1

INTRODUCTION

1.1 CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Chronic Obstructive Pulmonary Disease (COPD) imposes a significant burden on affected individuals and society. The 2008 World Health Statistics show that in 2004 COPD was the fourth leading cause of death worldwide and is predicted to become the third by 2030 (WHO 2008).

1.1.1 Definition

Chronic obstructive pulmonary disease (COPD) is defined as "*a preventable and treatable disease with significant extra-pulmonary effects that may contribute to the severity in individual patients. Its pulmonary component is characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive*

and associated with an abnormal inflammatory response of the lung to noxious particles or gases" (GOLD Guidelines 2009).

1.1.2 Classification of COPD severity

The airflow limitation is defined and quantified by spirometry measuring the forced expiratory volume in one second (FEV_1) and the forced vital capacity (FVC). The spirometric definition of COPD is the presence of FEV_1/FVC ratio of less than 70% and an FEV_1 <80% predicted. Spirometry is the gold standard for the diagnosis of COPD and for monitoring of disease progression. It is a standardized, easy to perform and reproducible measurement of the airflow limitation (GOLD 2009).
The degree of the airflow obstruction can be stratified by measuring the percentage predicted FEV_1 (%FEV_1). I will use the GOLD classification of COPD which is presented in **Table 1** to stratify disease severity in the remainder of this thesis.

Table 1: GOLD Classification of COPD based on post-bronchodilator FEV_1

Stage	Severity	Criteria
Stage I:	Mild	FEV_1/FVC<70 FEV_1> 80% predicted
Stage II:	Moderate	FEV_1/FVC<70 50%<FEV_1<80% predicted
Stage III:	Severe	FEV_1/FVC<70 30%<FEV_1<50% predicted
Stage IV:	Very severe	FEV_1/FVC<70 FEV_1<30% predicted or FEV_1< 50% predicted plus cor pulmonale and hypoxaemia

1.1.3 Aetiology

COPD is a heterogeneous disease, and the classic subdivision into predominantly bronchitic or emphysematous phenotypes does not capture its diversity well. The chronic exposure of a genetically susceptible individual to certain noxious environmental factors is believed to cause COPD.

Worldwide, cigarette smoke is the most common risk factor for COPD. It has been recognized since the description by Peto & Fletcher in the 1970's. The WHO estimates that 73% of COPD mortality is related to cigarette smoking in high income countries and 40% in middle and low income countries (Mannino et al 2007). However, not all smokers have COPD and some non-smokers also develop COPD.

Over the last decade several epidemiological studies have demonstrated other significant risk factors for the development of COPD. These factors are presented in **Table 2.**

At present alpha-1 antitrypsin deficiency is the only hereditary disease with a strong evidence for a causal relationship in the pathogenesis of COPD. It is present in about 1-3% of individuals with COPD (Mannino et al 2007). Alpha-1 antitrypsin is a major inhibitor of serine proteases. Individuals with the deficiency who smoke develop more severe COPD at a younger age than non-smokers with the disease. Multiple other gene polymorphisms are currently being studied for their links to COPD: genes coding for tumour necrosis factor (TNF)-α, interleukin (IL)-6, transforming growth factor (TGF)-β, haeme oxygenase and matrix metalloproteinases among others. Recent genome wide association studies in European ancestry populations have identified several single nucleotide polymorphisms in the following genes which are related to COPD: BICD1 (Kong et al 2010), CHRNA3/CHRNA5 (Pillai et al 2010), HHIP (Pillai et al 2010) and FAM13A (Pillai et al 2010, Cho et al 2010).

Exposure to biomass fuels such as animal dung, straw, crop residues, coal and wood used for cooking and heating is a major risk factor for COPD in low and middle income countries. The WHO estimates 35% of people with COPD developed it after exposure to these indoor air pollutants (Mannino et al 2007).

Occupational exposure to organic and inorganic dust, chemical fumes and vapours is recognized as an independent risk factor for developing COPD. This may account to up to 19% of all cases of COPD as recognized in the NHANES III survey (Hnizdo et al 2002). NHANES III is a large US population based survey of about 10000 adults aged 30-75 years which included lung function. The same study showed that occupational exposure can be responsible of 31% of cases of COPD in never smokers.

The contribution of outdoor pollution to COPD is much smaller and estimated at 1% in high income countries and 2% in low income countries according to the WHO (Mannino et al 2007).

Infections can contribute to both the development and the progression of COPD. Repeated infections in childhood can lead to impaired lung development and smaller lung volumes, when such an individual is exposed to another noxious agent COPD may appear. Recent epidemiological data from the Burden of Obstructive Lung Disease (BOLD) Initiative has identified a history of tuberculosis as a significant risk factor for the development of COPD (Buist et al 2007), even in non-smokers.

In-utero malnutrition can lead to smaller infants with smaller lungs and may predispose to COPD.

Table 2: Risk factors other than cigarette smoking associated with chronic obstructive pulmonary disease (Salvi et al 2009)

Risk Factor	Agent responsible
Indoor air pollution	• Smoke from biomass fuel: plant residues (wood, charcoal, crops, twigs, dried grass) and animal residues (dung) • Smoke from coal
Occupational exposures	• Crop farming: grain dust, organic dust, inorganic dust • Animal farming: organic dust, ammonia, hydrogen sulphide • Dust exposures: coal mining, hard-rock mining, tunnelling, concrete manufacturing, construction, brick manufacturing, gold mining, iron and steel founding • Chemical exposures: plastic, textile, rubber industries, leather manufacturing, manufacturing of food products • Pollutant exposure: transportation and trucking, automotive repair
Treated pulmonary tuberculosis	
Lower-respiratory-tract infections during childhood	
Chronic asthma	
Outdoor air pollution	• Particulate matter (<10 μm or <2・5 μm diameter) • Nitrogen dioxide • Carbon monoxide
Poor socioeconomic status	
Low educational attainment	
Poor nutrition	

1.1.3 Epidemiology

Acquiring reliable epidemiological data in COPD is hampered by imprecise definition of the disease, under recognition and under reporting of the disease. The existing prevalence data shows wide variation due to survey methods, diagnostic criteria and the various analytical methods used.

In recent years, the BOLD Initiative Study and the Latin American Project for the Investigation of Obstructive Lung Disease (PLATINO) Study have used spirometry and the GOLD diagnostic criteria to survey the prevalence of COPD in 17 countries worldwide. This data shows the prevalence of COPD is generally higher than previously reported and there is heterogeneity in prevalence and staging of COPD across sites and between men and women within sites (Buist et al 2007). This heterogeneity is likely related to the prevalence of different risk factors in the different areas. The prevalence of stage II or higher COPD in persons aged 40-80 is 0.1% worldwide. The prevalence rates increase with age and the pack years smoked.

It is also estimated that up to 2/3 of patients with COPD are undiagnosed (Shahab et al 2006).

1.1.5.1 Pathology

Our understanding of the anatomical changes present in the COPD lung has significantly improved in the last 20 years. Our knowledge is derived from studies from bronchial biopsies, broncho-alveolar lavage (BAL) fluid and lung tissue obtained after lung volume resection surgery or lung resection for cancer.

COPD is associated with increased inflammation throughout the airway. The upper airway, the trachea, the large bronchi, the medium and small airways as well the lung parenchyma are affected. However, small airways with a calibre <2mm are the main site of airway obstruction in COPD. The inhalation of cigarette smoke/other toxic causes an increase in the number of neutrophils, macrophages, natural killer cells and lymphocytes, in the COPD lung.

In a study of bronchial biopsies O'Shaughnessy et al reported an increase in macrophages and T lymphocytes, particularly CD8+ cells in patients with chronic bronchitis and COPD compared to normal subjects. They also found an inverse correlation between the number of airway CD8+ cells and $\%FEV_1$ in the COPD patients (O'Shaughnessy et al 1997). This relationship was also found by another group in a study of specimens obtained after lung resections for a pulmonary nodule. There was an inverse correlation between the number of CD8+ cells in the peripheral airways and the $\%FEV_1$ (Saetta et al 1998).

In another study of bronchial biopsies from smokers with severe and mild to moderate COPD, the number of sub-epithelial neutrophils, macrophages and natural killer cells were increased compared to smokers without COPD (Di Stefano et al 1998). This study also showed that the number of inflammatory cells in the bronchial sub-epithelium was inversely correlated with the $\%FEV_1$ and that the inflammation was quantitatively and qualitatively different between smokers with and without COPD.

Chronic sputum production is common in COPD. Saetta et al examined surgical specimens from lung resection for localized pulmonary lesions in chronic bronchitic smokers with and without COPD (Saetta et al 2000) and compared them with non-smokers. Smokers with chronic bronchitis and COPD had an increased number of goblet cells, macrophages and CD8 cells in their epithelium compared to those without COPD.

In another surgical resection study the same group also showed that smokers with severe COPD had increased number of leucocytes in the small airways (Turato et al 2002). Furthermore the number of leucocytes showed a positive correlation with the radiological emphysema score and the measured residual lung volume. There was a negative correlation between the number of leucocytes in the airway and the FEV_1 and the diffusion of carbon monoxide DLCO). The number of CD4 and CD8 T cells was increased in the airway wall and the number of macrophages was increased in the airway epithelium. The smoking history was also correlated with the airway wall and smooth muscle thickness.

In a study of lung volume reduction surgery (LVRS) specimens, Retamales et al showed that severe emphysema was associated with an absolute increase in the total number of inflammatory cells (neutrophils, macrophages, eosinophils, CD4 and CD8 T cells) in the lung tissue and airspaces. They demonstrated that the extent of emphysema detected on high resolution CT scans was directly related to the number of total macrophages in the lung tissue and airspaces (Retamales et al 2001).

The relationship between the severity of the airway obstruction as measured by the GOLD criteria and the pathological changes in the lung tissue was elegantly demonstrated in LVRS specimens by Hogg et al (Hogg et al 2004). Lung tissue from 159 patients with GOLD stage I to IV were examined. The progression of COPD was strongly associated with an increase in the volume of tissue in the airway wall and the accumulation of inflammatory mucous exudates in the lumen of the small airways. The percentage of the airways containing neutrophils, macrophages, CD4 cells, CD8 cells, B cells and lymphoid aggregates increased as the severity of COPD increased. Patients with stage III-IV COPD had lymphoid follicles and higher numbers of all the lymphocytes subsets in their airways (Hogg et al 2004).

