Chapter 2

General Introduction

2.1 Definition of COPD

“COPD is a disease state characterized by airflow limitation that is not fully reversible, usually progressive and associated with an abnormal inflammatory response of the lung airways in response to noxious particles and gases” (Barnes, 2003).

The term “chronic obstructive pulmonary disease” (COPD) now widely used was first used in the literature in 1964 (Mitchell & Filley, 1964). Later on in the 1970s and 1980s, sub-diagnosis such as emphysema, chronic bronchitis, chronic obstructive bronchitis and chronic bronchitis with emphysema were used and shortly recommendations and international guidelines became available on how to use these terms to define the disease, today called COPD (Larsson, 2007; Mitchell & Filley, 1964). Tobacco smoking is considered as the main etiological factor in this condition, at least in western countries. Why only a minority of smokers develop COPD is not known.

A number of different specific definitions exist for COPD in the literature but they are of course more or less similar. The American Thoracic Society (ATS) define COPD as “a disease state characterized by the presence of airflow limitation due to chronic bronchitis or emphysema; the airflow obstruction is generally progressive, may be accompanied by airway hyper-reactivity, and may be partially reversible” ("Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. American Thoracic Society," 1995). This, therefore, allows some overlap with asthma.
The European Respiratory Society (ERS) on the other hand defines COPD as “reduced maximum expiratory flow and slow forced emptying of the lungs, which is slowly progressive and mostly irreversible to present medical treatment” (Siafakas et al., 1995).

The Global Initiative for Chronic Obstructive Pulmonary Lung Disease (GOLD) report classified COPD as “a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases” (GOLD, 2007; Gomez & Rodriguez-Roisin, 2002; Pauwels, Buist, Ma, Jenkins, & Hurd, 2001). GOLD definition is the most widely recognised definition of COPD worldwide and is somewhat different in that it introduces an underlying pathological process. The GOLD criteria also further categorized COPD into four different stages on the basis of the physiological and clinical severity of disease as given below:

“GOLD Stage I is considered as mild COPD, characterized by mild airflow limitation (FEV1/FVC < 0.70; FEV1 ≥ 80 % predicted). Symptoms of chronic cough and sputum production may be present, but not always. At this stage, the individual is usually unaware that his or her lung function is abnormal” (taken from www.goldcopd.com).

“GOLD Stage II is considered as moderate COPD, characterized by worsening of airflow limitation (FEV1/FVC < 0.70; 50% ≤ FEV1 < 80 % predicted), with shortness of breath typically developing on exertion and cough and sputum production sometimes also present. This is the stage at which patients typically seek medical attention because of chronic respiratory symptoms or an exacerbation of their disease” (taken from www.goldcopd.com).

“GOLD Stage III is severe COPD, characterized by further worsening of airflow limitation (FEV1/FVC < 0.70; 30% ≤ FEV1 < 50 % predicted), greater shortness of breath, reduced exercise capacity, fatigue, and repeated exacerbations that almost always have an impact on patient’s quality of life” (taken from www.goldcopd.com).
“GOLD Stage IV is very severe COPD, characterized by severe airflow limitation (FEV1/FVC < 0.70; FEV1 < 30 % predicted or FEV1 < 50 % predicted plus the presence of chronic respiratory failure). Thus, patients may have Stage IV: Very severe COPD even if the FEV1 is > 30 % predicted where secondary complications are present. At this stage, quality of life is very appreciably impaired and exacerbations may be life threatening” (taken from www.goldcopd.com). Prognosis at this stage is also poor.

As included in ATS definition, COPD includes two distinct entities, chronic obstructive bronchitis characterized pathologically by airway inflammation and airway damage, and emphysema which consists of enlargement of peripheral airspaces and destruction of lung parenchyma, leading to loss of lung elasticity which then causes dynamic closure of small airways in expiration. “Chronic bronchitis” is a clinical entity defined symptomatic features of “chronic productive cough for 3 months in each of 2 consecutive years provided other causes of cough have been ruled out” (Mannino, 2003, 2007; Viegi et al., 2007). This reflects mucous hypersecretion and is not necessarily associated with airflow limitation though it frequently is (Barnes, 2003). There is no exact clinical definition of “emphysema” apart from the anatomical definition, but “clinically patient’s experiences progressive dyspnea and variable cough” (Mannino, 2003, 2007). However, it is reflected physiologically in decline in gas-absorption function of the lungs as reflected in measurement of the CO-transfer factor (diffusion capacity).

2.1.1 Social and economic burden
COPD is one of the leading causes of disability and death worldwide and is the fourth most common cause of death in the United States, Australia and Europe. In Australia nearly 12 % of the entire adult population is affected by the disease and due to inadequate diagnosis in its early stages related to its slowly progressive and insidious nature its prevalence is probably much higher (D. W. Reid et al., 2003). It is estimated that over one million individuals in the UK, 6% of men and 4% of women, suffer from COPD with around about 30,000 deaths annually and is anticipated that it will rank fifth by 2020 in burden of disease worldwide, according
to a study published by the World Bank/World Health Organization (Barnes, 1998b; Rabe et al., 2007; Thorley & Tetley, 2007).

COPD has very major social and economic consequences (Johnson, Campbell, Bowers, & Nichol, 2007). Thone and colleagues (Thone, Schurmann, Kuhl, & Rief) in a very interesting study reported spouses’ quality of life during the course of the disease. They showed that spouses also had poor quality of life and with increased psychological suffering. It has also been observed that patients with COPD feel disgrace and distancing from people around them and also from their physicians (Johnson et al., 2007). A study reported by Earnest and colleagues described patients feeling personal shame and embarrassment when they are carrying oxygen cylinders with them in public (Earnest, 2002).

The European Lung White Book in 2001 evaluated exhaustively the annual cost associated with COPD in Europe; they found that COPD costs €38.7 billion per annum (A$54.4 billion) including €4.7 billion (A$6.6 billion) for ambulatory care, €2.7 billion for drugs (A$3.7 billion), €2.9 billion (A$4.08 billion) for inpatient care and €28.4 (A$39.9 billion) for lost work days very year. The estimated cost for individual European countries annually ranged from €109-€541 million (A$153-A$761 million), and individual patient costs were estimated around from €151-€3,912 annually (A$212-A$5504) (Chapman et al., 2006; Loddenkemper R, 2003). The economic burden associated with COPD is probably underestimated as well, because the economic value of the care provided by family members of patients with COPD is not usually reported. A patient with very severe COPD needs long term home care which has serious negative effects not only on their own professional life but also on the life of engaged relatives and carers (Viegi et al., 2007).

### 2.1.2 Risk factors associated with COPD

Direct exposure to tobacco smoke is considered as the main risk factor associated with progression of COPD in western countries. The cigarette smoke which is inhaled while puffing is approximately 45% of the total while the remaining 55% is released into the surrounding environment (Yoshida & Tuder, 2007). Environmental tobacco exposure or “passive smoking” (Pinkerton & Joad, 2006; Sethi & Rochester,
(Yoshida & Tuder, 2007) is a growing centre of attention, given that such lifetime exposure could be associated with COPD (Eisner et al., 2005). The increasing burden of indoor pollution due to biomass fuel smoke (Fullerton, Bruce, & Gordon, 2008), outdoor air pollution, viral infections and occupational exposure to noxious gases and dust may also cause COPD (Blanco et al., 2009; Driscoll et al., 2005). Biomass fuel refers to burned animal or plant material; charcoal, wood, and even cow dung and crop residues, which provide more than one-half of domestic energy in most of the developing world and nearly 95% in low income countries (Fullerton et al., 2008). There is evidence in the literature now, and more data is accumulating, explaining the potential link between an increased risk of respiratory tract infections, inflammatory lung / airway diseases and increased levels of indoor air pollution especially due to increase in biomass fuel consumption in rural areas (Fullerton et al., 2008; Jaakkola & Jaakkola, 2006). Understanding the effects of burning biomass fuels on health is important for any physician practising in the developing world, but unfortunately not well represented in the literature and it is quite surprising how little published research there is to date on its health consequences (Jaakkola & Jaakkola, 2006). There are few studies reporting associations between biomass fuel combustion and physiological and structural pathological changes in the respiratory system and little hard data are available on relationships between biomass fuel consumption and COPD. However, a Chinese study reported that biomass fuels are the probable primary risk factor for COPD in rural South China; they found that the use of biomass fuel was higher in rural areas, with a strong association between COPD and exposure to biomass fuel used for cooking. They also reported higher concentrations of carbon monoxide, particulate matter, sulphur dioxide and nitrogen dioxide in the kitchen during biomass fuel combustion compared to LPG combustion (Liu et al., 2007).

In another study in Spain, Orozco and colleagues reported a strong association between wood or charcoal smoke exposure and COPD, suggesting that this is also a problem in less developed European countries (Orozco-Levi et al., 2006). In a systematic review and meta-analysis, Kurmi et al reported that even with enormous heterogeneity across different studies, exposure to biomass fuel is consistently associated with COPD and chronic bronchitis (Kurmi, Semple, Simkhada, Smith, & Ayres).
Despite the recognition of these environmental factors, the major cause of COPD worldwide/non-westernised countries is still a matter of debate (M. Roth, 2008). However, Salvi and Peter Barnes suggested in a recent review in the Lancet that about 3 billion people, which is nearly half of the total world population, are exposed to smoke from biomass fuel compared with 1.01 billion people who smoke tobacco, which suggests that exposure to biomass smoke, might be the biggest risk factor for COPD globally compared to tobacco smoke (Salvi & Barnes, 2009). However in westernised countries tobacco smoking is considered as the main etiological factor associated with COPD, but why only a minority of smokers develop COPD while others not, leads to a possibility that there may be a genetic susceptibility associated with COPD development interacting with environmental factors. Deficiency of protease inhibitor, alpha-1 antitrypsin is the only confirmed genetic risk factor associated with emphysema (Babusyte, Sitkauskiene, & Sakalauskas, 2006; Silverman, 2001), but this is a relative rare cause. Further discussion of the genetics of COPD is beyond the scope of this thesis although it is an area of much active research.

2.1.3 Tobacco smoke, components and airway epithelium

Cigarette smoke is a very diverse mixture of more than 4,700 different chemical compounds with a high concentration of free radicals and different oxidants and consists of a gas and a particulate phase or tar phase (MacNee, 2000). Free radicals are part of both gas and particulate phase in tobacco smoke. The gas phase of the cigarette smoke contains roughly $10^{15}$ radicals per puff mainly from alkyl and peroxyl categories. Nitric oxide is another main component of cigarette smoke, present at concentration of 500 to 1000 ppm, which has the capacity to react with superoxide anion to form peroxynitrites and can also react with peroxyl radicals to form alkyl peroxynitrites (MacNee, 2000; Pryor & Stone, 1993). Both peroxynitrite species are highly chemically reactive. The particulate phase, on the other hand, has more stable radicals like semiquinone radicals, hydrogen peroxide and hydroxyl radicals (Pryor & Stone, 1993), which add substantially to the oxidant burden.
The airway epithelium is one of the first tissue targets of cigarette smoke, which leads to the activation of a number of biological and cellular pathways characterising the development of COPD (Figure 2.1 and 2.2) (K. F. Chung & Adcock, 2008). Chronic exposure to reactive oxygen species (ROS) can cause lipid peroxidation in airway epithelium leading to disruption of cellular membranes and inactivation of membrane-bound receptors and enzymes (Rahman, 2003, 2005; Rahman, Marwick, & Kirkham, 2004). Cigarette smoke induces epithelial changes related to development of chronic bronchitis in large airways which may or may not be associated with COPD, depending on the degree and/or outcomes of epithelial inflammation (J. C. Hogg, 2006). It can lead to mucus hypersecretion which is a feature of both large and small airways in COPD. Mucus secreting pathways are associated with EGFR over-expression in the airway epithelium in response to cigarette smoke-generated reactive oxygen species (Figure 2.3). Over expression of EGFR is also considered as one of the earliest abnormalities found in smokers at high risk of developing lung cancer (K. F. Chung & Adcock, 2008; Mutch, 2002). In small airways, cigarette smoke induces goblet cell metaplasia (Saetta et al., 2000) and increased connective tissue in the airway wall (James C. Hogg et al., 2004).

**Figure 2.1:** Showing link between aetiology of COPD and clinical outcomes (K. F. Chung & Adcock, 2008)
Figure 2.2: Outlining different inflammatory and cellular interactions linking chronic cigarette smoke exposure to chronic inflammation in COPD (K. F. Chung & Adcock, 2008).
Increased oxidative stress due to cigarette smoke inhalation also initiates the process of airway remodelling which is defined as alterations in structural components of the airways leading to (gross) thickening of the airway wall causing airway obstruction (P. K. Jeffery, 2001b). Increased oxidative stress also disturbs the protease-antiprotease balance of the lung, activates macrophages and neutrophils (both of which can secrete various proteolytic enzymes) and also CD8+ T cells which release perforin (cytolytic protein) and granzymes (serine proteases). In addition oxidative
stress decreases VEGF signalling and alveolar repair and increased apoptosis of cells is also observed. All these pathways are associated with development of emphysema (Figure 2.4) with increased proteolytic load leading to elastic fibre damage (Kasahara et al., 2001; Morissette, Parent, & Milot, 2007; Sharafkhaneh, Hanania, & Kim, 2008).

Figure 2.4: Mechanism of cigarette smoke induced airway remodelling and emphysema (Sharafkhaneh et al., 2008).

Numerous studies have reported changes in gene expression in epithelial cells in smokers and non-smokers (Carolan et al., 2006; Chari et al., 2007; N. R. Hackett et al., 2003; Harvey et al., 2007; K. M. Lee et al., 2007). Epithelial cells from small airways demonstrated an increase in the activation of pro-apoptotic gene pirin, and in anti-oxidant genes including glutathione peroxidase, and ubiquitin carboxyl-terminal hydrolase (UCH) L1, which is a member of ubiquitin proteasome pathways, but in
contrast the expression of IL-4 receptor, CX3C chemokine ligand (CX3CL) 1 and the extracellular matrix protein spondin 2 were inhibited (Harvey et al., 2007).

Genes up-regulated during current smoking and then down-regulated with smoking cessation included those for trefoil factor 3, calcium-binding tyrosine-(Y)-phosphorylation-regulated protein, CXCL6, CX3CL1 and S100 calcium-binding protein A9 (S100A9). Partial reversibility with quitting was shown for genes transcribing for mucin (MUC) 5 subtypes A and C (also called as MUC5AC), but an irreversible gene was that for glycogen synthase kinase 3β. The authors suggested that irreversible changes may account for the persistent lung cancer risk despite smoking cessation (Chari et al., 2007).

2.2 Pathology of COPD

The pathological changes in lungs associated with COPD essentially involve two anatomical compartments; the airway component of the disease, and that involving the parenchyma. They frequently coexist, but with some people having predominantly the airway component of the disease and some the parenchymal. The airway component includes inflammation of the central airways and small (peripheral) airways, and the parenchymal component of the disease involves the destructive process of emphysema.

2.3 Airway component

2.3.1 Central airways

Tobacco smoking is a critical factor in the pathological changes and inflammatory response associated with central airways in COPD. Inflammation may present as “chronic bronchitis”, a clinical-epidemiological entity defined by cough and sputum production which reflects mucous hypersecretion in response to tobacco smoke, noxious gases and dust which may not be associated with airflow limitation (Barnes, 2003; J. C. Hogg, 2004). Pathologically, in COPD when associated with airflow limitation, chronic bronchitis involves mucus gland hypertrophy and mucus hypersecretion, squamous metaplasia, loss of epithelial cilia, goblet cell hyperplasia
and hypertrophy, and infiltration of airway wall with inflammatory cells (Figure 2.5) (Caramori et al., 2004 32; Saetta et al., 2000 143).

![Image](image.png)

**Figure 2.5:** Pathologic changes of the central airways in COPD. (A) Central bronchus from the lung of a physiologically normal current smoker, which shows that only small amounts of bronchial smooth muscle are present and the glands are small, compared to a subject (B) with chronic bronchitis with thick bronchial smooth muscle and enlarged bronchial glands. (C) The enlarged bronchial glands at a higher magnification. It also indicates a chronic inflammation involving leukocytes (see arrows) and mononuclear cells, including plasma cells (see arrows) (Macnee, 2007).
Inflammation observed in airways/lungs from patients with COPD is multifaceted and not well understood. In chronic bronchitis inflammation is mainly associated with the epithelium of the larger airways and extends to mucus glands, and involves infiltration of neutrophils and macrophages with increased number of CD8⁺ T-lymphocytes in the bronchial glands (Mullen, Wright, Wiggs, Pare, & Hogg, 1985; Saetta et al., 1997).

