groups.

The phosphorylation of S226 on the GR is mediated by C-Jun terminal kinase, a member of MAPK (mitogen activated protein kinase), which blocks GR transcriptional activation (Rogastsky *et al*, 1998). Ito *et al* (2002) revealed that S226 phosphorylation controls GR export from the nucleus upon hormone withdrawal. Our statistical analysis showed that the percentage of macrophages expressing S226 in peripheral lung tissue in all patients is more or less the same. However, GR phosphorylation at S226 was expressed in the epithelial cells only of healthy non-smokers, suggesting that S226 activity can be altered by smoking.

Moreover, we investigated a novel expression of TTC5 in the peripheral lung tissue. For different groups of patients, our statistical analysis shows that TTC5 expression in peripheral lung tissue is altered by smoking status and the progression of the disease. TTC5 expression in macrophages is inversely proportionate to the FEV1/FVC ratio and the linear regression curve shows a positive curve line (P value < 0.5).

We also investigated the expression of TTC5 in the epithelial cells of heathy non-smokers, healthy ex-smokers, heathy current smokers and both ex-smokers and current smokers with COPD. Our data shows that TTC5 is expressed in all the candidate groups.

4.3 Pro-inflammatory cytokines and COPD

Frequent contact of the epithelial surface with noxious antigens may precipitate the inflammatory process by means of inflammatory mediators including TNF-α, IL-1b and IL6, secreted from the inflammatory cells such as macrophages, neutrophils, T-lymphocytes, and pulmonary epithelial cells. The epithelial surface plays a role in airway remodelling (Perotin *et al*, 2014). In this research, we have evaluated the expression of pro-inflammatory cytokines IL-6 in the epithelial cells of COPD patients compared to normal healthy individuals. Our data revealed that IL6 is not expressed in healthy non-smokers, whereas in both heathy ex-smokers and COPD patients epithelial

cells express IL6, suggesting that exposure to cigarette smoke stimulates pulmonary epithelial cells to release IL-6.

C**onclusion**

Glucocorticoids play a pivotal role in both lung development and treatment and act by binding to intracellular protein glucocorticoid receptors (GR). Glucocorticoids are among the most widely prescribed drugs in the world for inflammatory diseases, such as rheumatoid arthritis, COPD and cases of malignancy like leukaemia, skin and numerous other conditions. They are regularly used in the treatment of lung diseases, such as asthma, because of their anti-inflammatory properties. However, resistance and side effects remain problems, highlighting the need for further research.

The aim of this project is to understand the basis of glucocorticoid resistance in COPD and in non-small cell lung cancers (adenocarcinoma). The glucocorticoid receptor function is controlled at multiple levels, including covalent modifications, the GR interaction with transcriptional co-factors such as TTC5 that is involved in control of its target genes and ligand type. In this project, glucocorticoid receptors phosphorylation and interaction with its cofactors were investigated in A549 cell lines and COPD patients. The samples were obtained from Wythenshawe hospital after surgical procedures and the analysis was far from the affected area. The results indicated that the GR and the cofactor TTC5 were expressed in lung cancer cell line A549, that GR is phosphorylated in that cell line, and that it can interact with TTC5. Immunohistochemistry analysis of different stages in COPD patients (categorised according to whether they smoke, or have COPD or cancer) was carried out to detect the total GR, GR phosphorylated at serine 211 and serine 226, and the expression of TTC5 and interleukin 6. The expression of these proteins was then correlated with the clinical picture for TTC5, in which TTC5 was highly expressed in heathy non-smokers and the level of expression decreased gradually among the candidate groups, with a significant difference between healthy non-smokers and current smokers with COPD. Moreover, the level of TTC5 expression in all the candidate groups was directly proportionate to FEV1/FVC ratio. The results of this research may improve therapy efficacy and help minimize the unwanted effects of steroids used in lung pathologies.

Future prospectus

In this thesis, we examined the expression of TTC5 in macrophages in different stages of COPD. However, the effect of dexamethasone on TTC5 expression in macrophages needs to be investigated. The interaction between TTC5 and glucocorticoid receptors in macrophages is unknown. Our future prospectus is the identification of TTC5 compared to the degree of inflammation in peripheral lung tissue, using real time PCR and isolation of macrophages from COPD patients to determine the interaction between TTC5 and GR, compared with healthy candidates.

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