In summary the changes present in the COPD lung include: destruction of cilia, infiltration of the respiratory epithelium and airspaces by inflammatory cells, goblet cells hyperplasia, mucus hyper-secretion, smooth muscle cell hypertrophy, increased connective tissue, fibrosis, remodelling of the airways with narrowing of airways (leading to increased lower airway resistance) and emphysematous destruction of the lung parenchyma leading to decreased elastic recoil of the lungs. In the pulmonary vessels there is thickening of the vascular wall, infiltration of inflammatory cells and smooth muscle cell hypertrophy. Several studies have demonstrated a direct relationship between the number of T cells present in the lung and the severity of the airway obstruction (O'Shaugnessy et al 1997, Saetta et al 1998). The severity of COPD as measured by the GOLD classification is directly associated with the accumulation of inflammatory exudates in the small airway lumen and infiltration of the wall by inflammatory immune cells that formation of lymphoid follicles in severe disease (Hogg et al 2004). These lymphoid follicles could represent be the response of the lower airways to the bacterial colonization often seen patients with more severe degrees of COPD.

1.1.6 Role of inflammation

Patients with COPD have increased airway and systemic inflammation compared to healthy controls, both in the stable state and at exacerbation. This inflammation is believed to represent an amplification of the normal inflammatory response of the airways to chronic irritants as depicted in **Figure 1**. The airway inflammation is further amplified by oxidative stress and the proteinase-antiproteinase imbalance.

The various inflammatory cells present in increased numbers in the COPD lung produce numerous mediators/cytokines capable of damaging and sustaining the inflammatory process. These cytokines can be measured in the broncho-alveolar lavage fluid, in the sputum and the blood of COPD patients and healthy controls. A selection of frequently measured cytokines is shown in **Table 3.** The exact origin of the systemic inflammation in COPD is unknown. It could be due to a spillover from the lung into the systemic circulation or local synthesis due to the increased numbers of inflammatory cells present in the circulation in COPD patients. It could also be due to the direct effect of smoking on other tissues such as the vascular endothelium.

The inflammatory markers assessed in this thesis are interleukin(IL)-6, IL-8 and C-reactive protein (CRP).

Interleukin 6

Interleukin-6 is produced by a variety of cells including bronchial epithelial cells, macrophages, fibroblasts, synovial cells, endothelial cells, glia cells, adipocytes and keratinocytes (Kishimoto T 2005). IL-6 production can be induced by a variety of stimuli including: IL-1, tumour necrosis factor (TNF), platelet-derived growth factor (PDGF), bacterial and viral infection and microbial components such as lipopolysaccharide (LPS). IL-6 stimulates the production of CRP and fibrinogen by hepatocytes. It also has a haematopoietic role as it stimulates leucocytosis, thrombocytosis and lymphocytes differentiation (Kishimoto T 2005). The secretion of IL-6 is inhibited by glucocorticoids.

Interleukin 8

Interleukin-8 is a 16 kDa protein produced by bronchial epithelial cells, monocytes/macrophages and neutrophils. Its secretion is stimulated by TNF-alpha, cigarette smoke and bacteria. It is involved in the chemotaxis of neutrophils into the airways and their activation. The concentration of sputum IL-8 is related to the severity of the airway obstruction in patients with COPD (Yammamoto 1997).

In an in-vitro model, when bronchial epithelial cells derived from patients with COPD are cultured and stimulated by TNF-alpha, they produce higher concentrations of IL-6 and IL-8 than cells derived from smokers & ex-smokers without COPD (Patel et al 2003). This response is attenuated in COPD patients taking inhaled corticosteroids.

C reactive protein

C-reactive protein is an acute phase protein produced by hepatocytes and IL-6 is the main stimulus for the release of CRP into the blood. CRP is increased in many inflammatory disorders, in response to infection and tissue damage. Epidemiological studies have demonstrated a relationship between increased levels of CRP and increased cardiovascular mortality. However, it is not yet established if CRP contributes directly to the increased cardiovascular mortality or if it is solely a marker of the systemic inflammation present in these subjects. In cross-sectional studies the concentration of CRP is related to FEV_1 (Barnes et al 2009).

Figure 1: Cellular and pathological processes that link the aetiology of COPD to clinical outcomes (Chung et al 2008).

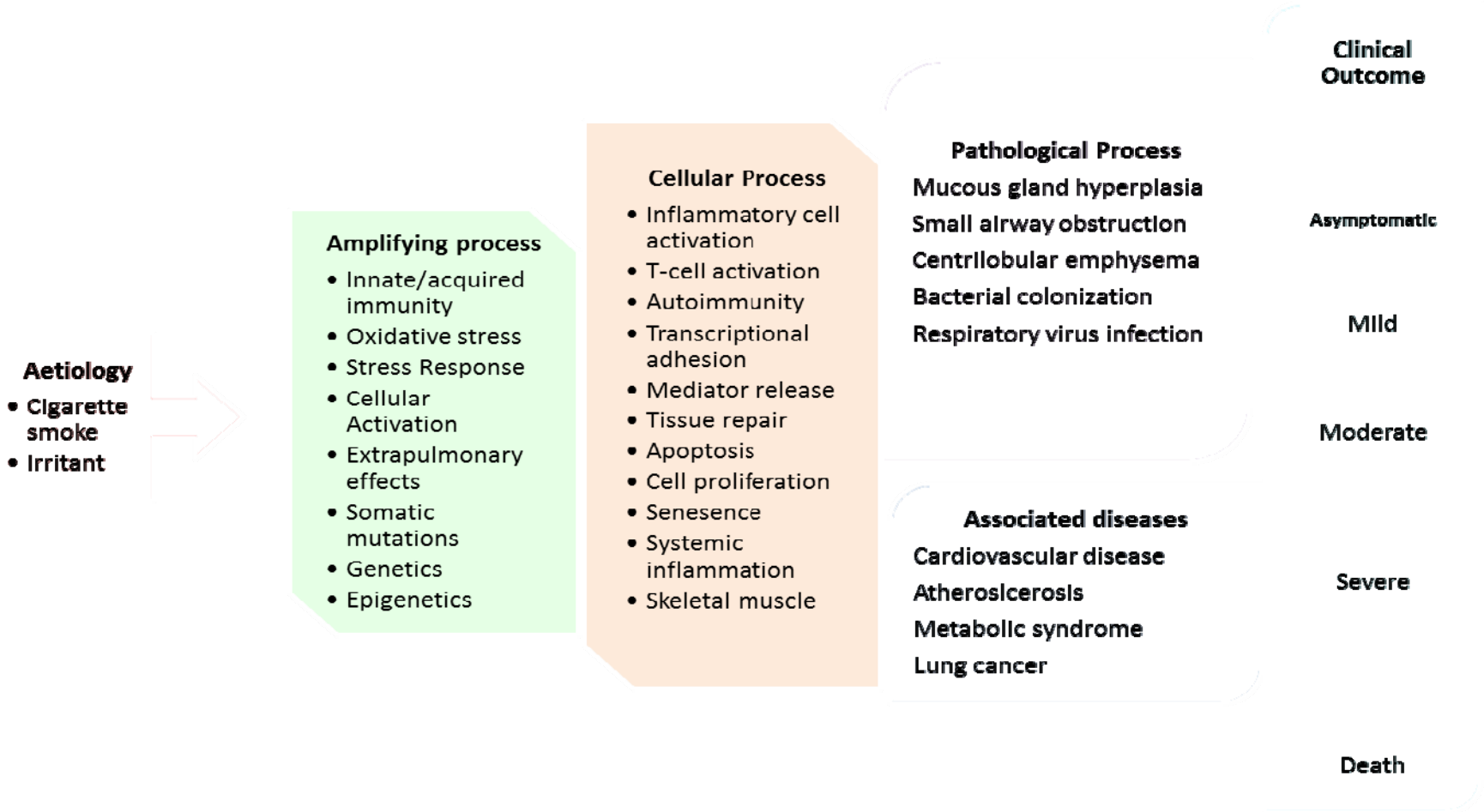


Table 3: List of commonly studied markers of airway and systemic inflammation in COPD

AIRWAYS	SYSTEMIC
Interleukin (IL)-6, IL-8	IL-6, IL-8
Leukotriene (LT)-B4	Fibrinogen
Tumour necrosis factor (TNF)-α and Receptors	Tumour necrosis factor (TNF)-α and receptors
Myeloperoxidase (MPO)	C-reactive protein(CRP)
Secretory leukoprotease inhibitor (SLPI)	Soluble intracellular adhesion molecule(ICAM)-1
Endothelin(ET)-1	ET-1
Matrix metalloproteinase 9 (MMP-9)	MMP-9
Granulocyte and macrophage colony stimulating factor (GM-CSF)	GM-CSF
Neutrophil elastase (NE)	
Total cell count and neutrophil count	Total cell count and neutrophil count
Eosinophilic cationic protein (ECP)	

1.1.7 Systemic Effects of COPD

COPD is associated with systemic manifestations such as cachexia/ loss of fat free mass, osteoporosis, muscle wasting and dysfunction. Patients with COPD have an increased risk of the following comorbidities compared to age matched healthy controls: cardiovascular disease, depression, gastro-oesophageal reflux, anaemia, lung cancer, metabolic syndrome, diabetes and obstructive sleep apnoea. Systemic inflammation is a key contributors to these comorbidities and systemic manifestations of COPD (Gan et al 2004, Agusti 2005, Barnes et al 2009, Sinden et al 2010). The systemic inflammation present in COPD patients can be quantified by measuring the circulating levels of leucocytes, C-reactive protein (CRP), fibrinogen, IL-6, IL-8, tumour necrosis factor and its receptors (Gan et al 2004).

Cachexia

Body composition is altered in patients with COPD and the fat-free mass index (FFMI) provides more relevant information than the body mass index (BMI). Vestbo et al assessed the FFMI, BMI and their relationship to all cause and COPD related mortality in 1898 subjects from the Cohpenhagen City Heart Study. The majority of the patients had mild disease, only 117 patients had an FEV_1<50% predicted (Vestbo et al 2006). The proportion of patients with low FFMI increased with COPD severity. In patients with GOLD stage 3 & 4 disease, 50% of patients with normal BMI had a reduced FFMI.