Mucus gland hypertrophy was first described in 1954 by Reid (L. M. Reid, 1954), she observed that bronchial glands were enlarged in chronic bronchitis and used the ratio of mucosal gland to the thickness of bronchial wall for the diagnosis of chronic bronchitis, known as Reid’s index (Caramori et al., 2004; L. Reid, 1960; L. M. Reid, 1954). Mucus hypersecretion associated with chronic bronchitis is still a matter of debate as to whether it is related to airway flow limitation or not (Maestrelli, Saetta, Mapp, & Fabbri, 2001).

It has been suggested that up-regulation/activation of the epidermal growth factor receptor (EGFR) on epithelial cells may be associated with abnormal epithelial cell growth and proliferation leading to goblet cell hyperplasia and hence to mucus hypersecretion in COPD (de Boer et al., 2006; Innes et al., 2006). Cigarette smoke is considered the main etiological factor associated with COPD and in humans chronic exposure to tobacco smoke may activate EGFR signalling pathway leading to mucus hypersecretion(Yoshida & Tuder, 2007).

2.3.2 Small airways (obstructive bronchiolitis)

COPD is widely accepted as a pan airway disease, but most attention has been given to the small airways (≤2mm in diameter) where most of the obstruction occurs, characterized by an obliterated lumen with accumulation of inflammatory mucous exudates (Figure 2.6) (Szilasi et al., 2006).

Macklem and colleagues in 1967 introduced the idea of peripheral or small airway disease (J. C. Hogg, 2006; Macklem & Mead, 1967). Soon after, Hogg and colleagues suggested that small airways represent the “quiet zone” where disease can progress for a long time without being clinically diagnosed (James C. Hogg et al., 2004; J. C. Hogg et al., 1968; M. Saetta, 2006). The key pathological changes related
to progression of COPD in small airways involve inflammatory cell infiltration (James C. Hogg et al., 2004; Saetta et al., 1998), goblet cell metaplasia (Saetta et al., 2000) and increased connective tissue in the airway wall (James C. Hogg et al., 2004).

Figure 2.6: Small airway obstruction
(A) Normal small airway. (B) Small airway containing plug of mucus (C) Acutely inflamed airway with thickened wall in which the lumen is partly filled with an inflammatory exudates of mucus and cells (D) Airway surrounded by connective tissue and B cell follicles (J. C. Hogg, 2004).
Severe obstruction is associated with presence of B cells, CD4 and CD8 lymphocytes organized into follicles (James C. Hogg et al., 2004). The increase in the lymphocyte population is also associated with bronchial associated lymphatic tissue (BALT) which is hardly seen in normal-lung function non-smokers but frequently seen in smokers (J. C. Hogg, 2004). The existence of lymphocyte and BALT in COPD suggests that the adaptive immune system is involved and might be associated with microbial colonisation and infections of the lower respiratory tract in COPD (J. C. Hogg, 2004; J. C. Hogg & Timens, 2008).

Alveolar attachments to small airways, which provide support during expiration preventing the airways from collapsing, are though to be damaged as the inflammation progresses. It is still not well understood whether small airway obstruction in COPD is because of bronchiolitis or due to obliteration of surrounding parenchyma and the loss of alveolar attachments, but probably both contribute to small airway obstruction (Szilasi et al., 2006).

2.4 Lung parenchyma component

The parenchymal component of the disease involves the process of emphysema which is further divided into two major types; centrilobular or centriacinar and panlobular or panacinar emphysema.

2.4.1 Emphysema

Emphysema in COPD has been defined as the enlargement of the distal airspaces, beyond the terminal bronchioles, caused by destruction of the alveolar walls (Figure 2.7) (“The definition of emphysema. Report of a National Heart, Lung, and Blood Institute, Division of Lung Diseases workshop,” 1985; MacNee, 2005).

Laennec was the first to explain the lesions produced by emphysema characterized by dilation and destruction of the lung parenchyma (J. C. Hogg, 2004; Kligfield, 1981; Laennec, 1834.) leading to a decrease in maximal expiratory airflow by reducing the elastic recoil force available to drive air out of the lungs (J. C. Hogg, 2004; Macnee, 2007).
The two major types of emphysema referred to above (Figure 2.8) are differentiated according to the region of lung acinus being destroyed. Centrilobular or centriacinar emphysema results from destruction and dilation of respiratory bronchioles in the central portions of the acinus. This type of emphysema is usually related to tobacco smoke and is especially severe in the upper lobes of the lung. Panlobular or panacinar emphysema on the other hand involves a more even destruction and dilation over the entire acinus and is usually associated with the lower lobes of the lungs, for example in α1 anti-trypsin deficiency. α1 anti-trypsin acts as anti-elastase to protect the lungs from destruction (J. C. Hogg, 2004; W. D. Kim et al., 1991; M. Saetta, 2006), its genetic absence or low levels may lead to lung destruction at young age and can also lead to liver disease in children (P. Lee, Gildea, & Stoller, 2002).

A diverse variety of processes seems to be involved in the pathogenesis of emphysema related to tobacco smoking, but the most widely accepted hypotheses relate protease-antiprotease or oxidant/anti-oxidants imbalance. The hypothesis states that the destruction of alveolar walls results from protease activity leading to obliteration of extracellular matrix tissue (ECM), but the various proteases involved and their specific targets is still a matter of debate (Abboud & Vimalanathan, 2008; J. C. Hogg, 2004; J. C. Hogg & Senior, 2002).

Even though the protease-antiprotease hypothesis has predominated it still doesn’t completely explain the pathogenesis of emphysema because if that was the case then all inflammatory lung diseases should be associated to emphysema (Walters, Reid, Soltani, & Ward, 2008). However, there is growing evidence for the vascular hypothesis which states that reduced vascularity might be the primary defect leading to development of emphysema. In 1959 Liebow reported a scarcity of vessels in the lungs of people with emphysema (Liebow, 1959) and later it was reported that inhibition of VEGF receptors can cause emphysema in rats (Kasahara et al., 2000). The same investigators later demonstrated that the process involved a progressive loss of capillary endothelial cells and epithelial cells due to apoptosis secondary to a decrease in vascular endothelial growth factor (VEGF) and VEGF receptor 2, the active VEGFR (Kasahara et al., 2001). Thus, VEGF might be playing a key role in COPD pathogenesis but unfortunately very little data is available on vascular
changes in human COPD, compared for example to work in asthma (Walters et al., 2008).

Figure 2.7: Pathology of emphysema.
Scanning electron micrographs of (A) a normal alveoli and (B) early emphysema with holes in alveolar walls. (C) Histologic section of a normal airway with surrounding alveolar attachments. (D) Enlargement of distal airspaces and reduced alveolar attachments in collapsed airway in emphysema (Macnee, 2007).
Figure 2.8: Photomicrograph showing the microscopic appearance of the different types of emphysema. (A) Normal lung; (B) Centriacinar emphysema showing an area of destruction and end enlargement of airspaces around a bronchiole in the central portion of the acinus surrounded by areas of grossly normal lung parenchyma; and (C) panacinar emphysema demonstrating homogenous enlargement of all air spaces beyond the terminal bronchiole (M. Saetta, 2006).
2.5 Airway inflammation in COPD

Inflammation observed in the respiratory tracts (airways and lungs) from patients with COPD is multifaceted and not well understood; even though it involves the activation of both innate and adaptive immune responses. Thus, the inflammatory response observed in COPD is very diverse tautological and recruitment of various inflammatory cells and mediators contribute significantly to the lung injury in patients with COPD, and appears to serve as a self-perpetuating stimulus for further enhancement of the immune response. The progression of disease involves an active migration of "leukocytes" (mainly neutrophils and macrophages) associated with production of inflammatory mediators and potentially destructive pro-inflammatory cytokines, proteases and various growth factors, which lead to structural changes in the airways termed as "airway remodelling" (explained in detail latter on) (V. Kim, Rogers, & Criner, 2008; Rumora et al., 2008). Chronic inflammation in COPD in response to chronic cigarette smoke and then other exposures, (Figure 2.2) is mainly characterised by accumulation of neutrophils, macrophages, B-cells lymphoid aggregates and CD8+ T-cells, especially in small airways and inflammation becomes worse with disease severity (Sutherland & Martin, 2003).

COPD is predominantly a neutrophilic disease at least in the airway and airspace lumen. Neutrophils are usually found in bronchial epithelium, glands and also in airway smooth muscle bundles but mainly in the airway lumen of both small and large airways, as reported in sputum and BAL (bronchoalveolar lavage) (K. F. Chung & Adcock, 2008). Saetta et al assessed number of neutrophils, eosinophils, mast cells, macrophages, CD4+ and CD8+ T-lymphocytes, and the ratio of CD4+ to CD8+ cells in the bronchial glands, epithelium, and lamina propria by immunohistochemistry. They found that smokers with symptoms of chronic bronchitis had an increased number of neutrophils and macrophages and a decreased CD4+/CD8+ ratio in the bronchial glands when compared to smokers without symptoms, and smokers with chronic bronchitis also had increased number of epithelial neutrophils, whereas the numbers of macrophages and CD4+ and CD8+ T-lymphocytes in the epithelium and lamina propria were similar in the two groups of smokers (Saetta et al., 1997). In another study it was shown that current smokers with COPD had increased number of neutrophils and CD8+ T cells in the airway
smooth muscle of peripheral airways compared with non-smokers, and they also reported that smokers with normal lung function also had a neutrophilic infiltration in the airway smooth muscle, but to a lesser extent compared to current smokers with COPD (Baraldo et al., 2004). When all the groups were analysed as one group neutrophilic infiltration in the smooth muscle of peripheral airways inversely related to FEV1% predicted (forced expiratory volume in one second) (Baraldo et al., 2004). Stanescu et al reported increased levels of neutrophils in sputum of smokers and showed that airways obstruction, chronic expectoration, and rapid decline of FEV1 in smokers are associated with increased levels of sputum neutrophils (Stanescu et al., 1996).

Different studies have variably reported increased levels of eosinophils in the airway wall and BAL and also in induced sputum, but the role of eosinophils in COPD are not very clear. It is suggested that eosinophilia may represent a different sub-group of COPD (K. F. Chung & Adcock, 2008). Interestingly, a few studies have reported that an increase in BAL and sputum eosinophils in COPD patients is related to good clinical response to steroid therapy (Chanez et al., 1997; Fujimoto, Kubo, Yamamoto, Yamaguchi, & Matsuzawa, 1999; Pizzichini et al., 1998), but this was not replicated by our group who could also not relate eosinophils to bronchodilator responsiveness in COPD (D. W. Reid et al., 2008).

The situation of mast cells in COPD is not very clear either, with some studies reporting an increase in airway mast cells while others not. Pesci et al investigated BAL and bronchial biopsies with immunohistochemical techniques for mast cells in current smokers and ex-smokers with chronic bronchitis, and found that subjects with “chronic bronchitis” (both current and ex-smokers with cough and sputum) had higher numbers of mast cells both in the epithelium and in the bronchial glands compared to normals, whereas the numbers of mast cells in BAL and in the lamina propria were similar in the two groups. In current smokers with bronchitis an increase in mast cell numbers was observed in the epithelium and lamina propria, and in BAL compared to ex-smoker bronchitis (Pesci et al., 1994). In another study, it was reported in small airway samples from COPD patients (both current smokers and ex-smokers) that numbers of epithelial mast cells and macrophages increased in the airway wall of smokers with airflow limitation, suggesting a potential role for mast
cells in development of COPD (Grashoff et al., 1997). On the other hand, Saetta et al reported no change in mast cell numbers in the bronchial mucosa of subjects with chronic bronchitis (Saetta et al., 1993). The same group later reported normal mast cell numbers in current smokers with COPD and also in normal lung function smokers (Di Stefano et al., 1998). In a similar study, Shaughnessy et al reported no change in sub-epithelial mast cells in subjects with chronic bronchitis (O'Shaughnessy, Ansari, Barnes, & Jeffery, 1997).

More consistently, CD8+ T cells are reported increased in COPD airways and also in the lung parenchyma (Peter J. Barnes, 2008). Shaughnessy et al reported that the number of CD8+ T cells increase in the sub-epithelial zone (lamina propria) of bronchial biopsies from patients with chronic bronchitis and inversely correlated to FEV1 (O'Shaughnessy et al., 1997). Baraldo et al reported increased numbers of CD8+ T cells localised within the smooth muscle of peripheral airways of COPD patients (Baraldo et al., 2004). Chrysofakis et al also reported increased levels of CD8+ T cells in sputum of smokers with and without COPD compared to non-smokers, and interestingly they also reported that they are highly active and expressed high levels of lytic substances such as perforin which may lead to parenchymal damage in COPD lung (Chrysofakis et al., 2004). Saetta et al also reported increased CD8+ T cells in peripheral airway wall of smokers who develop COPD, suggesting potential role in COPD pathogenesis (Saetta et al., 1998).

Gosman et al reported increased number of B-cells in the sup-epithelial region of bronchial biopsies from COPD patients (Gosman et al., 2006). Severe airway obstruction is associated with presence of B cells, organized into follicles in small airways (James C. Hogg et al., 2004). As also described earlier, increase in the lymphocyte population is also associated with bronchial-associated lymphatic tissue (BALT) which is hardly seen in physiologically normal non-smokers but frequently seen in smokers (J. C. Hogg, 2004). The existence of lymphocytes and BALT in COPD suggests that the adaptive immune system is involved and might be associated with microbial colonisation and infections of the lower respiratory tract in COPD (J. C. Hogg, 2004; J. C. Hogg & Timens, 2008). Dendritic cells along with B-cells have also been reported to play a role in adaptive immunity in COPD (K. F. Chung & Adcock, 2008). Van der et al reported lymphoid follicles consisting of B cells and
folicular dendritic cells with adjacent T cells both in the parenchyma and in bronchial walls of patients with emphysema (van der Strate et al., 2006). Demedts et al reported increased numbers of dendritic cells in the epithelium of small airways of patients with COPD compared with never-smokers and smokers without COPD (Demedts et al., 2007). Additional evidence for recruitment of dendritic cells by tobacco smoke exposure comes from studies done in mice in which chronic cigarette exposure led to an increase in CD11c+ dendritic cells (D’Hulst A et al., 2005).

It is suggested that COPD may also have an auto-immune component as it is a chronic inflammatory disease, albeit with a much better recognised aetiology than other such illness e.g. rheumatoid arthritis (RA). But like RA, once initiated, the inflammatory response COPD seems to be self-perpetuating even after smoking cessation (Agusti, MacNee, Donaldson, & Cosio, 2003). In a very interesting study, Lee et al showed that emphysema is an autoimmune disease, which could be characterised by circulating anti-elastin antibodies and Th1-type immune responses, which correlated with disease severity (Peter J. Barnes, 2008; S. H. Lee et al., 2007). Feghal et al reported increased levels of IgG auto-antibodies with increased avidity for pulmonary epithelium, and the potential to mediate cytotoxicity in patients with COPD (by using immuno-fluorescence and immuno-precipitation and immuno-histochemistry techniques), suggesting that auto-reactive adaptive immune responses may be important in the etiology of COPD (Feghali-Bostwick et al., 2008). The jury is still out on whether these observations are centrally important, or whether they just represent an epi-phenomenon related to release of airway antigens by tissue proteolytic damage.

Macrophage numbers are increased in COPD, to a greater extent compared to asthma (Peter J. Barnes, 2008). Saetta et al observed increased numbers of CD68+ macrophages in the sub-epithelial lamina propria of patients with chronic bronchitis (Saetta et al., 1993). In another study clusters of macrophages were observed in small airways associated with peri-bronchial fibrosis seen in smokers and ex-smokers and it was suggested that they may be related to small airway fibrosis and/or development of centrilobular emphysema (Fraig, Shreesha, Savici, & Katzenstein, 2002). Finlay et al showed that macrophages from BAL of patients with emphysema express higher levels of mRNA for MMP-1 and MMP-9 (matrix metalloproteinases),
and they demonstrated that alveolar macrophages from the emphysematous lung produced large amounts of matrix-degrading enzymes which may play an important role in development of COPD (Finlay et al., 1997). Others have also suggested that alveolar macrophages could be orchestrators of COPD (Barnes, 2004).