In this large sample of COPD patients, being in the lowest 10th percentile of the general population for FFMI was associated with a hazard ratio of 1.5 (95% confidence interval, 1.2–1.8) for overall mortality and 2.4 (1.4–4.0) for COPD-related mortality. FFMI was a predictor of overall mortality. In another study of 412 COPD patients with GOLD stage II-IV disease, the FFMI was found to be an independent predictor of mortality (Schols A et al 2005). The fat mass was not related to mortality in the same study. The FFMI has been shown to be related to the six minutes walking distance (6MWD) in all GOLD stages of COPD (Ischaki et al 2007). The FFMI was also related to the MRC dyspnoea score in patients with GOLD stage 2 or above in the same study.

Cachexia can be defined as a FFMI< 17 kg/m^2 in males or less than 14 kg/m^2 in females. It is estimated that about 25% of COPD patients develop cachexia (Wagner

P 2008). The exact mechanisms causing cachexia in COPD patients are unknown. Several hypotheses exist. Patients with COPD have an increased basal metabolic rate which can lead to weight loss. The increased work of breathing, tissue hypoxia, deficit of certain anabolic hormones, systemic inflammation and certain drugs (β-agonists) used in COPD can all be responsible for the increased basal metabolic rate seen in COPD (Agusti 2005, Wagner 2008).

Muscle dysfunction

The fat free mass is mainly constituted by skeletal muscle. In patients with COPD the skeletal muscle weakness mainly affects the locomotor muscles such as the quadriceps (Man et al 2009). The strength of the diaphragm, adductor pollis and abdominal muscles are preserved in COPD patients when compared with age matched controls (Man et al 2009). The quadriceps abnormalities observed in COPD patients include increased fatigability, reduced endurance, reduced mid-thigh cross section area, reduction in type I fibres and increased type IIx fibres (Man et al 2009). The skeletal muscle dysfunction is multi-factorial and likely related to the systemic inflammation, oxidative stress and increased immobility present in COPD patients. Other contributing factors include the recurrent courses of oral steroids, tissue hypoxia, hypercapnia, nutritional depletion, genetic factors and an imbalance of anabolic and catabolic hormones (Man et al 2009).

Cardiovascular disease

COPD increases the risk of cardiovascular disease. This could be attributed to the common risk factor of cigarette smoking or to the persistent low grade systemic inflammation and the endothelial dysfunction present in COPD. A recent analysis of the primary care records of over 1 million individuals over 35 years of age demonstrated that a physician diagnosis of COPD was associated with increased risks of cardiovascular disease, stroke and diabetes. This increased risk persisted after correction for age, sex and smoking status (Feary et al 2010).

Osteoporosis

A high proportion of patients with COPD have osteoporosis and low bone mineral density, even in the early stages of the disease (Jorgensen et al 2008). The loss of bone mineral density is directly related to the loss of fat free mass (Bolton et al 2004).

Osteoporosis could be due to the increased production of pro-inflammatory cytokines that alter bone metabolism and other features common in COPD such as repeated courses of oral steroids, immobility, nutritional depletion and old age.

Depression

The prevalence of depression is high in COPD patients and is multi-factorial (Norwood 2007). The progression of COPD is often associated with a reduction in the level of independent function and worsening quality of life due to recurrent exacerbations. There is also some evidence that the systemic and physiologic changes associated with COPD have direct effects on the brain's vasculature that lead to areas of sub-cortical hypo-densities that are more common in depression (Norwood 2007).

Lung cancer

Patients with COPD have a 3-4 times higher risk of lung cancer than smokers without COPD. The higher risk persists even in never smokers with COPD (Turner et al 2007). Lung cancer is a common cause of death in patients with COPD (Sin et al 2006). The association could be related to exposure to the same noxious agent or the underlying lung inflammation and structural changes.

Obstructive sleep apnoea

Sleep apnoea and COPD can co-exist and is called overlap syndrome. There is evidence of increased systemic inflammation in both diseases (McNicholas 2009).

Diabetes and the metabolic syndrome

COPD is associated with an increased prevalence of diabetes and metabolic syndrome (Mannino et al 2008, Watz et al 2009, Feary et al 2010). The systemic inflammation is again believed to contribute to the association. Pro-inflammatory cytokines such as TNF-alpha and IL-6 are known to induce insulin resistance (Barnes et al 2009). It has also been demonstrated that the presence of hyperglycaemia on admission to hospital with an exacerbation of COPD was associated with a poor outcomes (Baker et al 2006).

Gastro-oesophageal reflux disease

Several studies have reported an association between gastro-oesophageal reflux symptoms and more frequent exacerbations of COPD (Hurst et al 2010, Terada et al 2008, Rascon-Aquilar et al 2006).

1.2 EXACERBATIONS OF COPD

COPD is characterized by recurrent episodes of worsening respiratory symptoms called exacerbations. They are important events in COPD, both for the patient and for society. Exacerbations contribute considerably to the morbidity, mortality and increased healthcare costs associated with COPD. Exacerbations are a major cause of health care utilization, both as GP visits and hospitalizations. Exacerbations of COPD were responsible for around 1 million bed days in the UK (Donaldson et al 2006).

1.2.1 Definition

Exacerbations can be defined by respiratory symptoms alone (symptom based definition) or by healthcare utilization events such as GP visit, A&E attendance or hospital admission for an exacerbation (health care utilization definition). The health care utilization often involves additional treatment for increased respiratory symptoms. Both have advantages and disadvantages.

In a study of 70 patients with moderate to severe COPD followed with daily diary cards for one year, our group previously demonstrated that up to 50% of exacerbations are not reported to the study team (Seemungal et al 1998). There was no difference in symptom scores, exacerbation severity and peak expiratory flow rate (PEFR) changes between reported and unreported exacerbations. The INSPIRE study was the first large scale trial to use in parallel a symptom based and a healthcare utilization based definition of a COPD exacerbation (Seemungal et al 2007 and Wedzicha et al 2008). It showed that if a symptom based definition was used the

exacerbation rate was 3 per year, if a healthcare utilization definition was used the exacerbation rate was 50% less at about 1.5.

To date there is no single biomarker that can diagnose an exacerbation with sufficient sensitivity and specificity. Our group assessed the use of 36 plasma biomarkers in paired baseline and exacerbation samples from 90 patients with moderate to severe COPD to confirm the diagnosis of exacerbation and to predict severity (Hurst et al, 2006). CRP was the most selective biomarker for an exacerbation, however, its sensitivity and specificity on its own was insufficient. Associating any major symptom recorded by the patient to the CRP level increased the sensitivity. No biomarker was useful to predict the severity of the exacerbation.
A frequently used consensus definition of an exacerbation of COPD is "*an event in the natural course of the disease characterized by a change in the patient's baseline dyspnoea, cough, and/or sputum production that is beyond the normal day-to-day variation, is acute in onset, and may warrant a change in the regular medication in a patient with underlying COPD"* (GOLD 2009).

The definition of COPD exacerbation used in this thesis is the symptom based definition used in all the work from our department. The definition was developed from that of Anthonisen, used in his landmark study of antibiotics at exacerbation of COPD (Anthonisen et al 1987). The London COPD Cohort definition of an exacerbation is summarized in **Table 4**.

TABLE 4: The London COPD Study Definition of Exacerbation.

MAJOR SYMPTOMS	DEFINITION	MINOR SYMPTOMS
Dyspnoea		Cough
Sputum Volume		Wheeze
Sputum Purulence		Sore Throat
		Coryza*
	EXACERBATION	
Two or more new or worsening symptoms, on two or more consecutive days, at least one symptom being MAJOR		

*Coryza is defined as one or more of runny or blocked nose, post-nasal drip or sneezing.

The patients record changes in their respiratory symptoms on a diary card. They are asked to record symptoms only when they are new or worse than usual. The symptoms are categorised as major (increased dyspnoea, increased sputum volume or sputum purulence) and minor (increased cough, wheeze, and presence of a sore throat or a cold).

An exacerbation is defined as:

- the onset of two or more new or worsening symptoms
- on two or more consecutive days
- at least one symptom being major

This definition has been validated against important outcome measures in COPD including the rate of decline in FEV_1 (Donaldson et al 2002), and quality of life (Seemungal et al 1998).

1.2.2 Epidemiology of exacerbations

COPD exacerbations occur throughout the year but are about 50% more frequent in the winter months and contribute to the winter bed pressures each year. The increased prevalence of respiratory viral infections during the winter months could explain this seasonality.

1.2.3 Concept of exacerbation frequency

The exacerbation frequency is the number of exacerbations experienced by a patient in a year. It is an important outcome measure in COPD. Exacerbation frequency is directly related to quality of life (Seemungal et al 1998), lung function decline (Donaldson et al 2002), activities of daily living (Donaldson et al 2005), airway inflammation (Bhowmik et al 2000), lower airway bacterial colonisation (Patel et al 2002) and mortality (Soler-Cataluna et al 2005).

Exacerbations tend to become more frequent and more severe as the underlying disease progresses (Donaldson et al 2003, Hurst et al 2010). However, in each

individual the exacerbation frequency remains relatively constant over a period of 3-6 years (Donaldson et al 2003, Hurst et al 2010).

In the study by Donaldson et al, 132 patients with moderate to severe COPD were followed for 6 years with daily diary cards and 1,111 exacerbations were identified during the study period. Patients with GOLD stage III disease had a 0.75/year higher exacerbation rate than patients with GOLD stage II disease. Patients with severe disease (GOLD stage III) had a higher total symptom scores at exacerbation, reflecting more severe exacerbations as severity of COPD increased. The symptom and lung function recovery time was longer for patients with GOLD stage III disease compared to stage II disease (0.32 and 0.55 days/year respectively).

In the study by Hurst et al, 2138 patients were prospectively followed up for 3 years. Exacerbations were defined as an event that led to the prescription of antibiotics, steroids, both or admission to hospital. As a healthcare utilization definition was used the exacerbation frequency may be an underestimation of the true rate. Exacerbations were more frequent and severe as the severity of COPD increased. Exacerbation frequency was 0.85, 1.34 and 2 for patients with GOLD stage II, III and IV disease. The single best predictor of exacerbations in this large cohort and across all GOLD stages of COPD was a previous history of exacerbation. In this large study the frequent exacerbator phenotype was independently associated with a history of gastro-oesophageal reflux, poor quality of life and an elevated white cell count.