2.6 Airway remodelling in COPD

Airway inflammation is the response of a fully vascularised tissue to injury and the rationale behind the inflammatory response is to protect the host and to restore normal tissue functioning. It is widely assumed that accelerated decline in lung function in COPD is a consequence of acute inflammation leading to parenchymal and airway wall remodelling respectively. However, whether remodelling is part of the inflammatory process, or whether it is a separate and parallel entity is still a matter of debate and there is no convincing evidence either way available in the literature (P. K. Jeffery, 2001b). In reality there are very few data available on airway remodelling in COPD, even in comparison to asthma where many questions remain to be answered.

During lung development, the lung undergoes extensive “modelling” and then “remodelling” changes and this whole process is very tightly regulated by different signalling pathways, growth factors and various cytokines. It may be possible that the perception of remodelling in airway disease is a reactivation of these embryological mechanisms as most of the cytokines and growth factors thought to be detrimental in COPD and other respiratory disorders are expressed normally during lung morphogenesis (P. K. Jeffery, 2001b; Warburton et al., 2001).

Currently airway remodelling is defined as an alteration in size, mass, or number of tissue structural components leading to gross thickening of the airway wall occurring in response to oxidant or other injury and/or inflammation (P. K. Jeffery, 2001b). In COPD, remodelling may occur as a response to smoking-induced damage to the airways, but the mechanisms of damage and initiation, and even the structural changes themselves are poorly described compared to work published in asthma (Churg et al., 2006; Saetta et al., 2000). Structural changes mainly described in COPD are: small airway wall thickness(James C. Hogg et al., 2004), metaplasia of
the epithelium, loss of epithelial cilia, increase in goblet cell size and number, submucosal gland and smooth muscle hypertrophy (Figure 2.9 & 2.10) (Bergeron & Boulet, 2006; Saetta et al., 2000).

**Figure 2.9:** Airway structural changes in COPD. 
(A) Epithelial metaplasia (white arrow) and submucosal gland hyperplasia (black arrow). (B) Increase in smooth-muscle mass (black arrow) (Bergeron & Boulet, 2006).
2.6.1 Epithelium

Respiratory epithelium is described as pseudostratified “columnar”, consisting of eight different types of epithelial cells classified into three main categories: basal, ciliated and secretory. It is a highly active part of the airways and acts as a chemical, physical, and immunological barrier. It is also the site of first contact for cigarette smoke and plays an important role in protecting the lung (Davies, 2009; Knight & Holgate, 2003; Thorley & Tetley, 2007).
Epithelial shedding is not widely reported in COPD; however denuded epithelium has been reported in young smokers in small airways associated with increased inflammatory cell infiltration (M. G. Cosio, Hale, & Niewoehner, 1980). Thick epithelium, loss of cilia, goblet cell hyperplasia and squamous metaplasia (transformation of a columnar epithelium into a squamous epithelium) are also frequently observed and reported in COPD (Figure 2.9 & 2.10) (P. K. Jeffery, 2001b; V. Kim et al., 2008).

Chronic exposure to cigarette smoke activates various oxidative pathways leading to the secretion of a diverse variety of cytokines and chemokines and various epithelial derived growth factors including; granulocyte- macrophage colony stimulating factor (GM-CSF), which is secreted by a wide variety of cells in the airways including epithelium, airway smooth muscle, fibroblasts, T lymphocytes, mast cells, eosinophils and macrophages(Saha et al., 2009) These can lead to alterations in the reticular basement membrane (Rbm) by activating fibroblasts/myofibroblasts, sub-mucosal glands, smooth muscle and the vascular bed (P. K. Jeffery, 2001b). However, few studies have shown potential for GM-CSF in COPD, and no direct evidence is available (Saha et al., 2009).

EGFR expression and function has been studied broadly in asthma and reported in several studies, but fewer data are available in COPD. Cigarette smoke has the potential to activate signaling associated with EGFR which can further lead to mucus hypersecretion (Shao, Nakanaga, & Nadel, 2004). EGFR can induce abnormal epithelial cell growth, differentiation, migration and proliferation leading to goblet cell hyperplasia and hence to mucus hypersecretion in COPD (de Boer et al., 2006; Innes et al., 2006).

Transforming growth factor-β1 (TGF-β1) expression has been observed in the epithelium in bronchial biopsies from COPD patients (Kokturk, Tatlicioglu, Memis, Akyurek, & Akyol, 2003) and is also considered as one of the major growth factors driving the process of airway remodelling generally in inflammatory airway disease. Some studies have reported that interactions between TGF-β and its second messenger, Smad signalling pathway play an important role in airway remodelling
by regulating extracellular matrix (ECM) turnover (Figure 2.11) (Postma & Timens, 2006). Springer and colleagues demonstrated that Smad signalling is altered in COPD due to a decrease in TGF-β inhibitory Smad-6 and Smad-7 observed in epithelium in bronchial biopsies from COPD patients compared to normal controls. This may suggest that inhibitory pathways are distorted in COPD leading to lack of control of fibrotic changes (Springer, Scholz, Peiser, Groneberg, & Fischer, 2004).

Figure 2.11: Main components of the Smad pathway (Gosman et al., 2006).

Increased vascular endothelial growth factor (VEGF) expression in the epithelium has also been reported in COPD compared to normal non-smokers, which may suggest a role for VEGF in epithelial repair in response to cigarette smoke exposure (Kranenburg, de Boer, Alagappan, Sterk, & Sharma, 2005). The exact role of VEGF in epithelium is not clear but a significant body of literature suggests a decrease in VEGF in lung parenchyma may be associated with emphysema (Liebow, 1959).
Cigarette smoke increases the oxidative stress in the lungs which also leads to increased proteolytic load in the lungs of smokers through infiltrating neutrophils and macrophages, but there is very convincing evidence available now that the epithelium itself has the capacity to secrete a variety of proteases, mainly matrix-metalloproteinases (MMPs). MMPs are the largest family of proteases with more than 25 members identified so far. They have the capacity to degrade all the components of the ECM, (Thorley & Tetley, 2007) playing a key role in matrix turnover, remodelling and angiogenesis (Kian Fan Chung, 2005).

Immunohistochemical studies (Baraldo et al., 2007; Thorley & Tetley, 2007) and sputum profiles (Mercer et al., 2005) in COPD have shown that MMP-2 and MMP-9 levels are elevated during disease progression. On the other hand it was also shown that epithelial anti-proteases like SERPIN2 (serine protease inhibitor) which codes for a thrombin and urokinase inhibitor (Demeo et al., 2006), tissue inhibitors of metalloproteinases TIMPs (Mercer et al., 2005) and secretory leukocyte protease inhibitors (SLPI) (Thorley & Tetley, 2007) are elevated during chronic inflammation in COPD. This suggests activation of defensive mechanism of the epithelium against the enhanced proteolytic activity induced by smoking. It is the balance of this "combat" between competing proteolytic forces that may decide the fate of the airway and lung in smokers.

2.6.2 Reticular basement membrane (Rbm)

The human airway epithelium is attached to a true basement membrane, which further consists of a lamina rara and a lamina densa. The reticular basement membrane, also known as lamina reticularis, is located just below the true basement membrane extending superficially (Postma & Timens, 2006; Saglani et al., 2006), and separates the epithelium from the underlying lamina propria mesenchymal structure.

Immunohistochemical studies have confirmed that true basement membrane is comprised mainly of type IV collagen (Montes, 1996) and is attached strongly to the underlying Rbm by collagen VII strands (Liesker et al., 2009). In asthmatics Rbm is homogenous and hyaline in appearance (Peter K. Jeffery, 2004) (Figure 2.12) and
comprises mainly collagen I, III, V, fibronectin, tenascin (Postma & Timens, 2006) and laminin (Liesker et al., 2009) and is thickened (P. K. Jeffery, 2001b). Thickening of the Rbm in asthma has a strong positive correlation with the number of subepithelial fibroblasts and myofibroblasts (Brewster et al., 1990). Rbm develops in normal healthy individuals during childhood, but thickening may develop early in individuals with asthma perhaps even before symptoms start (P. K. Jeffery, 2001b).
Figure 2.12: The airway mucosa as it appears by light microscopy (LM) of bronchial biopsies from (A) an atopic asthmatic showing loss of surface epithelium and early thickening and hyaline appearance of the reticular basement membrane Rbm and (B) a heavy smoker with COPD demonstrating epithelial squamous metaplasia and a thinner Rbm of normal thickness. Stained with haematoxylin-eosin (H&E) (P. K. Jeffery, 2001b).
In COPD, data on Rbm are rare and very ambiguous; there is no clear evidence on Rbm thickness, composition or appearance (Figure 2.12), although some studies have reported that tenascin is increased in COPD Rbm and there is also a trend towards stronger type IV collagen staining. Less collagen I and laminin is also reported in COPD Rbm compared to asthma (Kranenburg et al., 2006; Liesker et al., 2009). Rbm thickness in COPD is still a matter of debate, with some studies showing increased thickness while others report no difference (Liesker et al., 2009), but it appears that composition of reticular basement membrane is different in COPD subjects compared to asthmatics (Liesker et al., 2009; Postma & Timens, 2006).

Interstitial collagen (forming scar tissue), which lies deeper than the Rbm and reported as airway wall fibrosis, is usually considered as the feature of smokers who are more likely to develop COPD (especially in small airways), but no such evidence of fibrosis is available in large airways (Bosken, Wiggs, Pare, & Hogg, 1990; Peter K. Jeffery, 2004).

2.6.3 Extracellular matrix
Increase in airway wall tissue has been observed due to increase in volume of epithelium, lamina propria, muscle and adventitial compartments of small airways and may lead to fixed airflow limitation in COPD (James C. Hogg et al., 2004). In asthma matrix deposition is mainly observed in the subepithelium Rbm area but in COPD the whole airway wall seems to be affected. However, there are actually very few studies reporting matrix changes and their exact role in COPD, this needs further investigations (Kian Fan Chung, 2005), which we aim to do.

Van Straaten et al reported immunohistochemical studies in lung tissues from patients with mild and severe emphysema, and from patients with lung fibrosis. They looked at collagens, laminin, fibronectin, proteoglycans and beta1-integrins and reported that majority of the patients with severe emphysema showed a decrease in staining for the interstitial proteoglycans decorin and biglycan in the peribronchiolar area, compared to controls and patients with fibrosis. Very few patients with mild emphysema showed this pattern of reduced staining. In comparison, decorin and biglycan were strongly positive in the perivascular area of all of the sections from
patients with emphysema. There was no change to collagen type I, III and IV and also no abnormality was observed in laminin or fibronectin expression in the three pathological groups. They also suggested that specific loss of interstitial proteoglycans may be essential for elastic recoil loss and following bronchiolar obstruction, as seen in patients with smoking-related emphysema (van Straaten et al., 1999).

Somewhat contradictory to these findings, Kranenburg et al. (Kranenburg et al., 2006) in another immunohistochemical study observed that COPD has increased expression of collagen type I, III and IV, fibronectin and laminin and also increased expression of matrix proteins in the Rbm, lamina propria and adventitia of the bronchial walls and vessels. At the site of damaged bronchial epithelium in the surface epithelial basement membrane, enhanced matrix protein deposition was observed. They also observed that collagens I and III were dominant in COPD patients in the reticular layer near the lamina propria, when compared to normal controls, and there was also increased expression of fibronectin and laminin observed in bronchial vessels. FEV1 correlated inversely with the collagen in the Rbm region, fibronectin in bronchial vessels and laminin in the airway smooth muscle layer. These findings may indicate a pathogenic role for matrix proteins in airway remodelling in COPD, but these studies are limited by the fact that tissue was obtained from COPD patients who had undergone surgery for lung cancer. There is no direct evidence from airway biopsies in otherwise healthy non-cancer COPD patients (Kranenburg et al., 2006).

2.6.4 Vascularity

The literature on vascular remodelling and angiogenesis in chronic airway disease is scant, although there are studies reported in asthma (Feltis et al., 2006; McDonald, 2001). Vascular remodelling involves structural changes, usually enlargement of arterioles, capillaries or venules without the formation of new vessels whereas angiogenesis is the growth of new blood vessels from the existing ones (Feltis et al., 2006; McDonald, 2001).

Dunnill et al in 1960 (Dunnill, 1960) were the first to describe enlargement of the capillary bed in the airway wall of patients who died of chronic asthma. Increased
vascularity was later reported in mild asthmatics as well, but perhaps especially in severe corticosteroid dependent asthmatics (Peter K. Jeffery, 2004). Bronchial mucosal blood vessel dilation, congestion and wall edema are reported as hallmarks of chronic asthma and may account for substantial swelling and stiffening of the airway wall (Peter K. Jeffery, 2004). In another study, Khor, et al suggested that airway vascular leakage is a major pathophysiologic feature of early asthma deterioration, occurring before recrudescence of cellular inflammation (Khor et al., 2009).

Several angiogenic growth factor cytokines have been recognized including members of the fibroblast growth factor (FGF) family, vascular endothelial growth factor VEGF, TGF-α and TGF-β, angiogenin, platelet derived growth factor, tumor necrosis factor-α (TNF-α), GM-CSF, hepatocyte growth factor (HGF), and various interleukins and chemokines such as IL-8. Angiopoietin 1 and 2 are also involved (Postma & Timens, 2006; Puxeddu, Ribatti, Crivellato, & Levi-Schaffer, 2005), perhaps more in a vessel stabilisation/maturation role. Among all the different angiogenic factors, VEGF has been recognised as the key growth factor involved in regulation of angiogenesis (Figure 2.13) and is highly expressed during chronic inflammation (Postma & Timens, 2006).
Figure 2.13: Schematic representation of the main regulatory roles of vascular endothelial growth factor (VEGF). ECM = extracellular matrix; TGF = transforming growth factor; MMP = matrix metalloproteinase; TIMP = tissue inhibitor of metalloproteinase (Gosman et al., 2006).
Imbalance between VEGF and endostatin (a potent anti-angiogenic factor) levels has been observed in the sputum of patients with asthma, this suggests that this disparity may trigger proangiogenic factors leading to abnormal new blood vessel formation in asthma (Asai et al., 2002). Hoshino and colleagues reported that VEGF expression increases in the airway mucosa of patients with asthma compared to normal controls (Hoshino, Nakamura, & Hamid, 2001). A very interesting study reported higher levels of VEGF in bronchoalveolar lavage fluid and in bronchial biopsies in subjects with asthma which were related to number of vessels (Feltis et al., 2006). Angiogenic sprouts (i.e., early-forming vascular structures) were also observed and their number increased in subjects with asthma (Feltis et al., 2006).

Yet again, data on COPD are much more limited. Vascular abnormalities have been reported in COPD, and associated with disease progression (Postma & Timens, 2006). Kranenburg and colleagues reported enhanced expression of VEGF in the bronchial, bronchiolar and alveolar epithelium and macrophages. They also observed VEGF expression in airway smooth muscle and vascular smooth muscle cells in both the bronchiolar and alveolar compartments (Kranenburg et al., 2005). They suggested that VEGF and its receptors VEGFR1 (decoy) and VEGFR2 (active) may be involved in epithelial and endothelial cell repair and maintenance in response to injury caused by cigarette smoking and may be associated with airway remodelling in COPD (Kranenburg et al., 2005; Siafakas, Antoniou, & Tzortzaki, 2007).

In a very interesting study, Calabrese and colleagues (Calabrese et al., 2006) evaluated the contribution of vasculraity in airway remodelling in smokers with normal lung function and smokers with COPD. They performed an immunohistochemical analysis involving vessels positive for integrin αvβ3. High αvβ3 expression was observed in bronchial vessels which was associated with higher cellular expression of VEGF, suggesting that these two molecules might be playing an important interacting role in angiogenesis. Enhanced bronchial vascularity was also observed as both number of vessels and vascular area of lamina propria in smokers with normal lung function and smokers with COPD compared to normal healthy controls. These data from normal lung function smokers may suggest that
vascular changes might be independent of the airway obstruction and severity of the disease (Calabrese et al., 2006).

Inflammation, which is one of the hallmarks of COPD, also has the potential to promote angiogenesis in numerous ways (Figure 2.14) (Siafakas et al., 2007). Airway inflammation in COPD mainly involves infiltration of neutrophils, macrophages and CD8+ T cells in the lumen, bronchial and bronchiolar airway wall and lung parenchyma (P. K. Jeffery, 2001a; Siafakas et al., 2007). These inflammatory cells in the lungs can release various mediators such as TNF-α which can promote angiogenesis. Hypoxia can also occur in COPD either globally or focally in inflamed tissue, which may also induce angiogenesis (Siafakas et al., 2007).

Figure 2.14: Schematic representation of an angiogenetic process in COPD (Siafakas et al., 2007).
Hypoxia has also been considered a key factor involved in pulmonary vascular remodelling leading to pulmonary hypertension in COPD (Nilsson, Shibuya, & Wennstrom, 2004; Wright et al., 1983). However, vascular remodelling of pulmonary arteries is not only the characteristic of severe COPD but has also been reported in patients with mild COPD without arterial hypoxemia and also in smokers with normal lung function (Barbera et al., 1994). It is reported that VEGF could play a major role in remodelling of pulmonary arteries in smokers and COPD (Peinado et al., 1999; Siafakas et al., 2007).

2.6.5 Airway smooth muscle

Bronchial smooth muscle mass (Figure 2.10) and its contribution to airway wall remodelling has been predominantly studied in small airways compared to large airways in COPD (P. K. Jeffery, 2001b). Whether airway smooth muscle mass is a prominent cause of airflow limitation, is still a matter of debate, with no convincing evidence available (V. Kim et al., 2008).

However, an increase in airway smooth muscle in small airways was inversely correlated with lung function in one study (Kian Fan Chung, 2005). In another it was reported that muscle mass was increased by 50% in patients with severe COPD in small airways (James C. Hogg et al., 2004). There is little information available on airway smooth muscle cells, but they may be functionally altered in proximal airways in COPD. Unfortunately there is no information available on function of the airway smooth muscle in small airways. It is not clear whether any increase in muscle mass in COPD is due to an increase in number of muscle cells, or increase in airway smooth muscle size, or a combination of both. In asthma, airway smooth muscle predominantly increases in large airways whereas in COPD this seems to occur mainly in small airways (Kian Fan Chung, 2005).

Abnormalities associated with smooth muscle mass in large airways have not been reported in COPD, although internal airway wall thickness has been associated with reduced FEV1/FVC ratio (Kian Fan Chung, 2005; Peter K. Jeffery, 2004). Even biopsy studies from large airways reported no increase in smooth muscle area and size; moreover, smooth muscle protein isoforms were not increased, but there was a
slight increase in myosin light chain kinase but with no increase in phosphorylated myosin light chain. Thus, more data are required on airway smooth muscle remodelling in large airways in COPD (Kian Fan Chung, 2005).

2.6.6 Goblet cells, submucosal glands and mucus

Goblet cells are mucus-producing cells which are dispersed widely in the airways, reproductive tract and alimentary canal. In the airway their major function is to hydrate, lubricate and to clear the particulate matter and pathogens from the lumen by secreting mucus, which forms a slimy film covering mainly the luminal surface of epithelial cells. Goblets cells are the major secretory cells of the airways and the major source of luminal mucus (Davis & Dickey, 2008; Yoshida & Tuder, 2007).

Goblet cell hyperplasia has been associated with both asthma and chronic bronchitis in large airways (P. K. Jeffery, 2001b). Their number is usually less in small airways, but an increase in peripheral airways (diameter < 1 mm) in COPD subjects has been associated with an influx of neutrophils into the airways promoting the concept that neutrophils might be playing a role in goblet cell degranulation through release of secretagogues neutrophils elastase and cathepsin G (Sommerhoff, Nadel, Basbaum, & Caughey, 1990). In another study done by M. Saetta, et al there was an increase in the number of goblet cells and inflammatory cells in the epithelium of peripheral airways of smokers with chronic bronchitis and airflow limitation (Saetta et al., 2000).

Submucosal gland hypertrophy was described by Reid in 1954 (L. M. Reid, 1954). Mucus gland hypertrophy is observed in both asthma and COPD. In COPD submucosal gland hypertrophy is mainly seen in large airways (P. K. Jeffery, 2001b). Glands are composed of serous and mucus secretory units or acini. In asthma normal proportions of mucus and serous units are retained, whereas in chronic bronchitis this balance is disturbed and there is a disproportionate increase in mucous units and decrease in serous units (Glynn & Michaels, 1960). Serous units in the submucosal glands secrete a wide range of antibacterial substances such as the secretory component of secretory IgA, lysozyme and lactoferin. Loss of these substances during airway remodelling may create a potential for chronic bacterial colonisation in
the airways (P. K. Jeffery, 2001b), but interestingly, mucus production and gland hypertrophy are poorly related to each other, whereas inflammation has been shown to be closely associated with mucus hypersecretion (Barbera et al., 1994).

There are very few studies in the literature reporting the composition of mucus in COPD and the various types of mucins involved (Kian Fan Chung, 2005) and data regarding mucus hypersecretion are contradictory. Innes and colleagues reported that epithelial mucin stores are elevated in the large airways of habitual smokers with airflow limitation, with predominant increase in MUC5AC gene expression (gene which encodes for mucin-5AC protein in humans, also commonly known as MUC5AC in literature). They reported significant decrease in MUC5B gene expression (gene which encodes for mucin-5B protein in humans, also commonly known as MUC5B in literature) while other authors found no change (Innes et al., 2006). In peripheral airways Caramori and colleagues found increased expression of MUC5B in the bronchiolar lumen and increases in MUC5AC expression in the bronchiolar epithelium of patients with COPD (Figure 2.15) (Caramori et al., 2004).

How mucus secretion is controlled is not well understood. However, limited studies have reported that cellular signaling initiated by epidermal growth factor receptor (EGFR) may be playing a key role in mucus production and regulation (Yoshida & Tuder, 2007). Cigarette smoke is considered as the main etiological factor associated with COPD and in humans chronic exposure to tobacco smoke may activate EGFR signalling pathways leading to mucus hypersecretion(Yoshida & Tuder, 2007). EGFR can be activated in two different ways ie, involving a ligand-dependent or a ligand-independent mechanism (Figure 2.16) (Burgel & Nadel, 2004). In ligand-dependent EGFR tyrosine phosphorylation, EGFR ligands bind to their receptor and activate it, but on the other hand in a ligand independent mechanism EGFR tyrosine phosphorylation can occur directly in response to increase in oxidative stress due to cigarette smoke and inflammation (Burgel & Nadel, 2004).

In COPD there are few studies reporting EGFR expression. Immunohistochemical analysis done by De Boer et al revealed that EGFR expression is increased in biopsies from COPD patients (de Boer et al., 2006). EGFR family has four different types of receptors termed ErbB1, ErbB2, ErbB3 and ErbB4 and EGFR itself is also
known as ErbB1. Donnell and colleagues reported enhanced expression of EGFR, ErbB3 and MUC5AC in the airways of long term current smokers; they also observed that this increase was not associated with neutrophilic inflammation and suggested that ErbB3, may be playing an important role in mucus hypersecretion (O’Donnell et al., 2004).
Figure 2.15: Representative photomicrographs of (A) bronchiolar epithelium immunostained for MUC5AC (brown) and (B) bronchiolar lumen immunostained for MUC5B (brown) from smokers with chronic obstructive pulmonary disease (COPD) (Caramori et al., 2004).
2.6.7 Airway remodelling and lung function consequences

Small airways have been reported as the major site of obstruction in COPD in various studies (James C. Hogg et al., 2004; J. C. Hogg et al., 1968). Airway remodelling and chronic inflammation in these airways has been associated with airflow limitation (Bosken et al., 1990; M. Cosio et al., 1978; James C. Hogg et al., 2004). In addition, a strong association has been observed between disease progression and increased structural abnormalities in components of the airways including epithelium, lamina propria, airway smooth muscle and adventitia in COPD (James C. Hogg et al., 2004). Furthermore, chronic inflammation in peripheral airways has been associated with abnormal connective tissue deposition in the airway wall and with emphysema (M. Cosio et al., 1978). With increasing abnormalities in
the peripheral airways, lung function declined progressively and small airway function tests helped to differentiate patients with minimal but progressive pathologic and physiological changes from patients with normal airways (M. Cosio et al., 1978).

Emphysema in COPD may also be play an important role in airflow limitation, depending on the type of the emphysema involved (Kian Fan Chung, 2005). Centrilobular or centriacinar emphysema results in destruction and dilation of respiratory bronchioles in the central portions of the acinus, is usually related to tobacco smoke and is primarily severe in the upper lobes of the lung and associated with small airway obstruction. Panlobular or panacinar emphysema on the other hand involves a more uniform destruction and dilation over the entire acinus and is usually associated with the lower lobes of the lungs (for example in α1 anti-trypsin deficiency) (J. C. Hogg, 2004; W. D. Kim et al., 1991; M. Saetta, 2006) and its major impact is on lung diffusing capacity.

Panacinar emphysema is also related to loss of lung elasticity and higher compliance, whereas centrilobular emphysema is associated with chronic airway inflammation and airway hyper-responsiveness (Kian Fan Chung, 2005). Hale K. A. et al reported that severe pathological changes, including emphysema, correlated with the degree of airflow limitation as measured by FEV\textsubscript{1} (Hale, Ewing, Gosnell, & Niewoehner, 1984). However in emphysema the loss of elastic recoil is the cause of airflow limitation rather than changes in airway wall remodelling directly on the airway lumen (J. C. Hogg et al., 1968). Where diffusing capacity is reduced in addition to airflow limitation, symptoms are appreciably greater (Burgess et al., 2008).

Recent detailed data on airway remodelling in COPD is very sparse, and how these changes lead to decline in lung function are not well understood. It is likely that in COPD the epithelium may get damaged by cigarette smoke and airway wall remodelling may occur in response to this, but the mechanisms and detailed structural changes are poorly described. In other COPD-like airway disease, especially bronchiolitis obliterans syndrome (BOS, a manifestation of chronic rejection post lung transplant), a new component of remodelling is emerging in which alveolar epithelial cells undergo a transition to a mesenchymal phenotype with
myofibroblast characteristics and then migrate through the Rbm to the sub-epithelial lamina propria, a process termed “epithelial-mesenchymal transition” (EMT) (Borthwick et al., 2009; Ward et al., 2005). There are no reported studies on EMT in COPD, but a significant body of literature shows evidence of EMT in BOS (Borthwick et al., 2009; Ward et al., 2005) and also in idiopathic pulmonary fibrosis (IPF) (Brigham C. Willis et al., 2006). Hackett et al. recently reported that alveolar epithelial cells from asthmatics can undergo a transition to a mesenchymal phenotype when stimulated with TGF-β1, but when the actual airway biopsy sections from asthmatics were stained for markers of EMT they were negative and in addition the thickened homogenous Rbm in asthma shows none of the characteristic fragmentation, characteristic of EMT (T. L. Hackett et al., 2009).

2.7 Epithelial - mesenchymal transition (EMT)

From a general perspective, epithelial mesenchymal transition is a biological process in which epithelial cells undergo extensive molecular reprogramming allowing epithelial cells to undergo numerous biochemical changes and acquire a mesenchymal phenotype. This is accompanied by progressive loss of epithelial markers, gain in migratory potential and invasiveness, and enhanced capacity to produce extracellular matrix components (Figure 2.17) (Raghu Kalluri & Neilson, 2003; R. Kalluri & Weinberg, 2009). The digestion of underlying basement membrane facilitates the process of EMT and the fragmented anatomical changes in the Rbm have been reported as a marker of completion of the process of EMT (R. Kalluri & Weinberg, 2009). This, of course is what first attracted my attention to EMT being a possibility in COPD.

Elizabeth Hay from Harvard University was the first to describe the process of “epithelial mesenchymal transformation” in 1982 and then in 1995 as an important process involved in embryogenesis and organ development (Guarino, Tosoni, & Nebuloni, 2009; Hay, 1982, 1995). In the intervening time, the term “transformation” has been replaced with the term “transition”, indicating potential induction and reversibility of the process, even a two-way process (Raghu Kalluri & Neilson, 2003). The reverse process has been termed as mesenchymal-epithelial transition
(MET), but there are very few studies reporting MET in the literature and most are concerned with embryogenic kidney formation (R. Kalluri & Weinberg, 2009).

**Figure 2.17:** Epithelial mesenchymal transition, with progressive loss of epithelial markers and gain of mesenchymal markers (R. Kalluri & Weinberg, 2009).

The literature on these different terms is somewhat conflicting; they are often used inappropriately. Thus, the terms “epithelial mesenchymal transformation, interaction and transition are often confused and inappropriately used with the term epithelial mesenchymal transdifferentiation” (Raghu Kalluri & Neilson, 2003). “Transformation” is typically associated with the oncogenic conversion of the epithelium and is not usually plastic over time, whereas “transdifferentiation” is associated with differentiated cells changing into other differentiated cells. Also, epithelial mesenchymal “interaction” involves paracrine cross-talk between epithelial cells and stromal fibroblasts and is totally separate from the concept of epithelial mesenchymal “transition” (Raghu Kalluri & Neilson, 2003). Epithelial mesenchymal transition is considered as a type of transdifferentiation, but the term epithelial mesenchymal transition is preferred over transdifferentiation as the latter is associated with differentiated cells changing into other differentiated cells. This issue is still a matter of debate with no clear explanation available (Raghu Kalluri &
Neilson, 2003). For my work in COPD, I have maintained use of the term epithelial mesenchymal “transition” or EMT.

Recently, Raghu Kalluri et al (R. Kalluri, 2009; R. Kalluri & Weinberg, 2009; Zeisberg & Neilson, 2009) also from Harvard, suggested that EMT could be divided into three different types (Figure 2.18) based on the biological conditions in which they occur and associated consequences:

- **Type 1 EMT** occurs during implantation, embryogenesis and organ development, this type of EMT is not associated with organ fibrosis and metastasis, and however type 1 EMT has the potential for MET to form secondary epithelia.

Figure 2.18: Different types of EMT. (A) Type 1 EMT (B) Type 2 EMT (C) and Type 3 EMT, (R. Kalluri & Weinberg, 2009).
• Type 2 EMT is implicated in wound healing, organ fibrosis and tissue regeneration. It occurs in response to injury and contributes to the repair process by generating fibroblasts and other cells to repair the tissue. This type of EMT is usually associated with inflammation and may stop once the inflammation ceases, but if the inflammation is long lasting due to repeated injury, type 2 EMT could well lead to organ damage and may have very severe consequences. Type 2 EMT could potentially be picked up using very reliable mesenchymal markers available like fibroblast-specific protein (also called S100A4), collagen I, along with vimentin, desmin, E-cadherins and cytokeratins.

• The last type of EMT, type 3 is associated with cancer progression and metastasis. Cells produced by this type of EMT may invade through and into the circulation and produce metastases. A significant body of literature explains the signalling pathways involved with type 1 and type 2 EMT, but we don’t know much about type 3 signalling pathways which are mainly involved with cancer progression.

2.7.1 Mechanism of EMT

EMT can be considered as a marker of profound epithelial plasticity engaged by disaggregating and reshaping epithelium for movement (B. C. Willis & Borok, 2007). The epithelium is basically a sheet of cells, in the airways one cell deep, with epithelial cells joined to each other in a uniform way. Tight junctions and adheren junctions between epithelial cells hold the epithelial cells strongly together as a single layer and restrict the movement of individual cells away from the epithelium. The epithelial cells are polarized, from which we understand that in epithelium apical and basal surfaces are likely to be visually different and may carry different functions. Mesenchymal cells compared to epithelial cells are lacking uniform composition and strong adhesion properties; they are of a more elongated and irregular shape allowing the cells to gain migratory potential for movement (J. M. Lee et al., 2006).

During transition the epithelium loses cell polarity, tight junctions like zonula occludins, adheren junctions like E-cadherins and desmosomes, cytokeratin filaments
Cytokeratins are keratin containing intermediate filaments found in epithelial cells and are vital for regular tissue function and structure) and reorganize their actin fibres, emission of filopodia and lamellipodia and then finally go through a transition into an elongated and irregular morphology associated with gain of mesenchymal markers (e.g.: S100A4 and vimentin) simultaneously (Savagner, 2001; B. C. Willis & Borok, 2007).