1.2.4 Impact of exacerbations

Exacerbations are responsible for a large amount of the burden of COPD, both to the patient and to society. The way exacerbations impact on patients and society will be examined below.

a. Patients

Quality of life

Our group was the first to describe the impact of frequent exacerbations on quality of life (Seemungal et al 1998). Seventy patients with moderate to severe COPD were followed with daily diary cards for one year. Patients recorded changes in their respiratory symptoms and their morning PEFR. Patient's quality of life was assessed by the St Georges Respiratory Questionnaire (SGRQ) which is a validated measure of health impairment in chronic airflow limitation. Patients with frequent exacerbations had significantly higher total SGRQ scores compared with patients with infrequent exacerbations. The mean difference for the total SGRQ score was 14.8, $p<0.001$, on a scale where a 4 unit change is considered clinically significant. The factors predictive of frequent exacerbations in this study were the presence of a daily cough, daily wheeze, daily sputum production and frequent exacerbations in the previous year.

Activity levels

As COPD becomes more severe patients become less mobile and they become housebound. This was demonstrated by Donaldson et al in a study of 147 patients followed for a median of 8 years with daily diary cards in our Cohort (Donaldson et al 2005). Patients recorded the time spent outdoors and completed the SGRQ annually. The time spent outdoors decreased by 0.16 hours/day per year and the decline was faster in patients with frequent exacerbations.

Lung function decline

Patients with frequent exacerbations have a faster decline in lung function (Kanner et al 2001, Donaldson et al 2002). The Lung Health Study (LHS) was a 5 year prospective, randomized multicentre clinical trial that followed 5,587 smokers with GOLD stage I & II COPD. It was designed to determine whether a smoking cessation intervention and regular use of a bronchodilator could slow FEV_1 decline. One of the reports from the LHS specifically examined the impact of exacerbations on lung function (Kanner et al 2001). Sustained quitters had fewer exacerbations than continuing smokers. Over 5 years, exacerbations had a significant effect on the rate of decline in FEV_1 in smokers. The occurrence of one exacerbation per year was

associated with an additional decline in FEV_1 of 7ml/year in smokers. Patients with more frequent exacerbations had greater declines in FEV_1. In another study from our Cohort, Donaldson et al showed that patients with frequent exacerbations had faster decline in FEV_1 (-40 ml/year) compared to infrequent exacerbators (-32ml/year) (Donaldson et al 2002). In this study, frequent exacerbators were also admitted to hospital more often and had a longer length of stay than infrequent exacerbators.

Airway inflammation

Our group has previously demonstrated that patients with frequent exacerbations have higher levels of airway inflammation in the stable state compared to those with infrequent exacerbations (Bhowmik et al 2000). In this study 57 patients with moderate to severe COPD were followed up with daily diary cards and had samples of blood and sputum taken in the stable state and at exacerbation. Exacerbation frequency was correlated with stable IL-6 and IL-8 levels. Infrequent exacerbators had median (95% CI) IL-6 and IL-8 levels of 22(12-93) and 1628 (607-4812) pg/ml versus frequent exacerbators who had IL-6 and IL-8 levels of 110 (11-215) and 6694 (3120-11995) pg/ml. The differences were statistically significant with $p<0.05$ for both.

Lower airway bacterial colonisation

In an analysis of 29 patients with moderate to severe COPD followed up for a median of 529 days, the colonisation by any possible pathogen was associated with an increased exacerbation frequency (Patel et al 2002).

Mortality

Patients with frequent exacerbations of COPD have higher mortality rates compared to those with infrequent exacerbations as demonstrated by a Spanish prospective observational study of 305 men with COPD followed over 5 years (Soler-Cataluna J et al 2005). In this study a multivariate analysis of survival was performed correcting for age, co-morbidity index, body mass index, severity of COPD and use of long term oxygen therapy. This study showed that patients with 3 or more exacerbations had a survival rate of 30% at 5 years compared to 80% for those without exacerbations. The survival rate was influenced by the need for recurrent hospital

admissions. Patients requiring readmission only had a 20% survival rate at 5 years compared to those with exacerbations not requiring admissions.

b. Society

COPD imposes a considerable burden on healthcare systems and society and exacerbations are the major contributor to this burden. The Confronting COPD Survey estimated the total cost of COPD at up to $ 4.119 per patient per year in North America and Europe (Wouters E 2003). Hospitalizations accounted for 54% of costs, medications accounted for 16%, unscheduled care accounted for 6%, investigations for 5% and oxygen therapy accounted for 3% of the direct costs. A review of data from the United States estimated the direct per patient costs for COPD to be in the range of $ 2,700 to $ 5,900 per year in 2005 (Foster et al 2006).

In the UK the direct cost of COPD to the healthcare system is estimated to be between £ 810-930 millions per year (NICE COPD Guidelines 2010). More than 50% of these costs are due to hospital admissions. COPD is among the most costly inpatient conditions treated by the National Health Service (NHS). Indirect costs are also substantial and incompletely quantified. COPD is estimated to cause 24 million lost working days per annum (NICE 2010).

A recent department of health analysis has estimated the direct cost associated with COPD by disease severity to be as follows:

GOLD Stage I: £120 - £130
GOLD Stage II: £270 - £290
GOLD Stage III: £910 - £980
GOLD Stage IV: £3,000 - £3,200

The estimated cost of an exacerbation in the UK in 2004 was (NICE 2010):

- £8 to £15 for a person with mild COPD
- £23 to £95 for a person with mild to moderate COPD
- £1,400 to £1,600 for a person with severe COPD

The National COPD Audit 2008 showed that COPD accounts for around 1.4 million consultations in general practice per year. In general, 74% of patients admitted to hospital with an exacerbation had made contact with their GP in the 4 weeks prior to admission and 31% had 3 or more contacts in the preceding 4 weeks.

Although only a small proportion of patients with COPD are admitted to hospital each year, 1/8 emergency admissions are now due to COPD and they account for more than one million bed days in the UK (NICE 2010).

1.2.5 Risk factors for exacerbation

A previous history of exacerbations is a consistent risk factor for further exacerbations. As the major costs related to COPD are due to hospital admissions with exacerbations, most of the data on risk factors for exacerbations come from studies examining risk factors for hospital admission. The different reports from the EFRAM study demonstrate that a history of frequent exacerbations, under prescription of long term oxygen therapy, reduced physical activity and lower FEV_1 are all risk factors for exacerbations of COPD and admission to hospital (Garcia Ameyrich et al 2000, 2001, 2003 and 2006).

The presence of increased fibrinogen level in the stable state has also been described as an independent risk factor for exacerbations in the subsequent year (Groenwald et al 2008). This study also found that a lower FEV_1 and a higher number of exacerbations in the previous year were risk factors for exacerbation.

1.2.6 Aetiology of exacerbations

COPD exacerbations are heterogeneous events likely reflecting the different aetiologies. An infection of the airways, either viral or bacterial is present in about 50-80% of exacerbations. Atmospheric pollution levels, temperature changes and pulmonary emboli have also been implicated as a cause of COPD exacerbations.

a. Role of bacteria

The role of bacteria during exacerbations of COPD was debated in the past due to the fact that the same bacteria were found in the airways of stable as well as exacerbated COPD patients. The fact that not all exacerbations responded to antibiotic therapy was another reason for the controversy. However over the last 20 years there have been significant improvements in our understanding of the role of bacteria in exacerbations of COPD. Up to 50% of exacerbations are bacterial in origin (Sethi et al 2002).

A more detailed analysis of the role of bacteria in COPD exacerbations will follow in section 1.3.3 .

b. Role of viruses

Respiratory viruses are an important cause of exacerbations of COPD.

Several studies have demonstrated that respiratory viruses are more frequently isolated from patients with exacerbations than from those with stable COPD, they are illustrated in **Table 5.** The table is the result of a literature search with the terms "viruses, COPD and exacerbation". The detection of respiratory viruses was by PCR, viral culture or serology.

Table 5: Studies detecting viruses at exacerbation of COPD

NPA=naso-pharyngeal aspirate, NPS= naso-pharyngeal swab, NL=nasal lavage, PBS=protected brush specimen, BAL=broncho-alveolar lavage

	Study setting	Samples taken	Virus detection by	n	Virus identified
Kherad O et al 2010	Hospital ED	NPS	PCR	86	51%
De Serres et al 2009	Hospital ED	NPA, Sputum	PCR	108	31%
Camargo et al 2008	Hospital ED	NPS	PCR	76	25%
Mc Manus et al 2008	Hospital IP	Sputum	PCR	136	37%
Ko et al 2007	Hospital IP	NPA	PCR	196	22.1%
Cameron et al 2006	ITU	NPA, NPS	PCR	105	43%
Papi et al 2006	Hospital IP	Sputum	PCR	64	23.5% and 25% Viral/bacterial
Wilkinson et al 2005	Outpatients	Sputum	PCR Rhinovirus	39	Rhinovirus 19.6%
Rohde et al 2003	Hospital IP	NL, sputum	PCR	85	56%
Bandi et al 2003	Outpatient	Sputum	PCR, serology, viral culture	24	46.7% 68.6% virus/bacteria
Tan et al 2003	Hospital IP	Sputum	PCR	14	64%
Aaron 2001	Outpatients	Sputum	PCR, serology, Viral culture	14	14% and 21% virus/bacteria
Seemungal et al 2001	Outpatients	NPA, NPS	PCR Rhinovirus Serology, culture	83	39.2% any virus , 58.2% Rhinovirus
Soler et al 1998	ITU	PBS, BAL	Serology	50	16%

The viruses most commonly detected at exacerbation of COPD are rhinovirus, influenza A & B (in non-vaccinated populations), coronavirus, parainfluenza virus and more rarely human metapneumovirus and bocavirus.

Respiratory syncytial virus (RSV) and Epstein Barr virus have both been detected in stable COPD and at exacerbation (McManus et al 2008a, Seemungal et al 2001, Wilkinson et al 2006 a). The recurrent isolation of RSV has been linked to faster FEV_1 decline (Wilkinson et al 2006 a).

Our group has previously described that the presence of a common cold at onset of an exacerbation is related to a prolonged recovery time (Seemungal et al 2001). Patients with symptoms of a cold or a virus isolated from the airways at exacerbation have more severe exacerbations (higher drop in FEV_1) and higher levels of serum IL-6, fibrinogen and higher levels of sputum IL-6 (Seemugal et al 2001, Bhowmik et al 2000) than those without a cold/ virus in airway secretions. The isolation of both a virus and a bacterium at exacerbation is associated with more severe exacerbations in terms of drop in FEV_1 and airway inflammation (Wilkinson et al 2006 b, Papi et al 2006).

c. Role of pollution

There is epidemiological evidence of a link between air pollution and admission to hospital with respiratory diseases (Dominici et al 2006) and in particular COPD (Ko et al 2007, Arbex et al 2009).