The process of EMT can simply be induced by a combination of different cytokines and growth factors associated with dismantling of the basement membrane by proteolytic activity (Raghu Kalluri & Neilson, 2003). The cells undergoing transition have the capacity to secrete proteolytic enzymes from the metalloproteinase family, which can start the proteolytic digestion of the underlying basement membrane assisting migration of the cells (J. Yang & Liu, 2001). A diverse variety of growth factors are involved in the process, mainly TGF-β, EGF, insulin growth factor-II (IGF-II), hepatocyte growth factor (HGF) and also fibroblast growth factor-II (FGF-II) (Figure 2.19); they have the capacity to induce EMT by binding to different receptors on epithelium cells (Raghu Kalluri & Neilson, 2003).
Figure 2.19: Schematic representation of different signalling pathways involved in EMT (Raghu Kalluri & Neilson, 2003).
TGF-β has been considered a prototypical driver of EMT; Fan et al (Fan et al., 1999) reported that TGF-β can induce the differentiation of tubular epithelial cells into α-SMA positive myofibroblasts. It was also reported that TGF-β and FGF-II expression is central to MMP-2 and MMP-9 expression; these proteolytic enzymes have the capacity to digest the basement membrane/Rbm to facilitate the migration of cells (Raghu Kalluri & Neilson, 2003; Ward et al., 2005). TGF-β can act mainly in two ways, through Smad-3 dependent pathway or other Smad independent pathway (B. C. Willis & Borok, 2007). Depending on the type of tissue all three isoforms of TGF-β could be involved during transition (Raghu Kalluri & Neilson, 2003). Other studies have also shown that TGF-β expression is up-regulated in lungs of patients with idiopathic pulmonary fibrosis (IPF) and can enhance EMT (B. C. Willis & Borok, 2007). On the other hand, a study reported by Zeisberg et al revealed that bone morphogenetic protein (BMP)-7 which is a member of the TGF-β superfamily has the ability to reverse TGF-β-induced EMT by restoring E-cadherin levels to normal through the Smad pathway, mainly through Smad 5, but Smad 1 and Smad 8 can also transduce BMP-7 actions (Zeisberg et al., 2003).

Citterio et al reported that EGFR expression increases in the epithelium during the process of EMT and interaction between TGF-β and EGFR might play a key role in EMT by assisting in migration and matrix turnover (Citterio & Gaillard, 1994). Furthermore, Lo et al reported that EGF/EGFR signalling pathways can lead to EMT via STAT3-mediated (signal transducer and activator of transcription 3) up-regulation of TWIST gene expression (Lo et al., 2007). IGF-II signalling pathways relocate the β-catenins (which belong to the family of cell adhesion molecules and is a subunit of the cadherin complex) from cells surface leading to the nuclear internalization resulting in intracellular degradation of the E-cadherin complex which further facilitates migration of cells as the epithelium becomes loose (Morali et al., 2001).

Janda et al (Janda et al., 2002) made an effort to differentiate true EMT from an epithelial phenocopy termed “reversible scatter”. Reversible scatter is similar to EMT but not true EMT, but more like a brief episode of EMT. For example Hackett et al, (T. L. Hackett et al., 2009), recently reported that alveolar epithelial cells from asthmatics can undergo a transition to a mesenchymal phenotype when stimulated
with TGF-β1, but when the actual airway biopsy sections from asthmatics were stained for markers of EMT they were negative. Thus, this is not true EMT, but probably reversible scatter as a result of TGF-β1 stimulation for a short period of time, and occurs only temporarily following cytokine stimulation with cells assuming spindle like cell morphology, loss of epithelial markers and gain of mesenchymal markers for a brief period of time. However, as the stimulus is removed the epithelium returns to its original shape and the whole process ceases. They also reported that true EMT is mainly induced by TGF-β and Ras signalling pathways, whereas EGF, HGF and FGF favour scattering only, and are not able to produce classical EMT.

2.8 Signalling pathways in EMT

The epithelial signalling which drives the process of EMT is very complicated (Figure 2.19). Further, these signalling pathways are linked to each other with broad cellular consequences (Raghu Kalluri & Neilson, 2003). Brigitte Boyer et al suggested that when EMT is not activated by an oncogenic stimulus, then it is primarily induced by specific growth factors or extracellular matrix components binding to their associated cellular receptors leading to a strategic intrinsic kinase activation which sets the process off (Boyer, Valles, & Edme, 2000). The major signalling pathways involved in EMT are: TGF-β signalling pathways, the Wnt/β-catenin signalling pathway, the Ras pathway and the Notch signalling pathway. All these pathways are extensively inter-related (Boyer et al., 2000; Guarino et al., 2009; Raghu Kalluri & Neilson, 2003).

2.8.1 TGF-β signalling pathway

I have already introduced transforming growth factor beta (TGF-β). It is a multifunctional protein playing a range of different roles through regulating tissue morphogenesis and differentiation by modulating cell proliferation, differentiation, cell adhesion, apoptosis, migration of cells, vasculogenesis and angiogenesis and is also involved in extracellular matrix turnover (Dennler, Goumans, & ten Dijke, 2002). TGF-β is a member of a large superfamily of structurally related proteins consisting of more than 30 members in mammals, including three types of TGF-β,
four activins and over more than twenty bone morphogenetic proteins (BMPs) (Dennler et al., 2002). TGF-β has been implicated as a master switch in the induction of fibrosis in various organs including the lungs (B. C. Willis & Borok, 2007). Signal transduction associated with TGF-β involves two different pathways in EMT, Smad-dependent signaling pathway and smad-independent signaling pathway (Dennler et al., 2002; B. C. Willis & Borok, 2007).

Smad-dependent pathway: Most members of the TGF-β superfamily signal through cell-surface receptors of the serine/threonine kinase category. A family of proteins known as Smads are involved in transducing the ligand-induced signals from the cell surface to the nucleus of the cell (Flanders, 2004). Thus, Smads are mainly signalling proteins that have the capacity to modulate the activity of TGF-β ligands. The name Smads was derived from a contraction of the names of TGF-β like ligand signalling intermediates first recognized in Drosophila (Mad) and Caenorhabditis elegans (Sma) (Flanders, 2004). Eight different types of Smads have been reported, further grouped into three subfamilies. The first five include receptor-regulated Smads (Smad 1, Smad 2, Smad 3, Smad 5 and Smad 8/9), termed as R-Smads; one common mediator Smad (Smad 4), named Co-Smads; and two inhibitory Smads (Smad 6 and Smad7), termed I-Smads (Flanders, 2004; Wikipedia).

The Smad-dependent pathways in the process of EMT in response to TGF-β are mainly mediated by Smad 3. In this pathway, TGF-β-generated signals are mediated by type I and type II serine-threonine kinase receptors and this process is initiated by binding of extracellular TGF-β ligands to the type II receptor on the cell surface. This binding further leads to the recruitment of type I receptors to form a heteromeric complex; at this stage the type I receptor is phosphorylated by the type II receptor, resulting in its activation. The type I receptor later phosphorylates Smad 2 and Smad 3 at serine residues inducing association with Smad 4 (Co-Smad) and forming a heteromeric complex leading to the translocation of this complex to the nucleus. In the nucleus this complex can act as a transcription factor and can also interact with other transcription factors leading to the activation or regulation of TGF-β responsive genes, which may include, Snail, connective tissue growth factor (CTGF), α-SMA (α-smooth muscle actin), collagen 1A2 and also plasminogen activator inhibitor-1.
Smad-independent pathway: The Smad-independent pathway is not as well studied compared with the Smad dependent pathway in EMT although a significant body of in-vitro evidence is available for the role of a Smad independent pathway in EMT. It is hard to differentiate these pathways clearly as there is potential for significant cross-talk between Smad proteins and non-Smad proteins during EMT. The Smad-independent pathway mainly includes RhoA, Ras MAPK (mitogen-activated protein (MAP) kinases), PI3 kinase (Phosphatidylinositol 3-kinases), Notch and Wnt/β-catenin signalling pathways. Although the Smad-dependent pathway is considered as the main pathway, depending on the type of tissue it may not be sufficient to induce full blown EMT without activation of other supplementary pathways (Zavadil & Bottinger, 2005). Major Smad-independent pathways are discussed separately below.

2.8.2 The Wnt/β-catenin signalling pathway

The Wnt signalling pathway is activated when Wnt proteins bind to specific cell surface receptors. “The name Wnt was coined as a combination of Wg (wingless gene, originally identified in Drosophila melanogaster) and Int (Int gene was originally identified as vertebrate genes near several integration sites of mouse mammary tumor virus)” (Wikipedia). The Wnt signalling pathway describes a network of Wnt proteins, which form a family of highly conserved secreted signaling molecules that regulate cell-to-cell interactions during embryogenesis and cancer. Wnt-mediated signalling (Figure 2.19) can inhibit phosphorylation of β-catenin by
glycogen synthase kinase 3β (GSK-3β), hence stabilizing cytoplasmic levels of β-catenin, this leads to the translocation of β-catenin to the E-cadherin complex or into the nucleus where it combines with the lymphoid enhancer factor-1 (LEF-1) and other transcription factors leading to the activation of a diverse variety of EMT-associated genes, including those encoding for fibroblast specific protein-1 (FSP-1 or S100A4), vimentin, fibronectin, MMPs and also members from the Snail family. On the other hand, in the absence of Wnt-associated signals most of the β-catenin is bound to the E-cadherin complex, while the unbound β-catenin undergoes phosphorylation by GSK-3β which further allows it to form a complex with APC suppressor protein and Axin (Adenomatosis polyposis coli or APC is a protein encoded by the APC gene in humans; it is suggested that APC protein plays an important role in cell migration by targeting β-catenin for proteasomal degradation) (Nathke, 1999).

p53 activates an APC-dependent pathway and both of these lead to the direct loss of β-catenin through proteasomal degradation. In this whole process free levels of β-catenin are regulated by E-cadherin complexes or by APC/β-catenin/Axin complexes, the latter shuttle β-catenin between its degradation through proteasomal pathways and adheren junctions in EMT. β-catenin plays a dual role in the EMT process: it has the potential for cell-cell adhesion when bound to the cadherin complex, and on the other hand can also act as a transcription factor when present in the nucleus. The cell adhesion due to β-catenin depends on binding of β-catenin to the α-catenin and further binding of the α-catenin to the cadherin. Furthermore, in addition to Wnt glycoproteins, IGF-II, Ras and integrin-linked kinase (ILK) all lead to β-catenin accumulation in the cytoplasm and also increased Snail, possibly by GSK-3β or by inhibition of other kinases to facilitate EMT (Guarino et al., 2009; Raghu Kalluri & Neilson, 2003; Nathke, 1999; Postma & Timens, 2006; B. C. Willis & Borok, 2007; Zavadil & Bottinger, 2005).

2.8.3 The Ras signalling pathway
Ras is a superfamily of small GTPases or G-proteins. All the signalling pathways involved with EMT can activate small GTPases through ligand-inducible receptor
kinase activation. These small GTPases are involved with changes in cell shape and gain of migratory potential. Transition of epithelium to mesenchymal phenotype depends considerably on different molecular switches under the control of small GTPases from the Ras superfamily. Small GTPases are activated by guanine nucleotide exchange factors and deactivated by GTPase activating proteins. There are more than a hundred proteins in the Ras superfamily but the 3 best studied small GTPases are, Rho, Rac and Cdc42, and they can activate or inhibit each other during the process of EMT.

Rho signal transduction pathways are linked to the actin cytoskeleton and are also helpful in rearranging the actin stress fibres and can stimulate actin-myosin contraction in the cell body. Furthermore, they also participate in regulation of cell polarity, gene transcription, cell cycle, vesicular transport pathways and are also involved with various enzymatic activities. Rac on the other hand has the capability to induce actin-rich surface extensions called lamellopodia. Cdc42 supports the development of actin-rich finger-like protrusions called filopodia and modulates cellular asymmetry. The small GTPase family is able to control or regulate different cellular properties including contraction, migration, proliferation, and phagocytosis. These cellular activities of small GTPases can potentially activate MAP kinases, altering gene transcription and are vital to change in cell phenotype during the process of EMT. (Bar-Sagi & Hall, 2000; Etienne-Manneville & Hall, 2002; Guarino et al., 2009; Raghu Kalluri & Neilson, 2003; Savagner, 2001).

2.8.4 The Notch signalling pathway

The Notch pathway plays an important role in EMT and functional interactions between Notch signalling and EGF, FGF, TGF-β and Smads have been suggested (Dasari, Gallup, Lemjabbar, Maltseva, & McNamara, 2006; Zavadil, Cermak, Soto-Nieves, & Bottinger, 2004). It is a highly preserved cell signalling system present in most multicellular organisms and is a family of four different types of receptors, Notch1-4. Different ligand proteins can bind to the extracellular domain of the Notch receptor and can induce proteolytic cleavage and release of the intracellular domain, which then enters the nucleus of the cell to alter the gene expression. The first mutant of Notch was identified in Drosophila melanogaster (Artavanis-Tsakonas, Rand, & Lake, 1999). Zavadil et al demonstrated that when keratinocytes were induced to
EMT by TGF-β, Notch signalling pathway activation occurred downstream of TGF-β, controlled by early up-regulation of the Notch ligand, Jagged1 and also the Notch target genes TLE3 (transducin-like enhancer) and HES1 (hairy enhancer of split) (Zavadil et al., 2001).

In 2004, the same group described the expression of the HES1-related (Dasari et al., 2006) transcriptional repressor family which included HEY1, HEY2, HES1 and HES5 and the Notch ligand Jagged1 being induced by TGF-β at the start of EMT in a panel of epithelial cells from kidney tubules, mammary glands and epidermis (Zavadil et al., 2004). They also reported that TGF-β-induced EMT could be inhibited by suppressing the expression of the Notch ligand Jagged1 and HEY1 and also by chemical inactivation of Notch (Zavadil et al., 2004).

**2.9 Transcriptional control of EMT**

Signalling pathways implicated in the process of EMT end up as a final common path in the nucleus of the cell where they regulate the transcription of EMT-related genes. In EMT a wide array of different proteins are gained, maintained or attenuated, which requires the coordinated expression of numerous set of genes. Therefore, it becomes quite critical to understand and identify the different molecular targets of these signalling pathways leading to EMT (Boyer et al., 2000). A range of different transcriptional factors are involved in regulating EMT; among these several transcription factors can act as master switches in regulating the whole process of EMT (Zavadil & Bottinger, 2005), with a degree of synergy and/or redundancy between them.

**2.9.1 The Snail family**

The *Snail family* of transcription factors has been implicated in EMT; it includes Snail or Snail1 and Slug or Snail2 zinc finger proteins, and recently Snail3 has also been found. The Snail family induces EMT mainly by repressing transcription by recognizing E-box elements (they are “enhancer” elements and play a regulatory role in control of gene transcription) in their associated target promoters. It is reported that Snail can repress transcription of the E-cadherin gene through several E-boxes located in the E-cadherin promoter in cultured cells and also during embryonic
development and tumour progression (Barrallo-Gimeno & Nieto, 2005; Xu, Lamouille, & Derynck, 2009; Zavadil & Bottinger, 2005). Snails themselves are regulated by a variety of signalling pathways and at numerous levels. For example, it is reported that selective phosphorylation by GSK3 leads to the inhibition of Snails by ubiquitination or degradation. On the other hand Wnt signalling pathway can inhibit GSK3 resulting in Snail activation and loss of E-cadherins.

Various growth factors or signalling molecules involved in EMT can also regulate Snails. For example, TGF-β has the potential to activate both Snail and Slug directly through a Smad3-dependent pathway (Zavadil & Bottinger, 2005). TGF-β1 can induce Snail1 expression in mesothelial cells, epithelial cells and hepatocytes and also during palate development (Xu et al., 2009). TGF-β2 and BMP-4, on the other hand, can induce expression of Snail2 or Slug (Zavadil & Bottinger, 2005). EGF has also been reported in several studies as an inducer of Snail and EMT. The EGF pathway promotes caveolin-dependent endocytosis which leads to loss in E-cadherin function and successive Snail and EMT activation (Lu, Ghosh, Wang, & Hunter, 2003). In addition, EGF can also activate STAT3 (“signal transducer and activator of transcription 3”, it is a transcription factor encoded by STAT3 gene in humans), which has the potential to enhance Snail function (Barrallo-Gimeno & Nieto, 2005).

Another Zinc-finger protein from the Snail family widely reported in EMT is Slug or Snail2, which can play an important role in regulating EMT (Savagner, 2001). Slug can be quickly induced in epithelial cells undergoing EMT by FGF and HGF and effectively regulates disassembly of desmosomes (Savagner, 2001; Zavadil & Bottinger, 2005). During EMT it is suggested that Slug appears to be activated through Ras/MAPK pathway and does not seem to be participating in E-cadherin suppression but rather contributes more to the maintenance of the mesenchymal phenotype (Boyer et al., 2000; Savagner, 2001). But interestingly it is also suggested that dissociation of E-cadherin junctions can lead to autoregulatory induction of Slug with successive suppression of E-cadherin transcription, although its exact role is still a matter of debate (Zavadil & Bottinger, 2005).
2.9.2 Role of Transcriptional repressors (DeltaEF1, SIP1 and Fos)
Delta-crystalline enhancer factor-binding factor 1 (DeltaEF1) (also known as ZFH1 and ZEB1), and Smad-interacting protein-1 (SIP1) (also known as ZFH2) are two closely related zinc finger transcriptional repressors. DeltaEF1 is a very close homologue of SIP1 and both have the capability to regulate E-cadherin expression during carcinogenesis associated EMT (Type 3 EMT) (Eger et al., 2005; Zavadil & Bottinger, 2005). SIP1 expression can be induced in response to TGF-β and has the potential to regulate the activity of Smad proteins. Comijn et al have shown that ectopic expression of SIP1 in MDCK cells (Madin-Darby canine kidney cell line) leads to the destruction of adheren junctions, especially E-cadherin, favouring invasion in malignant epithelial tumours (Comijn et al., 2001; Zavadil & Bottinger, 2005).

Eger and colleagues (Eger et al., 2005) strongly suggested that DeltaEF1 has the potential to directly inhibit E-cadherin transcription, being associated with the E-cadherin promoter at the chromatin level. They also suggested that although E-cadherin suppression is considered as a hallmark of EMT, it is still not itself sufficient to induce the expression of N-cadherin and vimentin, EMT-related mesenchymal markers. Thus DeltaEF1 may play an important role in regulating epithelial plasticity by controlling other proteins, but cannot induce EMT fully on its own.

Reichmann and colleagues showed that Fos transcription factor expression in mammary epithelial cells can lead to the process of EMT, by modulating epithelial phenotype and increasing invasiveness (Reichmann et al., 1992). Zavadil and colleagues reported that in human keratinocytes, TGF-β stimulates the ERK-dependent induction of c-Fos, which occurs very rapidly but for only a short period of time at the inception of sustained EMT (Zavadil et al., 2001), when it may have a very strategic role.

2.9.3 Id, Twist and E2A
Id proteins (Id1, Id2, Id3, and Id4) are recognized as inhibitors of differentiation, playing a vital role during carcinogenesis (Kowanetz, Valcourt, Bergstrom, Heldin, & Moustakas, 2004). Kowanetz et al did a microarray analysis of epithelial cells and
reported that TGF-β1 suppresses Id2 and Id3 for a long period of time, whereas BMP7 induced these factors. In addition, Id2 also inhibited the destruction of adheren and tight junctions in TGF-β-induced EMT (Zavadil & Bottinger, 2005). This suggests that TGF-β may favour EMT by suppressing these inhibitors of differentiation and BMP-7, but still the exact physiological role of these pathways is poorly understood (Kowanetz et al., 2004; Zavadil & Bottinger, 2005). Id proteins associate constitutively with the basic helix-loop-helix (bHLH) transcriptional regulator E2A, and maintain epithelial phenotype by inhibiting the role of E2A in E-cadherin suppression. Conversely, TGF- β-mediated inhibition of Id proteins can lead to activation of E2A (a transcription factor), maintaining the mesenchymal phenotype during EMT (Zavadil & Bottinger, 2005).

Another basic helix-loop-helix transcription factor that appears to be centrally associated with EMT is Twist, which is an important trigger factor involved in embryogenesis and carcinogenesis (Pozharskaya et al., 2009; Zavadil & Bottinger, 2005). Twist can be induced during EMT by the expression of EGFR, TGF-β and the transcription factor nuclear factor κB (NF-κB). Pozharskaya et al reported in a recent study that Twist contributes to EMT in the model of virus-induced lung fibrosis (Pozharskaya et al., 2009). Yang and colleagues reported that Twist suppression in mammary carcinoma cells specifically inhibits their ability to metastasize from the mammary gland to the lung; they also reported that ectopic expression of Twist in MDCK cells leads to loss of E-cadherin with subsequent expression of mesenchymal markers (e.g. vimentin, α-SMA and N-cadherins) and an increase in migratory potential, thus playing a very important role in tumour metastasis (type-3 EMT) (J. Yang et al., 2004; Zavadil & Bottinger, 2005). In another study, Kida et al reported that Twist was associated with renal tubular EMT leading to proliferation of myofibroblasts and subsequent renal fibrosis in an obstructed kidney model (Kida, Asahina, Teraoka, Gitelman, & Sato, 2007), but there are very few studies reporting role in EMT associated with chronic inflammation (Mironchik et al., 2005).

### 2.10 The EMT proteome

The EMT proteome is defined as the essential changes in proteins gained, maintained or attenuated during the EMT process (Figure 2.20). This involves a wide range of
proteins and most of the studies reported in the literature have been able to focus on a few specific markers and are far from complete. The majority of studies suggest that transcriptional factors, especially from the Snail family are the main players in the events leading to the loss of epithelial markers (e.g. E-cadherin, muc-1, cytokeratin and desmosomes) and gain of mesenchymal markers (e.g. FSP-1, or S100A4, vimentin, fibronectin and N-cadherin) with increased mobility (Raghu Kalluri & Neilson, 2003), but actually very little attention has been given to clearly identifying EMT in vivo and in vitro, and the best/most specific markers to use are still a matter of debate. Michael Zeisberg and colleagues have made an attempt to define the in vivo and in vitro criteria to identify EMT in this way (Figure 2.21) (Zeisberg & Neilson, 2009).

EMT has been widely studied in different cell culture systems and most of the attention has been given to the process of identifying cells undergoing transition to a fibroblast like phenotype. One of the benefits associated with an in vitro system is the ability to detect the phenotype and movement of the cells in the culture medium, but on the other hand it is much more difficult in an in vivo system because many of the tissue sites are disrupted by inflammation and cicatrisation (Zeisberg & Neilson, 2009). Unfortunately, because something can be demonstrated in a manipulated in vitro system and environment, does not necessarily mean that those change(s) are operative in vivo.
The EMT proteome

<table>
<thead>
<tr>
<th>Proteins gained or maintained:</th>
<th>Proteins attenuated:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snail</td>
<td>E-cadherin</td>
</tr>
<tr>
<td>Slug</td>
<td>β-catenin</td>
</tr>
<tr>
<td>Scratch</td>
<td>Desmoplakin</td>
</tr>
<tr>
<td>SIP1</td>
<td>Muc-1</td>
</tr>
<tr>
<td>E47</td>
<td>ZO-1</td>
</tr>
<tr>
<td>Ets</td>
<td>Syndecan-1</td>
</tr>
<tr>
<td>FTS binding protein</td>
<td>Cytokeratin-18</td>
</tr>
<tr>
<td>RhoB</td>
<td></td>
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<tr>
<td>FSP1</td>
<td></td>
</tr>
<tr>
<td>TGF-β</td>
<td></td>
</tr>
<tr>
<td>FGF-1,-2,-8</td>
<td></td>
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<tr>
<td>MMP-2</td>
<td></td>
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<tr>
<td>MMP-9</td>
<td></td>
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<tr>
<td>Vimentin</td>
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<td>αSMA</td>
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<td>Fibronectin</td>
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<td>Collagen type I</td>
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<td>Collagen type III</td>
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<tr>
<td>Thrombospondin</td>
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<td>PAI-1</td>
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**Figure 2.20:** The EMT proteome, showing proteins gained or maintained and lost during EMT (Raghu Kalluri & Neilson, 2003).
**In vivo criteria for EMT**

**Major criteria**
- Use of an epithelial cell reporter construct that appears locally in newly formed fibroblasts
- New expression of FSP1 and possibly DDR2 associated with disruption of basement membrane
- Increased expression of HSP47, collagen 1 (α1), collagen 2 (α2), N-cadherin, or vimentin
- Nuclear relocalization of CBF-A or β-catenin/LEF or new expression by in situ hybridization of one of the following transcription factors: Snail, Slug, or Twist
- Loss or partial loss of epithelial markers such as cytokeratin, E-cadherin, or ZO-1
- Spindle-shape morphology with redistribution of stress fibers and loss of polarity

**Minor criteria**
- Localized adjacency of transitioning cell near its epithelial compartment
- Exclusion of possible bone marrow-derived progenitor cells

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**In vitro criteria for EMT**

**Major criteria**
- New expression of FSP1 and possibly DDR2
- Increased expression of HSP47, collagen 1 (α1), collagen 2 (α2), or vimentin
- Cadherin switch
- Nuclear relocalization of CBF-A or β-catenin/LEF or new expression of one of the following transcription factors: Snail, Slug, or Twist
- Absence of epithelial markers; loss of cytokeratin or ZO-1
- Spindle-shape morphology with redistribution of stress fibers and loss of polarity
- Resistance to apoptotic stimuli
- Increased migratory capacity
- Phenotype stable upon removal of inducing stimulus

**Minor criteria**
- Abundant intermediate filaments and microfilaments
- Loss of chromatin condensation associated with gain of multiple nucleoli
- Gain of rough ER, abundant lysosomal granules, and loss of intercellular junctions on electron microscopy

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**Figure 2.21:** *in vivo* and *in vitro* criteria from EMT (Zeisberg & Neilson, 2009).

Thus, an ideal way to identify EMT associated with chronic inflammation is to use reporter genes in cultured epithelial cells that can be tracked to fibroblasts on transition. However, marking studies are very difficult to perform with human tissue, and in this setting the ideal approach is to identify epithelial cells positive for S100A4, a fibroblast specific protein-1 (also known as FSP-1) associated with partial or complete loss of cytokeratins or E-cadherins, lying adjacent to the typically disrupted and fragmented basement membrane or Rbm (Zeisberg & Neilson, 2009), evidence of the effects of the proteolytic activity of matrix metalloproteinases...
(MMPs) (Ward et al., 2005) which are an inherit part of this process. This basement membrane or Rbm change has been suggested as the best “hallmark” of EMT in vivo (R. Kalluri & Weinberg, 2009). Alpha-smooth muscle actin has also been observed in mature fibroblasts, but is non-specific and is not present in newly transitioning epithelial cells, whereas S100A4 is mainly confined to transitioning epithelial cells and is a more reliable and specific marker than alpha-SMA (Zeisberg & Neilson, 2009). Elizabeth Hay et al (Hay, 2005) argued that mesenchymal cells and fibroblasts shouldn’t be defined on the basis of alpha-SMA stress fibres, as myofibroblast phenotypes are not thought to migrate actively. Another marker of EMT widely reported is an intermediate filament protein called vimentin, which has the potential to induce changes in cell shape, motility, and adhesion during EMT (Mendez, Kojima, & Goldman). Vimentin has also been suggested to play a major role in breast cancer progression, an observation made both in vitro and in vivo (Kokkinos et al., 2007).

2.10.1 S100A4 (or FSP-1)
S100A4 belongs to a large family of Ca$^{2+}$-binding proteins collectively designated as S100. There are at least 21 described members in the S100 family which includes two newly discovered members, namely S100A14 and S100Z. Usually S100 proteins are low molecular weight proteins ranging between 9 to 13 kDa, sharing a common feature of two Ca$^{2+}$-binding EF-hand motives (helix-loop-helix), and most of the members exist as dimmers, including S100A4. S100 proteins do not have any enzymatic activity (Rosario, 2003; Schneider et al., 2008).

The most widely reported protein in EMT from the S100 family is S100A4, and it is considered as a potential mediator of EMT and organ fibrosis in diseases. S100A4 has both intracellular and extracellular effects leading to a wide range of biological actions including increasing contractility, motility, differentiation and cellular growth (Rosario, 2003; Schneider et al., 2008).

Raghu Kalluri et al suggested that S100A4 in the cytoplasm dimerizes and binds to the c-terminal of p53 (it is a tumour suppressor protein encoded by TP53 gene in humans), in the presence of calcium and this process may guard p53 from the APC-mediated destruction pathway and may raise levels of free β-catenin (Figure 2.19),
thus facilitating and maintaining the process of EMT. Therefore, the exact role of S100A4 is not clear but it also has the potential for angiogenesis (Raghu Kalluri & Neilson, 2003), and seems well fitted to be a core mediator of the changes associated with chronic inflammation as well as metastatic tumours. Indeed, it may be a link between the two processes in diseases such as COPD or IPF.

S100A4 has been suggested as a prototypical and very reliable cytoskeletal marker for detecting EMT in various pulmonary disorders and cancer (Lawson et al., 2005; Ward et al., 2005; Zeisberg & Neilson, 2009). Preliminary findings in idiopathic pulmonary fibrosis showed strong staining for S100A4 in fibroblasts (Schneider et al., 2008), Chris Ward et al showed that S100A4 localises to bronchial epithelial cells in human transplanted lungs indicative of EMT in the airways which may further lead to remodelling and fibrosis as part of chronic rejection (Ward et al., 2005). This was further confirmed by a study by Hodge and colleagues (Hodge et al., 2009).

There is emerging evidence that S100A4 can also activate expression and release of MMPs which in turn may lead to tissue remodelling and assisting in the migration of cells by degrading the basement membrane and Rbm and components of the extracellular matrix (Raghu Kalluri & Neilson, 2003; Schneider et al., 2008).

In a very interesting study (in prostrate cancer), Saleem and colleagues observed that S100A4 gene repression significantly decreased the expression of MMP-9, while the over expression of the S100A4 gene considerably increased MMP-9 expression. These expression changes were mirrored in changes in proteolytic activity of MMP-9. They also suggested that transcriptional activation of MMP-9 is regulated by the S100A4 gene, as knock down of the S100A4 gene decreased MMP-9 activity, while over expression lead to an increase in MMP-9 promoter activity (Saleem et al., 2006). Increased expressions of S100A4 and MMP-9 are also observed in human non-small cell lung cancer and have significant correlations with clinical and biological behaviour of these cancer cells (Chen, Wang, Zhang, Chen, & Sun, 2008).

Put together all these data suggests that S100A4 could be involved both in inflammation-related fibrosis and in cancer progression. Tissue fibrosis and cancer share common signalling pathways, molecular and biological programs, and
especially EMT. This raises a possibility that S100A4 could be a common mediator of both fibrosis and cancer by directing EMT (Hodge et al., 2009; Okada, Danoff, Kalluri, & Neilson, 1997; Saleem et al., 2006; Schneider et al., 2008; Ward et al., 2005; Zeisberg & Neilson, 2009). “Simply stated, S100A4 is central to EMT in diseases” (Schneider et al., 2008).

2.10.2 Matrix metalloproteinases-9 (MMP-9)

MMP-9 or matrix metalloproteinases-9 (MMP-9) is a proteolytic enzyme from a family of more than twenty zinc dependent proteases, they all share some structural similarities but differ from each other in their expression profile and substrate specificity (Chakrabarti & Patel, 2005; Elkington & Friedland, 2006). Since MMPs have a broad range of substrate specificity they have the potential to cause significant damage to the host tissue, so their expression is tightly regulated at transcriptional level. MMPs are secreted as proenzymes which further require proteolytic cleavage to become active, but in the tissue they are tightly regulated by tissue inhibitors of metalloproteinases (TIMPs). TIMPs block the enzymatic activity of the MMPs and equilibrium between these two determines matrix turnover (Chakrabarti & Patel, 2005; Elkington & Friedland, 2006). Another physiological inhibitor reported for MMPs is α-2 macroglobulin (Lagente et al., 2005).

MMP-9 is also known as gelatinase B, as gelatin is one of its major enzymatic substrate; it also has the potential to degrade other components of the ECM such as type IV collagen, aggrecan, elastin and vitronectin. Human MMP-9 is highly glycosylated with a molecular mass of 92 kDa as a pro-enzyme and 83 kDa when active (Atkinson & Senior, 2003; Chakrabarti & Patel, 2005). MMP-9 is secreted by a variety of cells in the lungs including bronchial epithelial cells, macrophages, eosinophils, mast cells, natural-killer cells, dendritic cells, neutrophils, fibroblasts, smooth muscle cells, alveolar type II cells and endothelial cells (Atkinson & Senior, 2003).

Increased MMP-9 expression has been reported in various respiratory disorders including asthma, COPD and IPF (Atkinson & Senior, 2003), although there are relatively few studies reporting MMP-9 levels in airways of COPD patients.
However, substantial evidence exists in smoking related emphysema leading to the protease/anti-protease imbalance theory of its aetiology (Atkinson & Senior, 2003; Elkington & Friedland, 2006). Juanita et al reported an increase in MMP-9 activity in induced sputum from mild-to-moderate COPD patients; in addition they also suggested an aetiological protease-antiprotease imbalance in COPD (Vernooy, Lindeman, Jacobs, Hanemaaijer, & Wouters, 2004). Mercer and colleagues reported increase in both MMP-9 and TIMP-1 levels in sputum of COPD patients during acute exacerbations (Mercer et al., 2005).