In the study by Ko et al the relationship between levels of ambient air pollutants (sulphur dioxide (SO_2), nitrogen dioxide (NO_2), ozone (O_3), particulates with diameter <10µm (PM_{10}) and 2.5 µm) and hospital admission for COPD was assessed retrospectively in 15 hospitals in Hong Kong between 2000 and 2004. In a multi-pollutant model, O_3, SO_2 and $PM_{2.5}$ were significantly associated with increased admissions for COPD. SO_2, NO_2 and O_3 had a greater effect on COPD admissions in the cold season than during the warm season.

In the second study the relationship between the daily number of emergency visits for COPD and the environmental air concentrations of PM_{10}, SO_2, NO_2, carbon monoxide and ozone were studied in the City of Sao Paulo, Brazil between 2001 and 2003. PM_{10} and SO_2 readings showed both acute and lagged effects on COPD emergency department visits (Arbex et al 2009).

In vitro studies show that exposure of alveolar macrophages to atmospheric pollution increases their phagocytic activity, oxidant production and release of pro-inflammatory cytokines such as IL-8 and TNF-α (Ling et al). This increase in airway inflammation could contribute to the epidemiological link between air pollution and exacerbations of COPD.

d. Role of temperature

Exacerbations are more common during the winter months in the Northern hemisphere. The higher prevalence of viral infections may be one of the reasons. In a study of 76 patients with moderate to severe COPD from our cohort, Donaldson et al demonstrated that a fall in outdoor temperature was associated with increased number of exacerbations (Donaldson et al 1999).

e. Venous thrombo-embolism

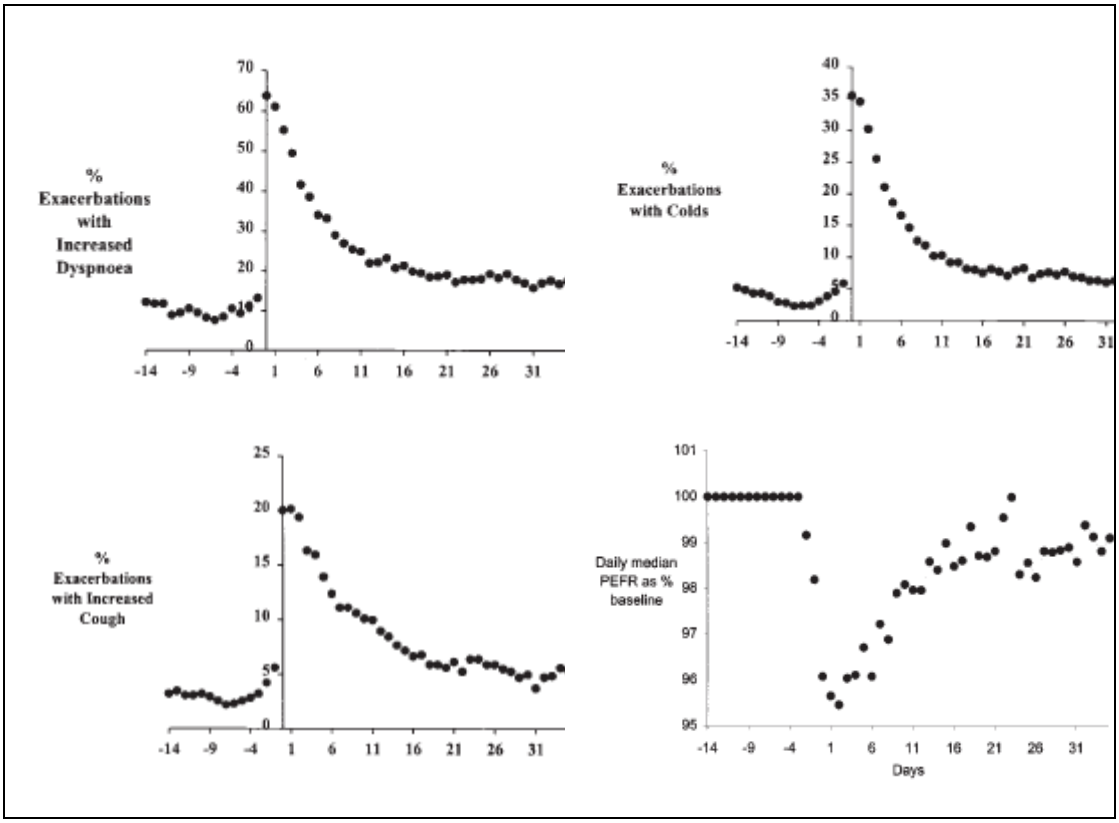
A recent systematic review and meta-analysis found that up to 25% of patients who require admission with symptoms of an exacerbation of COPD may have a pulmonary embolism (Rizkallah et al 2009). Patients with COPD exacerbations have increased systemic inflammation and reduced mobility, both could contribute to the increased risk of thrombus formation.

1.2.7 Time course of symptoms at exacerbation

Our group was the first to prospectively assess the time course of symptoms during an exacerbation of COPD (Seemungal et al 2000). One hundred and one patients were asked to record any change in the following respiratory symptoms on daily diary cards: dyspnoea, sputum colour, sputum volume, common cold, cough and wheeze or chest tightness. They were also asked to contact the study team when they noticed any change in their symptoms. The patients were followed for 2.5 years and 504 exacerbations were identified from diary cards. Only 50% of the exacerbations were reported to the study team or other health care professional. The respiratory symptoms worsen in the 7 days prior to the onset of the exacerbation, then on the day of onset there is a sudden increase in symptoms and drop in peak expiratory flow rate

(PEFR). Symptoms generally recover in median (IQR) 7 (4-14) days and PEFR recovers in 6 days (1-14). However, in this study 25% of patients had not recovered to baseline 35 days after the onset of the exacerbation. See **Figure 2.**

Figure 2: Symptoms and PEFR time course during an exacerbation of COPD (from Seemungal et al 2000)



1.2.8 Airway and systemic inflammation at exacerbation

Airway inflammation can be assessed non-invasively by measuring inflammatory cells and cytokines in the sputum. Our group has previously demonstrated that there is no difference between the cytokines levels and total cell count measured in induced and spontaneously expectorated sputum (Bhowmik et al 1998). However, induced sputum has a higher proportion of viable cells (Bhowmik et al 1998).

Patients with COPD have higher airway and systemic inflammation in the stable state compared to healthy controls. Several studies have demonstrated a further

increase in these inflammatory markers at exacerbation. These studies are summarized in **Table 6**.

Table 6: Changes in airway and systemic inflammatory markers at exacerbation of COPD

Paper	Increased at exacerbation	Unchanged at exacerbation	Design	Patient number	Comments
Bathoorn et al 2009	LTB4,MCP-1, ECP	IL-8, IL-6, MPO, TNF-α	Matched	45	
Sethi et al 2008	IL-8, NE, TNF-α		Matched	65	148 exacerbations in 65 patients
Hurst et al 2006	MPO	IL-6, IL-8	Matched	41	
Papi et al 2006	ECP				Exacerbation-convalescent samples
Wilkinson et al 2006	IL-8		Matched	39	
Tsoumakido et al 2005	IL-8, MPO		Matched	12	
Fujimoto et al 2005	ECP, elastase, IL-8, RANTES		Matched	30	
White et al 2003	Elastase	IL-8, LTB4, MPO	Matched	40	Baseline was a convalescent samples

Gompertz et al 2001	Elastase, LTB4, MPA	IL-8	Matched		Baseline was a convalescent samples
Aaron et al 2001	IL-8, TNF-α	MPO	Matched	14	
Roland et al 2001	Endothelin-1	IL-6, IL-8	Matched	14	
Seemungal et al 2001	IL-6	IL-8	Matched	30	30 exacerbations in 22 patients
Crooks et al 2000	Elastase, IL-8, LTB4, MPO		Matched & Unpaired	6	
Bhowmik et al 2000	IL-6	IL-8	Unpaired	37	
Hill et al 2000 b	Elastase, IL-8, LTB4, MPO, α1-antitrypsin		Matched	11	α1-antitrypsin deficient patients

1.2.9 Time course of airway and systemic inflammation at exacerbation

The study by Seemungal et al 2000 described the time course of symptoms at exacerbation. Three studies describe the time course of airway and systemic inflammation at exacerbation (Crooks et al 2000, Gompertz et al 2001 and Aaron et al 2001). These studies will be described in detail here.

a.

This study included a small group of patients (n=8) with very severe COPD who presented a mean (range) of 12 (2-28) days after they first noticed an increase in their respiratory symptoms (Crooks et al 2000). Their mean (range) exacerbation frequency in the 12 months prior to the sampled exacerabation was 4.6 (0-10). The 8 study patients had a mean (SEM) FEV_1 of 0.65 (+/- 0.09) litres and were all admitted to hospital with an exacerbation of COPD. An exacerbation was defined as an increase in dyspnoea and/or cough accompanied by an increase in sputum purulence, together with a positive bacterial culture from the sputum. All patients were treated with antibiotics. Patients were sampled on admission prior to antibiotics, after 3, 5, 14 days and when clinically stable. Sputum samples were taken at each visit and assessed for the levels of MPO, elastase activity, chemotactic activity, IL-8 and LTB-4 were. Systemic inflammation was assessed by measuring CRP and serum α1-proteinase inhibitor. All indices of airway and systemic inflammation were higher at presentation and decreased significantly by day 3 and remained low until they were seen in the stable state. No precise information was given as to when the stable state sample was taken.

b.

This study included 69 patients with chronic bronchitis and a clinical diagnosis of COPD who presented to their primary care physician with an exacerbation (Gompertz et al 2001). An exacerbation was defined as a combination of worsening respiratory symptoms (breathlessness, sputum volume, colour or viscosity, chough, wheeze or chest pain) with or without systemic symptoms (fever, malaise, rigors). Patients with purulent sputum were treated with antibiotics and those with mucoid sputum were treated with inhaled corticosteroids. Patients had sputum and blood

samples taken at presentation prior to antibiotics, after 5 days, at end of treatment (10 days for patients on ICS and 14 days for patients on antibiotics) , after 28 days and after 56 days. Sputum levels of MPO, NE, IL-8, LTB-4 were measured as well as serum CRP. Quantitative sputum cultures were also performed. Patients were grouped according to their sputum appearance into mucoid bronchitics and purulent bronchitics. Purulent bronchitics were more likely to grow a PPM, have higher bacterial loads and higher airway and systemic inflammation at presentation. The mucoid bronchitics showed little change in their inflammatory markers throughout the study. The purulent bronchitics had a significant drop in airway inflammation by day 5 but there was a wide variation in the range of values observed. The patients mean exacerbation frequency and the duration of symptoms prior to presentation were not recorded.

c.

Fifty patients with a mean FEV_1 of 39% predicted were recruited in the stable state and followed up prospectively for 9 to 15 months (Aaron et al 2001). An exacerbation was diagnosed according to criteria from Anthoniesen (Anthoniesen et al 1987). Patients were treated with antibiotics, oral steroids or both. They were sampled in the stable state, at exacerbation and one month after. Induced sputum samples and blood samples were taken at each visit. Viruses were detected by PCR and cultures in the sputum and serology. Quantitative bacterial cultures were performed and an exacerbation was deemed to be bacterial if a new organism was isolated at exacerbation. Airway inflammation was measured by IL-8, TNF-α and MPO. Systemic inflammation was not assessed. Only 14 patients experienced an exacerbation during the study period. All indices of airway inflammation increased significantly between baseline and exacerbation. After one month all indices had returned to baseline levels. One patient had a new bacterium in his sputum at exacerbation and two patients had evidence of a viral infection by both PCR and serology. The three patients with documented infection did not have significantly higher levels of airway inflammation at exacerbation compared to those without infection. The authors concluded that COPD exacerbations are associated with neutrophilic inflammation that resolves after one month and that this inflammation was independent of an acute infection.

All three studies confirm that exacerbations are associated with increases in neutrophilic airway inflammation that returns towards the baseline levels after about 5 days. If another exacerbation does not occur, airway inflammation remains low up to 30-56 days after the exacerbation. These studies are quite heterogeneous. The duration of symptoms prior to sampling and the exacerbation frequency are only documented in one of the studies. The first two studies only included patients with chronic bronchitis. The baseline sample was also taken after the exacerbation in both of them. In the third study, a bacterial aetiology was only considered if a new organism was isolated at exacerbation; the baseline airway colonization rate is not documented. The definition of exacerbation is also variable among these studies.

1.2.10 Severity of exacerbation

The severity of an exacerbation can be assessed in the following ways:

- counting the total number of symptoms present at exacerbation
- quantifying the deterioration in lung function parameters (FEV_1, PEFR)
- the need for additional treatments: antibiotics, steroids, both, oxygen
- quantifying the intensity of the airway and/or systemic inflammation
- the need for admission to hospital
- patient death

Different studies have used different combinations of the above to describe the severity of an exacerbation. In clinical practice it is difficult to disentangle the severity of the exacerbation from the severity of the underlying COPD and we usually talk about the severity of the presentation.

1.2.11 Recovery

An exacerbation is considered to have recovered when the patient's symptoms and/or lung function have returned to their pre-exacerbation levels. Our group has previously demonstrated that in 25% of exacerbations PEFR does not return to baseline after 5 weeks (Seemungal et al 2000). The reasons for this non-recovery are

unknown. The use of oral steroids at exacerbation is associated with a faster recovery in lung function and less treatment failures (Davies et al 1999, Niewohner et al 1999).

1.2.12 Recurrences

COPD exacerbations are often recurrent as demonstrated by several studies. However, the underlying mechanisms leading to recurrences are unknown.

The 2003 UK National COPD audit analysed data on 7529 patients admitted to hospital with an exacerbation. Thirty one per cent were readmitted within 3 months with another exacerbation (Price et al 2006). The previous audit in 1997 had found that 34% of patients were readmitted within 3 months (Roberts et al 2002).

In a randomized double blind placebo controlled trial of outpatient oral Prednisolone in the treatment of exacerbations of COPD, 27% of patients in the active drug group required another physician visit within 30 days for worsening symptoms (Aaron et al 2003).

In a study from our cohort, 297 patients were assessed for 904 patient-years. The timing of a second exacerbation after an initial exacerbation was compared with that expected should exacerbations occur randomly (Hurst et al 2009) by using a mathematical model. Twenty seven per cent of exacerbations were followed by a recurrent exacerbation within 8 weeks. The study showed that exacerbations are not random events and that they cluster together and the highest risk period is the 8 weeks immediately after the initial exacerbation.

1.2.13 Prevention of exacerbations

There are effective treatments that can reduce exacerbations. **Table 7** shows pharmacological agents that prevent exacerbations. The exact mechanism by which this reduction is achieved is not clear.

Pharmacological treatments

The ISOLDE study was the first long-term prospective double-blind placebo controlled study to demonstrate an effect of ICS in COPD (Burge et al 2000). The study included 751 patients with a mean (SD) FEV_1 of 50 (14.7) % who were followed up for 3 years. Exacerbations were a secondary outcome and were reduced by 25% in the treatment arm compared to the placebo arm. ISOLDE also showed that health status as measured by the SGRQ declined by 2 units/year in the ICS arm and by 3.2 unit/year in those in the placebo arm. It showed for the first time that treatment with ICS can modify the decline in health status (Spencer et al 2001 and 2004).

The Salmeterol versus-placebo arm of the TRISTAN (Calverley et al 2003 a) and TORCH (Calverley et al 2007) trials both show a reduction in exacerbation frequency of 15-25%. The role of combination therapy with ICS and LABA is now well established, they reduce exacerbation frequency by about 25%.

The long acting anti-muscarinic agent Tiotropium reduces exacerbation frequency by 14-20%. It is worth noting that in the UPLIFT study (Tashkin et al 2008), patients on the treatment arm were allowed to be on combination ICS-LABA.

Macrolides reduced exacerbation frequency by 35% in a randomized controlled study conducted by our group (Seemungal et al 2008).

Mucolytic agents have been shown to reduce exacerbation frequency in patients who are not on inhaled cortico-steroids (Zheng J et al 2008, Decramer M et al 2005).

Non-pharmacological therapies

The non-pharmacological interventions that reduce exacerbations and hospital admissions include pulmonary rehabilitation, vaccination and lung volume reduction surgery. A comparison of the exacerbation rate between the medically managed group and those undergoing LVRS in the NETT trial showed a 30% reduction in exacerbation frequency in the operated group (Washko et al 2008).

Table 7: Pharmacological agents that reduce exacerbations in COPD

Trial	Therapy	n=	% Reduction
UPLIFT	Tiotropium/placebo	5993	14%
TORCH	Salmeterol/placebo	1521	15%
TORCH	Fluticasone/placebo	1534	18%
TORCH	Combination/placebo	1533	25%
Casaburi	Tiotropium/placebo	921	20%
Vincken	Tiotropium/Ipratropium	535	20%
TRISTAN	Salmeterol/placebo	733	20%
TRISTAN	Fluticasone/placebo	735	19%
TRISTAN	Combination/placebo	719	25%
Szafranski	Budesonide-formeterol/placebo	413	24%
Calverley	Budesonide-formeterol/placebo	510	24%
ELECT	Erythromycin/placebo	109	35%
Rennard	Cilomilast/placebo	647	39%
Calverley	Roflumilast/placebo	3091	17%

1.3 ROLE OF BACTERIA IN COPD

In the fifties British investigators hypothesized that recurrent bronchial infections led some smokers to develop chronic airways obstruction (Fletcher et al 1977). The seminal study of Fletcher et al was designed to test this hypothesis. Their prospective observational study of working men in London measured the frequency of "chest illnesses", the daily respiratory symptoms of cough and sputum production and lung function over eight years. They studied the quality and quantity of sputum produced and measured the changes in FEV_1 of the subjects. They found that chronic cough, sputum production and recurrent "chest illnesses" did not correlate with lung function decline (Fletcher et al 1977). These findings led to the belief that chronic bronchitis resulted from an inflammatory response of the lung to chronic exposure to irritants. Cigarette smoke and air pollution were possible irritants and bacterial colonization was considered to be an epiphenomenon. Fletcher summarized this in his Philip Ellman lecture at the Royal Society of Medicine in May 1965 as follows:

> ***Pathogenetic Conclusions***
> **I have adduced evidence that air pollution and smoking may play a part, probably independently, in causing all three components of the bronchitis syndrome: hypersecretion, infection and obstruction. Infection by bacteria or viruses can apparently initiate prolonged hypersecretion and this may also increase susceptibility to infection so that a vicious circle may be started. Structural damage to the bronchi may eventually lead to persistent infection but this by itself carries no threat to survival. It is increase in airways resistance which eventually kills our patients. This I believe is neither a consequence of hypersecretion nor of bronchial infection but rather a primary reaction to environmental irritants in subjects whose bronchial reactivity is analogous to that of the asthmatic but different in that it is less obviously mediated through allergy and is much more persistent. In patients without airways obstruction recurrent exacerbations of infection have only a nuisance value, but in those with obstruction the increase of secretion and perhaps associated mucosal swelling may raise the resistance to air flow to dangerous levels and although it appears to be the infection that menaces life it is really the pre-existing obstruction from bronchitis or emphysema that is so dangerous.**

From Fletcher CM 1965.

Research conducted in the nineties renewed the interest in the role of bacteria in COPD pathogenesis and progression.

Five potential ways in which bacteria may contribute to COPD pathogenesis and progression were described by Sethi et al in 2001:

1. Recurrent childhood lower respiratory tract infections impair lung growth and lead to smaller lung volumes in adulthood.
2. Bacteria are the main cause of exacerbations of COPD and contribute to the morbidity and mortality of the disease.
3. The vicious cycle hypothesis: chronic colonization of the lower airways by bacteria amplifies the inflammatory response present in the COPD lungs and leads to progressive airway obstruction.
4. Bacteria invade and persist in the respiratory tissues and alter the host response to cigarette smoke. They can also induce a chronic inflammatory response and thus contribute to the pathogenesis of COPD.
5. Bacterial antigens induce hypersensitivity in the lower airways.