In EMT, MMP-9 expression is up-regulated in epithelial cells (Atkinson & Senior, 2003) and can act as a marker of epithelial and basement membrane damage (Figure 20) (Ward et al., 2005). Type IV collagen is the major component of the “true” basement membrane (BM) and MMP-9 has the potential to digest the BM collagen and hence can assist the migration of cells through the BM into the lamina propria (Atkinson & Senior, 2003; Raghu Kalluri & Neilson, 2003; Ward et al., 2005). MMP-9 is one of the major MMPs secreted during the process of EMT (Raghu Kalluri & Neilson, 2003). Chris Ward et al reported an increased expression of MMP-9 during EMT in clinically stable lung transplant recipients (Ward et al., 2005). Tan and colleagues reported that MMP-9 mediates EMT in vitro in murine renal tubular cells (Tan et al.). Another study suggested that MMP-9 can mediate EMT by disrupting the E-cadherin complex in renal tubular cells hence promoting migration (Zheng et al., 2009). MMP-9 also stimulates EMT during tumor development (Orlichenko & Radisky, 2008). MMP-9 expression has also been reported in IPF and may be involved in cell migration and activation of TGF-β, hence facilitating the process of EMT in that disease (Atkinson & Senior, 2003). MMP-9 expression has also been observed in human non-small cell lung cancer and was significantly associated with its biological behaviour (Chen et al., 2008). These studies suggest that MMP-9 can play an important role in the process of EMT.

2.10.3 Vimentin

Vimentin is a 57 kDa type III intermediate filament protein normally expressed in cells of mesenchymal origin, cells undergoing EMT (Raghu Kalluri & Neilson, 2003) and also in tumour invasion (Fuchs & Weber, 1994). Vimentin increases in abundance in epithelial cells undergoing EMT suggesting a particular role in
epithelial cell migration (J. M. Lee et al., 2006). Gilles et al reported that vimentin could be functionally involved with migration of mammary epithelial cells during EMT (Gilles et al., 1999). In breast cancer, observations made both in vitro and in vivo by Kokkinos et al suggested that during cancer-associated EMT, cell intermediate filament status changes from a keratin rich network to a vimentin rich network favouring migration and increased invasion (Kokkinos et al., 2007). In another study, co-expression of vimentin and keratin intermediate filaments was associated with increased motility in human melanoma cells (Chu, Seftor, Romer, & Hendrix, 1996). Increased vimentin expression was also reported during EMT in human kidney transplant patients (Vongwiwatana, Tasanarong, Rayner, Melk, & Halloran, 2005).

Data on vimentin in lungs is very sparse. However, Borthwick et al demonstrated an increase in vimentin with subsequent decreases in airway epithelial markers like E-cadherins during the process of EMT and airway remodelling in lung transplant recipients (Borthwick et al., 2009). In a recent study, Hackett et al found increased levels of vimentin in TGF-β-induced EMT in primary airway epithelial cell cultures from patients with asthma (T. L. Hackett et al., 2009). Increased levels of antivimentin antibody were also observed in sera of patients with IPF (Y. Yang et al., 2002) suggesting abnormal expression of this protein in this disease.

### 2.10.4 (Cyto) keratins

Cytokeratins are keratin-containing intermediate filaments found in epithelial cells and are vital for regular tissue function and structure. The term “cytokeratin” was used in 1970 when keratins were first identified inside cells and then later in 2006 was technically replaced by the term “keratins” but is still widely used (Franke, Schmid, Osborn, & Weber, 1979; Schweizer et al., 2006).

Keratins are the biggest and most complex family of intermediate filaments comprising three different types (type I, type II and type III). Types I and type II are the main members, whereas type III involves vimentin, desmin, peripherin and glial fibrillary acidic protein (GFAP). Type I keratins include acidic keratin with 11 epithelial proteins (K9-K20), and also four hair keratins (Ha1-Ha4). Type II keratins
on the other hand are more basic with 8 epithelial keratins (K1-K8) identified so far (Fuchs & Weber, 1994; Schweizer et al., 2006).

Data on (cyto) keratins are widely reported in the literature on EMT. They are one group of epithelial proteins which generally decrease in abundance during EMT, but with substantial increase in mesenchymal markers (vimentin) (Kokkinos et al., 2007; J. M. Lee et al., 2006). Cytokeratins are very tightly regulated during the process of EMT and these protein changes in the cell reflect specific transcriptional activity during EMT (Savagner, 2001).

Borthwick and colleagues reported decreased expression of cytokeratin-19 with subsequent increase in the mesenchymal markers (vimentin) in epithelial cells from lung transplant recipients during TGF-β1-induced EMT (Borthwick et al., 2009). Furthermore, in IPF a decrease in cytokeratin levels was also observed in alveolar epithelial cells during TGF-β1-induced EMT (Brigham C. Willis et al., 2005).

2.11 EMT in the lungs

The exact role of EMT in the respiratory tract’s response to injury and development of fibrosis is not clearly understood (B. C. Willis & Borok, 2007). Myofibroblasts have been implicated as key mediators of fibrosis in idiopathic pulmonary fibrosis (IPF) but the precise origin of these primary effector cells of fibrosis in the lungs is not yet established. However, there are several potential sources of fibrogenic cells within the respiratory tract: first, there may be expansion of a local fibroblast pool by in-situ proliferation in response to injury which may lead to pathological fibrosis; second, recruitment of circulating bone-marrow derived fibrocyte progenitors may occur; and third, transition of respiratory epithelial cells into a mesenchymal phenotype via EMT (Brigham C. Willis et al., 2006).

In pulmonary fibrosis it was recently reported that circulating bone-marrow derived mesenchymal progenitor cells (fibrocytes) can play an important role in the progression of pulmonary fibrosis (Strieter, Keeley, Hughes, Burdick, & Mehrad, 2009). Phillip et al demonstrated in a murine model of bleomycin-induced pulmonary fibrosis that CD45 (+) Col I (+) CXCR4 (+) fibrocytes contribute to the pathogenesis of pulmonary fibrosis (Phillips et al., 2004). Hashimoto et al
demonstrated using a chimeric mice model in which they transplanted of GFP+ (green fluorescent protein) bone marrow into a wild-type mice and then that bone marrow-derived GFP+ Col1+ cells were found in the lungs of mice exposed to bleomycin, suggesting that circulating fibrocytes may contribute to pathogenesis of pulmonary fibrosis (Hashimoto, Jin, Liu, Chensue, & Phan, 2004).

EMT has only recently been recognised in the human lung and airway, (T. L. Hackett et al., 2009; Hodge et al., 2009; Ward et al., 2005; Brigham C. Willis et al., 2006) but it is well described in lung embryogenesis, (J. M. Lee et al., 2006) metastatic malignant disease (Bjornland et al., 1999) and as part of the repair process in renal disease following tissue injury (Yanez-Mo et al., 2003). There are no reported studies in COPD associated with EMT, although Araya and colleagues suggested a key role for integrin-mediated TGF-β activation in amplifying squamous metaplasia and driving IL-1β-dependent profibrotic mesenchymal response in smokers with COPD (Araya et al., 2007).

2.12 Histone acetylation and deacetylation in COPD

Chronic inflammatory diseases like COPD, asthma, cystic fibrosis, interstitial lung disease, inflammatory bowel disease and rheumatoid arthritis are associated with a specific pattern of inflammation, which requires a coordinate expression of a wide range of different pro-inflammatory genes coding for various inflammatory mediators leading to infiltration and activation of a panoply of inflammatory cells (Barnes, 2006a, 2006b; Barnes et al., 2005). In COPD, the specific pattern of inflammation is mainly characterized by increased numbers of luminal, sputum and BAL (bronchial alveolar lavage) neutrophils and airway wall macrophages and T-lymphocytes, predominantly cytotoxic (CD8+) cells (Barnes et al., 2005). The increased expression of these inflammatory genes is at least partly regulated by acetylation of core histones around which DNA is wound, and on the other hand these activated genes can be switched off by deacetylation of these histones (Barnes et al., 2005). Transcription of various proinflammmtory genes in chronic inflammatory diseases including COPD, is regulated by transcription factors, such as nuclear factor-Kappa B (NF-κB), activator protein-1 (AP-1) and glucocorticoid receptors as well
(which act as transcription factors when activated by glucocorticoids) (Barnes, 2006d).

Compared to asthma, COPD responds relatively poorly to the anti-inflammatory corticosteroids. The potential molecular mechanism behind this relative corticosteroids resistance in COPD is now being revealed, as the details of the anti-inflammatory mechanisms of steroids are becoming better understood (Barnes, 2006b; Barnes & Stockley, 2005). It has been suggested that corticosteroids resistance in COPD is due to a decrease in the key nuclear enzyme histone deacetylase -2 or HDAC-2 (Barnes, 2006c; Ito et al., 2001). The exact reason behind HDAC-2 reduction in COPD is not clear, but it is suggested that increased oxidative and nitrative stress in the airways due to cigarette smoking might lead to inactivation or destruction of the enzyme (Barnes et al., 2005). Since 1960 it has been known that acetylation of DNA-associated histone proteins and remodelling of the tightly packed chromatin structure is associated with induction of a variety of genes (Littau, Burdick, Allfrey, & Mirsky, 1965). Indeed, it is now understood that histone and chromatin remodelling is central to gene expression and regulation through the process of acetylation, deacetylation and also methylation (Barnes et al., 2005; Rice & Allis, 2001).

### 2.12.1 Chromatin remodelling

In each human eukaryotic cell of average size (say 50-100µ in diameter), there are 46 chromosomes giving a total length of DNA per cell of 1-2 meters, packed into a nucleus millions of times smaller in diameter. A very convoluted, precise and sophisticated packing method indeed is needed to achieve this amazing phenomenon. DNA in cells exists as chromatin, a DNA-protein complex (Elliott; Peterson & Laniel, 2004). Chromatin is made up of a basic unit called a nucleosome, which further consists of 146 base pairs of genomic DNA wrapped around an octamer (8) of core histone proteins. The histone octamer consists of a central subunit of histones H3 and H4 tetramer, and two histones H2A and H2B dimers. Nucleosome are separated from each other by 10-60 base pairs of linker DNA, and the resulting nucleosomal array comprises a chromatin fibre 10 nm in diameter (Figure 2.22) (Barnes et al., 2005; Rahman, 2003).
In a resting cell, chromatin is a tight compact structure with DNA tightly wound around the core histones, which makes DNA inaccessible to various transcription factors and RNA polymerase II, which has the potential to induce messenger RNA formation. This architecture or conformation of the chromatin is described as “closed” and is associated with gene suppression. Gene transcription can only occur if the chromatin structure becomes loose and is “opened” by unwinding of the DNA wrapped around the histone proteins, so that the DNA becomes accessible to the various transcription factors and RNA polymerase II enzyme to initiate the process of transcription of respective genes (Barnes et al., 2005; Rahman, 2003).

This alteration in the chromatin architecture is known as “chromatin remodelling”, which means the effective removal of nucleosomes from the promoter site of the gene to be activated. However, it is not well understood whether a nucleosome is physically dissociated from the DNA during the whole process or just changes its attachment or is completely lost, so as to permit the transcription machinery to assemble on the promoter site of the gene to be activated. The term remodelling avoids implications of what exactly is happening in molecular terms. It is not known how many nucleosomes need to be remodelled during this gene activating procedure (Elliott; Rahman, 2003). However, it is understood that the chromatin structure is strongly controlled by post-translational modification of histones, which may involve methylation and/or phosphorylation, but the two best studied modifications are histone acetylation and histone deacetylation which involves opposing types of enzymes, respectively (de Ruijter, van Gennip, Caron, Kemp, & van Kuilenburg, 2003).
2.12.2 Histone acetylation

Acetylation is the best understood histone modifications. Transcriptionally active genes are regulated by acetylation of lysine residues on N-terminal histone tails. All core histones become acetylated, but modifications to H3 and H4 are more characterised than H2A and H2B. Hypoacetylated histones are associated with gene suppression (de Ruijter et al., 2003). Acetylation leads to the opening of the chromatin structure, allowing recruitment and activation of proinflammatory transcription factors (P. J. Barnes, 2008).

When these transcription factors are themselves activated in response to extracellular stimuli, they bind to specific sites on DNA and start interacting with various coactivator molecules (Figure 2.23) such as CREB-binding protein (cyclic AMP response element binding protein), p300, p300/CBP-associated factor (PCAF) and signal transduction activated transcription factors (STATs). These act as molecular
switches to control gene transcription. All these coactivator molecules also have intrinsic complementary histone acetyltransferase (HAT) activity (P. J. Barnes, 2008; Ogryzko, Schiltz, Russanova, Howard, & Nakatani, 1996; S. Y. Roth, Denu, & Allis, 2001). Histone acetyltransferases or HATs are the enzymes responsible for acetylation of the lysine residues on histone tails and therefore the resulting opening up of the structure of chromatin, so that acetylation, opening of chromatin and DNA-RNA transcription are complementarily regulated events (S. Y. Roth et al., 2001). In COPD, expression of pro-inflammatory genes is predominantly regulated by increased acetylation of H4 which is induced by the binding of NF-κB and AP-1 transcription factors, which also go on sequentially to activate inflammatory mediator genes (P. J. Barnes, 2008).

In a resting cell, basic histone proteins (positively charged) are tightly associated with an acidic DNA backbone (negatively charged) thus reducing the accessibility of the DNA to transcriptional activators. Each histone protein has a long terminal rich in lysine residues, acetylation of which leads to decrease in overall positive charge of the tail, which further leads to reduced association between histone tails and DNA. It may also change the conformational state of the nucleosomes, further enhancing the accessibility to DNA of transcription factors (Rice & Allis, 2001). This further allows the binding of the TATA box-binding protein (TBP), TBP-associated factors and finally binding of RNA polymerase II enzyme leading to gene transcription. Ito and colleagues reported that there is an increase in acetylation of histones associated with the promoter region of inflammatory genes, such as IL-8 which are regulated by NF-κB. In COPD specimens of peripheral lung; airway biopsies and alveolar macrophages the rate of acetylation increases with diseases severity (Ito et al., 2005).
2.12.3 Histone deacetylation

In the process of histone deacetylation, histone deacetylase enzymes (HDACs) reverse the process of acetylation by removing the acetyl groups, and are hence associated with closed conformation of the chromatin leading to gene suppression (Barnes et al., 2005). Interplay between HATs and HDACs is central to control of chromatin structure and function (Peterson, 2002). HDACs induce gene suppression by recruitment of co-repressor proteins as such nuclear receptor co-repressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT). This leads to formation of a co-repressor complex associated with gene suppression (Figure 2.23) (Barnes, 2006b).

**Figure 2.23:** Histone acetylation leading to gene activation and deacetylation leading to gene suppression (Barnes et al., 2005).
There are 11 HDAC isoenzymes that deacetylase histones within the nucleus and specific HDACs appear to be differentially regulated to control different set of genes. They are divided into two major classes. Class I comprises HDAC1, 2, 3, 8 and 11 whereas class II includes HDAC4, 5, 6, 7, 9 and 10. Marked reduction in HDACs has been observed in a variety of chronic inflammatory diseases (Barnes et al., 2005; de Ruijter et al., 2003; Ficner, 2009). In COPD a marked reduction in HDAC2 expression and activity has been observed, with rather less reduction in HDAC5 and HDAC8 expression, and normal expression of other HDACs. It appears that for the regulation of inflammatory genes HDAC2 appears to be of critical importance (Barnes, 2009; Ito et al., 2005).

2.12.4 HDAC2 in COPD

Ito and colleagues showed (by using an HDAC fluorescence activity assay) that there was progressive reduction in total HDAC “activity”, HDAC2 mRNA and protein expression (measured by using quantitative reverse-transcriptase–polymerase-chain-reaction (qRT-PCR) and Western blotting) in samples of peripheral lung tissue, alveolar macrophages and bronchial biopsy specimens from patients with COPD in comparison to healthy non-smokers. They also reported an increase in IL-8 mRNA expression and H4 acetylation in COPD patients (by using a chromatin immunoprecipitation assay) and interestingly there was a positive correlation between H4 acetylation and HDAC activity indicating that the balance between the two had shifted toward the hyperacetylation of the histones in the peripheral lung of patients with COPD, but no change was observed with HDAC activity in patients with pneumonia or cystic fibrosis, or patients with mild asthma, which further shows that change in HDAC activity might be specifically related to COPD. They also reported that there was a marked decrease in HDAC2 activity with lesser reduction in HDAC5 and HDAC8 in COPD (Ito et al., 2005).