The three organisms most commonly isolated from patients with COPD are: Haemophilus influenzae, Streptococcus pneumoniae and Moraxella catarrhalis. Other frequently isolated pathogens include Haemophilus parainfluenzae, Staphylococcus aureus, Psuedomonas aeruginosa and Entoerobacteriacaeae. These organisms are commonly referred to as potentially pathogenic micro-organisms (PPM) (Sethi et al 2000). The bacteria that colonize the airways of COPD patients are less virulent, in the usually accepted sense of the word, than those that cause invasive disease such as pneumonia (Wilson et al 1996). These organisms persist in the lower airways in a state of equilibrium where their number is contained but not eliminated by the host defences. However, this persistence is associated with higher airway and systemic inflammation.

1.3.1 Lower airway bacterial colonisation in COPD

The lower airways of healthy non-smokers are usually sterile due to the innate lung defences. From the late fifties it was known that bacteria could be present in the lower airways of smokers in the absence of clinical symptoms of an infection (Fletcher CM 1965).This phenomenon is called lower airway bacterial colonization. The major papers on lower airway bacterial colonization in COPD relevant to this thesis are listed in **Table 8** and their main findings will be discussed in the next section.

a.

In the first study, 40 patients with an FEV_1 of 51% predicted had bronchoscopies and protected brush specimens (PBS) taken whilst in the stable state (Monso et al 1995). Bacterial cultures were considered positive if there were 1000 or more colony forming units per millilitre (CFU/ml). Twenty five per cent of samples were positive and the most commonly isolated bacteria were H influenzae and S pneumoniae.

In the second study the authors used PBS in 41 patients with stable chronic bronchitis to determine the prevalence of lower airway bacterial colonization and the risk factors associated with it (Monso et al 1999). The cut-off 1000 or more CFU/ml was used to declare a sample positive. Most patients had mild to moderate COPD with FEV_1 74% +/- 23% predicted. Active smoking was found to be a risk factor for lower airway colonization. H influenzae was again the most prevalent organism in the PBS samples.

b.

This study included 88 patients with stable COPD and 20 subjects with normal spirometry and chest x-ray (Zalacin et al 1999). All had bronchoscopies and PBS taken, cultures were considered positive if 1000 or more CFU/ml were present. There was significant bacterial growth in 40% of the patients and in 0% of the normal subjects. The most common organisms were H infulenzae, S viridans, S pneumoniae and M catarrhalis. In a multivariate analysis, severe airflow limitation and current smoking were associated with a positive culture. If only potentially pathogenic micro-organisms were considered, a significant bacterial growth was found in 30.7% of patients and severe airflow obstruction was the only associated risk factor.

Table 8: Papers on lower airway bacterial colonization in COPD

Paper	Samples analysed	n=	Colonization rate	Comments
Monso et al 1995	PBS	40	25%	
Monso et al 1999	PBS	41	22%	
Zalacin et al 1999	PBS	88	40% or 30.7%	30% if only PPM
Soler et al 1999	PBS & BAL	52	34.3%	33% if smokers and COPD patients counted together
Hill et al 2000	Sputum	160	74%	55 COPD & normal α1-antitrypsin level, 62 COPD with low α1-antitrypsin, 43 idiopathic bronchiectasis
Bandi et al 2001	PBS, BW & Sputum	23	9%, 18%, 35%	Detection rate of H influenza only
Patel et al 2002	Sputum	29	51.7%	
Wilkinson et al 2003	Sputum	30	53% and 56.6%	Sampled one year apart
Banerjee et al 2004	Sputum	67	40%	
Sethi et al 2006	BAL	26	34.6%	
Miravitlles et al 2009	Sputum	119	33.6%	
Marin et al 2010	Sputum	79	53.2%	

PBS= protected brush specimen, BW= bronchial wash, BAL= broncho-alveolar lavage.

c.

This study included 8 non-smokers, 18 smokers with normal lung function and 52 patients with COPD (28 mild, 11 moderate and 13 severe) (Soler et al 1999). All patients underwent bronchoscopy and had PBS and BAL samples taken. Quantitative cultures were done, samples were considered positive if 1000 or more CFU/ml were present in BAL and 100 or more CFU/ml were present in PBS samples. Among the COPD patients, a PPM was isolated in 34.6% of the cases. The presence of a PPM was associated with higher BAL neutrophil count and TNF-α concentration.

d.

This study tested the hypothesis that the number of organisms and bacterial species present in the lower airways modulate airway inflammation (Hill et al 2000). The study included 55 patients with COPD without α1-antitrypsin deficiency, 62 patients with COPD and α1-antitrypsin and 43 patients with idiopathic bronchiectasis. Patients provided up to 4 samples of spontaneously produced sputum whilst in the stable state. Seventy four per cent of sputum samples grew $\geq 10^5$ CFU/ml of bacteria. Airway inflammation was assessed by measuring IL-8, LTB4, myeloperoxidase and neutrophil elastase levels in the sputum. The sputum/serum albumin ratio was also assessed. The airway bacterial load correlated with sputum myeloperoxidase, IL-8, leukotriene-B4 levels and sputum neutrophil elastase activity. The type of bacteria isolated was also directly related to the degree of airway inflammation, P aeruginosa was associated with more airway inflammation than H influenzae, which in turn caused more airway inflammation than M catarrhalis. This study demonstrated a significant linear relationship between the lower airway bacterial load and the degree of airway inflammation.

e.

This study included patients with stable chronic bronchitis (n=23), normal subjects (n=26) having general anaesthesia for an operation and patients with exacerbations of chronic bronchitis requiring intubation and mechanical ventilation (n=15) (Bandi et al 2001). All subjects had samples of PBS, BAL and sputum sent for quantitative bacterial cultures. Samples were considered positive if more than 10 CFU/ml were isolated. Patients with stable COPD had H influenzae in 26% of specimens collected at bronchoscopy (PBS & BAL) and in 35% of sputum samples. The control subjects

did not grow any bacteria in the sputum or in the bronchoscopy specimens. In the patients with H influenza isolated, strain typing revealed the presence of multiple strains. Sixteen of the chronic bronchitis patients had a second bronchoscopy a mean of 6.4 months later; H influenza was isolated from bronchoscopy samples in 50% of samples. Strain typing revealed the same strains and new strains on the repeat bronchoscopy. This study demonstrated that the lower airways of patients with chronic bronchitis are frequently colonized by multiple strains of H influenza and the colonization is a dynamic process.

f.

Twenty nine patients with severe COPD (mean FEV_1% predicted 38.7) provided sputum samples at least 3 weeks away from an exacerbation (Patel et al 2002). One or more PPM was isolated from 51.7% of the patients. The most common pathogens were H influenzae (53%), S pneumoniae (33%), M catarrhalis (20%), H parainfluenzae (20%) and P aeruginosa (20%). Colonisation by any PPM was associated with a higher exacerbation frequency. Colonisation by H influenzae was associated with a higher total symptom count and more purulent sputum at exacerbation. This study demonstrated that lower airway bacterial colonisation in stable COPD modulates the character and the frequency of COPD exacerbations.

g.

Thirty patients with severe COPD (mean (SD) FEV_1 34.8 % +/- 13.6%) had sputum samples taken whilst in the stable state on two separate occasions one year apart (Wilkinson et al 2003). The study was prospective, quantitative and qualitative bacterial cultures were performed and lung function was monitored during the study period. The FEV_1 declined by 57.6 ml per year. All samples grew bacteria, total loads increased significantly from $10^{7.47}$ to $10^{7.93}$ CFU/ml after one year (p=0.01). FEV_1 decline was related to the increase in bacterial load. The FEV_1 decline was greater in patients who exhibited a change in the colonizing bacterial type compared to those with persistence of the same organism. This study showed a direct relationship between lower airway bacterial colonization and FEV_1 decline.

h.

This study included patients with severe COPD and assessed the impact of lower airway colonization by a PPM on airway inflammation, systemic inflammation and health status (Banerjee et al 2004). Sixty seven patients with a $FEV_1 \leq 50\%$ predicted had induced sputum and blood samples taken whilst in the stable state. Health status was assessed with the St George's Respiratory Questionnaire (SGRQ) and the 36-item Short-Form Health Survey questionnaire. Forty per cent of patients were colonized by a PPM. Patients colonized by a PPM had higher airway inflammation as measured by sputum IL-8, leukotriene-B4, tumour necrosis factor-α, neutrophil elastase levels and had increased neutrophil chemotaxis. They also had higher levels of plasma fibrinogen and worse health status compared to patients not colonized by a PPM. This study demonstrated that colonization by a PPM is associated with a worse health status and higher airway and systemic inflammation.

i.

This study included 26 ex-smokers with stable COPD, 20 ex-smokers without COPD and 15 healthy non-smokers (Sethi et al 2006). All subjects underwent bronchoscopy and had BAL samples taken for quantitative bacteriology and measurement of airway inflammatory markers. A PPM was isolated at a load ≥100 CFU/ml in 34.6% of the COPD patients. COPD patients colonized by a PPM had significantly higher neutrophil count, IL-8 levels, active metalloproteinase-9 levels and endotoxin levels in the BAL than non-colonized COPD patients. This study showed that lower airway bacterial colonization in ex-smokers with stable COPD is associated with neutrophilic airway inflammation.

j.

This cross-sectional study included 119 patients with a moderate to severe COPD who provided sputum samples whilst in the stable state for quantitative and qualitative bacterial cultures (Miravittles et al 2010). Patients' daily respiratory symptoms, smoking history, sputum colour and exacerbation frequency were also assessed. Bacterial colonisation was defined as the presence of a PPM at a load $\geq 10^2$ CFU/ml of sputum. The bacterial colonization rate was 48.7% in these patients; more than 80% of patients with dark yellow to greenish sputum samples grew a PPM compared with 5.9% of patients with white sputum samples.

In a multivariate analysis increased levels of dyspnoea and a darker sputum colour were associated with the presence of PPM's in the sputum.

k.

This study included 40 patients with moderate COPD who were infrequent exacerbators with no previous hospital admissions with COPD exacerbations (Marin et al 2010). Patients were sampled in the stable state on two separate occasions 8 months apart. Quantitative and qualitative sputum cultures were performed, strain typing of the PPM was performed with pulse field gel electrophoresis to ascertain if new strains were being acquired or the same strains were persisting. Airway inflammation and lung function indices were measured. A PPM was isolated in 53.2% of the baseline samples and 70.9% of the follow-up samples. The principal organisms isolated were H influezae, P aeruginosa and enterobacteria. Colonisation by these organisms was associated with sputum neutrophilia. For H influenza only, a relationship was observed between the bacterial load and airway inflammation. Long term persistence of the same strain was observed in 15.2% of the cases and was mainly due to P aeruginosa and enterobacteria.