In a prominent study it was reported that the decrease in HDAC activity is associated with the intensity of inflammation, as measured by IL-8 expression and the number of inflammatory cells in peripheral airways of COPD patients and also accounted for resistance to anti-inflammatory effects of corticosteroids, a typical feature associated with COPD (Barnes et al., 2005). Cigarette smoking has been considered as an main etiological factor associated to COPD, and Ito et al reported that cigarette smoking
reduced HDAC2 expression in alveolar macrophages and biopsies and enhanced the expression of inflammatory mediators (TNF-α and IL-8) and also reduced glucocorticoid responsiveness in alveolar macrophages (Barnes et al., 2005; Ito et al., 2001). Their immunohistochemical analysis suggested that HDAC2 expression is not different between non-smokers and normal lung function smokers in bronchial biopsies, and although localized to all airway cells, most intense staining was within the epithelium. However, this analysis was limited by lack of any analysis of the cellular profile of the biopsy sections. Looking now at the images they published in the paper it is quite clear that total cellularity of the lamina propria in normal lung function smokers was substantially less than in normal controls (Figure 2.24). This could lead to potential bias associated with results produced from molecular techniques and protein analysis (Ito et al., 2001). Using Western blotting they showed reduced HDAC2 protein content in bronchial biopsies from normal lung function smokers compared to normal controls (Ito et al., 2001), but as stated above, this did not take into account the overall decrease in cellularity and therefore total protein available.
Marwick et al reported that HAT activity is increased and HDAC2 activity is decreased in lungs of rats exposed to cigarette smoke, with increased NF-κB activation and inflammatory gene expression (Marwick et al., 2004), but again it is difficult to know if any cell density changes were taken into account. In another study Ito and colleagues reported that inducing HDAC2 over-expression in glucocorticoid-insensitive alveolar macrophages from COPD patients restored glucocorticoid sensitivity (Ito et al., 2006). Tomita et al reported that oxidative stress has the potential to acetylate H4 in epithelial cell lines and lead to elevated levels of inflammatory cytokines such as IL-8 (Tomita, Barnes, & Adcock, 2003).

2.12.5 Mechanism of HDAC2 reduction in COPD

The reason for the decrease in total HDAC activity in COPD, especially HDAC-2 is not well understood, but evidence is emerging gradually that this may be due to increase in oxidative and nitrative stress in the lungs with COPD (Barnes et al.,
Peter Barnes indicated (Figure 2.25) that cigarette smoke and inflammatory cells produce superoxide anions (O2-) and nitric oxide (NO), and this leads to the formation of peroxynitrite. Inducible nitric oxide synthase (iNOS) induces NO production from inflammatory cells during the process. Peroxynitrite nitrates HDAC2, possibly at tyrosine residues within the catalytic site of the enzyme, which may then obstruct enzymatic activity and may also mark the enzyme protein for ubiquitination, and so degradation proteasome which leads to a decrease in HDAC2 expression and intensification of the inflammatory response and increased resistance to corticosteroids in COPD.

HDAC activity may be restored using antioxidants, iNOS inhibitors, or by using peroxynitrite scavengers that potentially decrease tyrosine nitration. Theophylline may also act as an HDAC activator and has the potential to restore HDAC, but the mechanism behind it is not well understood (Barnes, 2006d; Barnes et al., 2005; Rahman, 2003). The oxidative pathway also has the potential to activate the phosphoinositide-3-kinase (PI3K) pathway leading to phosphorylation of serine residues, also resulting in inactivation of HDAC2 (Barnes, 2009).
2.13 Role of inhaled corticosteroids (ICS) in COPD

Corticosteroids or more fully glucocorticosteroids (though commonly used clinically in asthma and COPD simply as “steroids”) are considered as the most effective therapy for the treatment of non-infectious chronic inflammatory airway disorders, especially asthma. They are thought of as relatively less effective in other inflammatory disorders such as COPD, although are very widely used in this condition. Mechanistically, the main effect of steroids at the cellular level is to inhibit the expression of multiple inflammatory genes encoding for chemokines, cytokines and growth factors, activated as part of the inflammatory process (Barnes, 2006a).
As stated above, it is perhaps paradoxical that steroids are extensively used for the treatment of COPD even though their efficacy is still a matter of debate (Telenga, Kerstjens, Postma, Ten Hacken, & van den Berge). However, several studies have shown that short term use of ICS improves lung function and frequency of exacerbation in long term users (Chanez et al., 2004; Telenga et al.).

Pauwels et al in a double-blind, placebo-controlled study in 1277 subjects with moderate COPD who continued smoking, investigated the effects of budesonide 400µg, and reported that during the first six months of the study, FEV1 improved at the rate of 17 ml per year in the treatment arm, as compared with a placebo group (81 ml decline). However, from nine months of treatment to the end of treatment which was at 3 years there was no significant difference between the groups, suggesting that ICS is associated only with a short term improvement in lung function but does not appreciably affect the long-term progressive decline in lung function in patients with COPD (Pauwels et al., 1999).

A multicenter TORCH study (Toward a Revolution in COPD Health) performed with 5343 patients with COPD in 2004 evaluated the effects of fluticasone propionate combined with salmeterol (LABA, long acting β agonist) for 3 years. In the treatment arm a reduced rate of lung function decline was observed compared to the placebo group (Vestbo, 2004).

As explained above, it can be concluded that steroids do improve lung function but still today the type of inflammation and structural changes effected by ICS in COPD are not well understood, and there are very few studies reporting the effects of ICS on airway inflammation and even fewer on structural changes (airway remodelling) in COPD. Overall our knowledge is limited (Chanez et al., 2004).

Hattotuwa and colleagues reported a significant reduction in CD4/CD8 cell ratios in the epithelium, and in subepithelial mast cells in bronchial biopsies obtained from COPD patients in the active ICS arm treated with fluticasone propionate compared to placebo (Hattotuwa, Gизycki, Ansari, Jeffery, & Barnes, 2002). Reid and et al reported from observations made in bronchial biopsies and BAL from COPD
patients, that fluticasone propionate reduced BAL neutrophils and epithelial cell numbers and CD68+ macrophages, CD8+ lymphocytes and mast cells in bronchial biopsies, but interestingly noted increased neutrophils in bronchial biopsies (D. W. Reid et al., 2008). In another study, it was reported that three month treatment with fluticasone propionate significantly decreased mucosal mast cells and increased neutrophils in biopsies from COPD patients (Gizycki, Hattotuwa, Barnes, & Jeffery, 2002).

Recently, in a very interesting study, Lapperre et al reported the effects of fluticasone propionate on inflammation in airway biopsies and sputum from COPD patients, and found that fluticasone significantly decreased the number of mucosal CD3+ cells, CD4+ cells, CD8+ cells and mast cells after 3 months, with effects maintained to 30 months. They also reported that treatment with fluticasone for 30 months reduced the number of mast cells, increased number of eosinophil and increased the percentage of intact epithelium (This is the only study to my knowledge reporting effects of ICS on epithelium). There was also a decrease in sputum neutrophils, macrophages, and lymphocyte accompanied by improvements in FEV1 decline, dyspnea, and quality of life. The decrease in inflammatory cells correlated with clinical improvements. Discontinuing fluticasone for 6 months on the other hand increased CD3+ cells, mast cells, and plasma cells and thus was accompanied by deterioration in clinical outcomes (Lapperre et al., 2009).

The baseline Rbm thickness in COPD is also debatable, with some studies reporting abnormally thick Rbm and others not (Chanez et al., 2004; Liesker et al., 2009; Postma & Timens, 2006). The effects of ICS on the Rbm have not been specifically studied in COPD. Zanini and et al, (Zanini et al., 2009), however reported in a cross-sectional study that bronchial vascular remodelling may have potential for change with ICS, but we need further longitudinal studies to confirm that.

So, careful review of the literature is fairly convincing that COPD is not strictly steroid resistant as frequently stated. However, It is not clear exactly how steroids work in COPD, but in general (this is not just COPD) it is suggested that they can work in two different ways; at higher concentration they are associated with the activation of anti-inflammatory genes and at low doses they are associated with gene
suppression by recruiting HDACs to the sites of pro-inflammatory transcription, details are given below (Barnes, 2006b).

2.13.1 Gene activation by corticosteroids

At high dose the activation of anti-inflammatory genes (Figure 2.26) (Barnes, 2006b) is mainly through binding to glucocorticoid receptors (GRs) localized in the cytoplasm of the target cell; the complex acts as a transcription factor to control the transcription of several steroid responsive genes. In the cytoplasm of the cells GRs are usually attached to proteins recognized as molecular chaperons, which include heat shock-proteins-90 (hsp-90) and FK binding protein (FK or also called as FK506 is a family of proteins that have prolyl isomerise activity which suggests that they have the capacity of interconverting the cis and trans isomers of peptide bonds) (Wikipedia). These proteins when bound to the receptor prevent its nuclear localisation by covering the sites of the receptor that are essential for transportation into the nucleus (Wu et al., 2004).

Binding of corticosteroids to GR leads to changes in the receptor structure which further leads to dissociation of these inhibitory molecular chaperons proteins, by exposing the sites essential for nuclear localisation. This results in rapid transport of active GR-complex into the nucleus, where it binds to glucocorticoid response elements (GRE) in the promoter region of steroid responsive genes, including several types of anti-inflammatory genes and thus leading to increase in synthesis of anti-inflammatory proteins such as annexin-1 (lipocortin-1), secretory leuko-protease inhibitor (SLPI), IL-10, the inhibitor of NF-κB, IκB-α, glucocorticoid-induced leucine zipper protein, which inhibits both NF-κB and AP-1 and mitogen-activated protein (MAP) kinase phosphatase-1, which inhibits p38 MAP kinase (Barnes, 2006b; Barnes et al., 2005).

Activation of anti-inflammatory genes by high dose of corticosteroids is associated with a selective acetylation of lysine residues 5 and 16 on H4, resulting in increased gene transcription, whereas in response to inflammatory stimuli differential acetylation of residues 8 and 12 is involved. However, the relevance of this mechanism is in doubt. It seems hard to explain the anti-inflammatory actions of
corticosteroids by inducing the transcription of a small number of anti-inflammatory genes, as high doses of corticosteroids are generally required for these types of responses, whereas on the other hand in clinical practice, corticosteroids are able to suppress inflammation at low doses (Barnes, 1998a, 2006b, 2006d, 2009; Barnes et al., 2005).

![Diagram of gene activation by corticosteroids at high dose](image)

**Figure 2.26:** Gene activation by corticosteroids at high dose (Barnes, 2006b).

### 2.13.2 Gene suppression by corticosteroids

Corticosteroiod at lower doses leads to suppression of inflammatory genes by recruiting HDAC2 to activated pro-inflammatory transcriptional complexes (Figure 2.27). Initially it was believed that gene suppression by corticosteroids is generally induced by binding of GR to negative GRE sites in their promoter region, but later it
was confirmed that this process is applicable to only a small number of genes, and this mechanism does not include genes encoding most inflammatory proteins (Ismaili & Garabedian, 2004). It was observed in asthma that most of the genes which are activated during the inflammatory process do not have GRE sites, but are still effectively repressed by corticosteroids (Barnes et al., 2005). There is convincing evidence now that anti-inflammatory actions of corticosteroids are due to inhibition of transcription factors such as AP-1 and NF-κB (by inhibition of histone acetylation and stimulation of histone deacetylation), which are transcription factors actively involved in regulation of many genes coding for a host of pro-inflammatory proteins (Barnes, 2006d; Barnes & Karin, 1997).

**Figure 2.27:** Gene suppression by corticosteroids at low dose (Barnes et al., 2005).

Inflammatory genes get activated in response to inflammatory stimuli (Figure 2.28) which may then be potentially reinforced by production of interleukin (IL)-1β and/or tumour necrosis factor (TNF)-α which lead to activation of I-κB kinase (IKK)2 which further activates NF-κB, a heterodimer made up of proteins p50 and p65 (Barnes, 2006d). NF-κB proteins move to the nucleus and bind to the specific κB recognition sites and also bind to co-activator proteins like CBP or PCAF. All of these factors have intrinsic HAT activity, which leads to acetylation of lysine...
residues in H4, and thereby augmented expression of genes encoding for inflammatory proteins, for e.g. GM-CSF, cyclo-oxygenase-2 (COX-2), and numerous cytokines, chemokines and receptors.

**Figure 2.28:** Role of transcription factors and gene suppression by corticosteroids at low dose, for details please see text (Barnes, 2006d).
Thus, this whole integrated process leads to activation of numerous inflammatory genes. As already described, corticosteroids at low concentrations activate GRs which rapidly move to the nucleus and bind to co-activators such as CBP or PCAF to directly inhibit intrinsic HAT activity, so that HDACs are recruited leading to histone deacetylation and suppression of inflammatory genes. So in very general terms, corticosteroids at low concentrations recruit HDACs to the transcription complex and convert the process of acetylation to deacetylation to suppress the transcription of inflammatory genes (Barnes, 2006a, 2006b, 2006c, 2006d; P. J. Barnes, 2008; Barnes, 2009; Barnes et al., 2005; Barnes & Karin, 1997).

2.13.3 Proposed mechanism of relative CS resistance in COPD

Even though corticosteroids have been highly effective in asthma, the inflammatory process in COPD has been at least partly and relatively resistant to the anti-inflammatory effects of corticosteroids, regardless of the fact that active airway and lung inflammation is present. Although this resistance has perhaps been over stated by some, it is true that the inflammation in COPD is not fully suppressed nor to the same extent as in asthma, by corticosteroids. (Culpitt et al., 1999; Culpitt et al., 2003; Keatings, Jatakanon, Worsdell, & Barnes, 1997; Loppow et al., 2001). Furthermore, Hogg and colleagues reported in a histological analysis of peripheral airways of patients with COPD that despite treatment with high dose steroids intense inflammatory responses still existed (James C. Hogg et al., 2004), and evidence is available that COPD has active steroid resistance mechanisms (Culpitt et al., 1999; Culpitt et al., 2003; Keatings et al., 1997; Loppow et al., 2001). Corticosteroid resistance in COPD may be explained on the basis of increased oxidative and nitrative stress due to cigarette smoking leading to impaired HDAC2 function in COPD (Figure 2.25) and resistance to corticosteroids as explained before in previous sections (Barnes, Ito, & Adcock, 2004).

The oxidative pathway also has the potential to activate the phosphoinositide-3-kinase (PI3K) pathway leading to phosphorylation of serine residues, which results in inactivation of HDAC2 (Barnes, 2009). PI3K are a family of enzymes involved in cellular functions such as cell growth and migration, inflammatory cell function, proliferation and differentiation, motility, survival and intracellular trafficking. They are divided into three different classes: the primary class reported to be associated
with impairment of steroid responsiveness in COPD is class I PI3K, including the isoforms PI3Kγ and PI3Kδ, and especially PI3Kδ (Ito, Caramori, & Adcock, 2007).

Marwick et al investigated this system in a study designed to evaluate the role of PI3K in the development of cigarette smoke-induced steroid insensitivity in a model of wild type, PI3Kγ knock out and (dead) PI3Kδ kinase knock-in transgenic mice. They showed that cigarette smoke reduced HDAC2 activity and impaired steroid function in mice, which was blocked by elimination of PI3Kδ kinase as shown in (dead) PI3Kδ kinase knock-in transgenic mice. The overall data suggested that activation of the PI3Kδ isoform might be playing an important role in the development of impaired steroid function in an oxidative environment (Marwick et al., 2009).

In another study, using peripheral lung tissue and blood monocytes obtained from patients with COPD, smokers with normal lung function and healthy never smokers, the same group reported that PI3Kδ and Akt (protein kinase) phosphorylation was increased in macrophages from patients with COPD and smokers compared to controls in vivo. Their in vitro data revealed that oxidative stress-induced phosphorylation disappeared by selective inhibition of PI3Kδ but not of PI3Kγ. However, the study was limited by the fact that COPD subjects were also suffering from lung cancer (Marwick et al.) and material was obtained from cancer-containing lung resections. However, these studies taken together suggest that PI3K might be playing an important role in steroid unresponsiveness in COPD and PI3Kδ might be a future therapeutic target.

Perhaps the important question that the Barnes group, from which these data have mainly emerged, have not asked, is how/why ICS do (undoubtedly) have some positive effects both clinically and also on inflammation in COPD airways, at least over 6-9 months, and what important elements of inflammation, and indeed remodelling could be made more responsive if the PI3K system was suppressed? In other words, if COPD really is a corticosteroid-resistant disease, what are the strategically important resistant elements of its pathology?