1.3.2 Mechanisms involved in bacterial colonization

The lower airways are usually kept sterile by the coordinated efforts of the innate and adaptive immune responses. The main components involved are:

- The mucociliary escalator
- Protein components of the innate lung defences: lysozyme, lactoferrin, defensins, secretory leukoprotease inhibitor, cathelicidin and pentraxin family
- Surfactant proteins
- Alveolar macrophages and dendritic cells
- Airway epithelium and all the mediators and antimicrobial substances it produces
- The pattern recognition receptors

- Adaptive immune response: B cells, plasmocytes, T cells, bronchus associated lymphoid tissue and lymphoid follicules

Bacteria have developed multiple strategies to evade lung defences. They are presented in T**able 9.**

Table 9: Bacterial strategies to evade clearance from the airways (From Wilson et al 1996)

Bacterial exoproducts impair mucociliary clearance	**Stimulate mucus production** **Slow and disorganize ciliary beat** **Affect ion transport** **Damage epithelium**
Bacterial enzymes break down local immunoglobulins	**Proteases cleave antibodies to create non-functional or "blocking" antibodies** **IgA proteases**
Bacterial exoproducts alter immune effector cell function	**Neutrophils: inhibit chemotaxis and phagocytosis, enhance oxidative metabolism** **Lymphocytes: impair cytokine production, activate suppressor T cells** **Macrophages: reduce viability**
Bacterial adherence to epithelium	**Increased by certain environmental factors and in certain disease states** **Increased by damaged epithelium** **Increased as not cleared from secretions** **Enhances the effect of toxins released in the epithelial microenvironment** **Increases the availability of nutritional factors for bacterial growth**
Bacteria avoid immune surveillance	**Antigenic heterogeneity of the bacterial surface** **Growth of biofilms** **Microcolonies of bacteria surround themselves with a polysaccharide gel** **Endocytosis: bacteria hide within epithelial cells**

1.3.3 Role of bacteria at exacerbations of COPD

The role of bacteria at exacerbations of COPD was controversial for several reasons including the lack of a consensus definition of an exacerbation and the fact that the same organisms were isolated in stable and exacerbated COPD patients. Numerous antibiotic trials further clouded the issue with their design flaws and variable results. Most studies recruited the patients at exacerbation and followed them up until resolution and the stable state or baseline sample was a post exacerbation sample. Prospective studies in patients with well characterized COPD, a clear definition of an exacerbation and pre-exacerbation baseline sampling are scarce.

Recent studies with better design and newer diagnostic methods have established that bacteria are responsible for about 50% of exacerbations of COPD (Sethi et al 2008). Our group and others have demonstrated that exacerbations are characterized by an increase in the number of organisms present in the lower airways and an increase in airway and systemic inflammation. Other studies have linked exacerbations to a change in the type of organisms present or the apparition of a new strains of the same organism. We and others have also demonstrated that both bacteria and viruses can be isolated together at exacerbation. The reduction of airway inflammation associated with bacterial eradication after antibiotic treatment of exacerbations has also been described and favours a causal link.

Several studies have demonstrated the apparition of a strain specific immune response in the sputum and serum of patients with COPD which again favours a role of bacteria in exacerbations (Sethi et al 2003, Murphy et al 2005).

The next section with describe the most relevant of these studies.

a. Change in bacterial load

Three studies have reported a higher rate of isolation of bacteria & PPM in patients with exacerbations of COPD and one study showed no relationship between change in bacterial load and COPD exacerbations. These studies are presented in the following section.

Rosell et al reported data from a pooled analysis of 6 studies using PBS sampling of the lower airways. The study included 70 healthy volunteers, 181 patients with stable COPD and 86 patients with exacerbations. In patients with COPD, they found $\geq 10^2$ CFU/ml of PPMs in 29% of stable patients and 54% of exacerbated patients. Patients with exacerbations had significantly higher bacterial loads than patients in the stable state. The predominant organisms at exacerbation were H influenzae and P aeruginosa (Rosell et al 2005). Patients with more severe airway obstruction were more likely to be colonized than those with mild disease.

In a study of 69 patients with chronic bronchitis presenting with an exacerbation, patients were grouped according to the appearance of their sputum: mucoid or purulent. The mean FEV_1 of the group was 52% and they had samples of sputum and blood taken at exacerbation, after 5, 10-14, 28 and 56 days for analysis of inflammatory markers as well as quantitative bacterial cultures. At exacerbation 19% of mucoid samples and 90% of purulent samples had bacterial loads $\geq 10^7$ CFU/ml. At day 56 the majority of sputum samples were mucoid and only 24% of the purulent samples had high bacterial loads (Gompertz et al. 2001).

Our group prospectively studied 56 COPD exacerbations in 39 patients with moderate to severe COPD (mean (SD) FEV_1 % predicted 40.6(15.6)) (Wilkinson et al 2006 b). There was a significant rise in the total bacterial load and the prevalence of PPM rose from 48.2% to 69.6% between baseline and exacerbation.

In another prospective study of 104 patients during 81 months, patients were seen during 560 exacerbation visits and 2449 stable visits (Sethi et al 2008). Spontaneously produced sputum samples were used for quantitative bacteriology and strain typing.

The authors found that at exacerbation there was no significant increase in the load of pre-existing strains of H influenzae, there was a decrease in the pre-existing load of M catarrhalis and S pneumonia. They also found that the appearance of a new strain of H influenza and M catarrhalis was associated with a small increase in the load. However, they did not feel this increase was of significant magnitude to be the cause of an exacerbation.

b. Acquisition of new strains

One group has demonstrated a link between the acquisition of a new strain of the most common PPM and the occurrence of an exacerbation of COPD (Sethi et al 2002).

In a prospective study of 81 patients with COPD followed monthly and during exacerbations for a total of 56 months, Sethi et al performed molecular typing of sputum isolates of H influenzae, M catarrhalis and S pneumonia. A total of 1353 stable visits and 302 exacerbations visits were included in the analysis. Isolation of a new strain of any of the above organism was associated with a significant risk of an exacerbation. The relative risk (95% confidence interval) was 1.69 (1.37-2.09) for H influenzae, 2.96 (2.39-3.67) for M catarrhalis and 1.77 (1.14-2.75) for S pneumonia. Although not all exacerbations were due to a strain change and not all strain changes resulted in exacerbations; the authors concluded that the apparition of a new strain was more likely to lead to an exacerbation. This type of study is time consuming and laborious, therefore unlikely to be reproduced.

c. Co-infection of bacteria and viruses

Several studies have isolated both bacteria and viruses at exacerbation (Bandi et al 2003, Wilkinson et al 2006 b, Papi et al 2006, De Serres et al 2009). These exacerbations were characterized by a more severe exacerbation (higher drop in FEV_1 and higher symptom score) and were associated with higher airway and systemic inflammation.

d. Antibiotics at exacerbation and bacterial eradication

Exacerbations are associated with increased airway inflammation and frequent isolation of PPM. However, distinguishing colonization from acute infection can be clinically difficult. Despite that antibiotics are frequently prescribed at exacerbation of COPD and several meta-analyses have confirmed their utility in exacerbations associated with increased dyspnoea and sputum purulence (Ram et al 2006, McCrory et al 2001, Saint et al 1995).

A recent randomized controlled trial assessed the utility of Doxycycline in addition to systemic corticosteroids for exacerbations of COPD (Daniels et al 2010). They sampled 265 exacerbations in 223 patients; exacerbations were defined as increased dyspnoea and sputum volume with or without increased sputum purulence. The use of Doxycyline was associated with clinical success and cure at day 10, symptom resolution and with microbial response. The study did not detect any difference in recovery of lung function, resolution of systemic inflammation or time to treatment failure between the treatment groups. At day 30 there were no significant differences in clinical outcome between the 2 groups.

Another way to demonstrate a causal role of bacteria is to demonstrate a relationship between the reduction in airway inflammation and the success or failure of antibiotics to eradicate bacteria (White et al 2003). In this study 58 patients were sampled at exacerbation, after 10 days and 2 months. All exacerbations were treated with antibiotics. Airway inflammation was assessed by measuring IL-8, neutrophil elastase, LTB-4 and MPO. At exacerbation 48/58 patients had a PPM. At day 10, 41 of these patients were able to provide a sputum sample and after 2 months 46 patients provided a sputum sample. The patients who had eradicated the PPM by day 10 had significantly lower levels of MPO, IL-8, LTB4 and neutrophil elastase than those who had not eradicated the PPM. After 2 months, IL-8 was not significantly different in the two groups but the other three markers remained lower in patients who eradicated the PPM. Those with persistence of a PPM had higher airway inflammation.

1.3.4 Traditional methods for detection of bacteria and their limitations

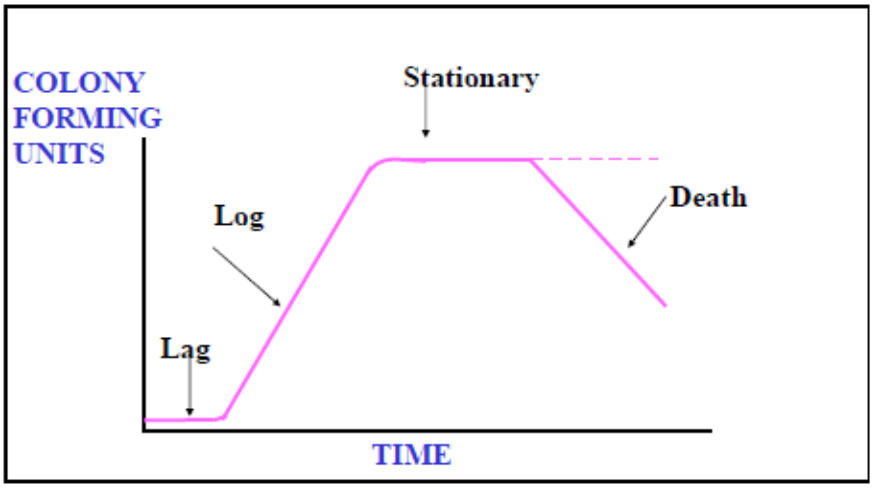
The traditional methods for detection of bacteria are microscopy, microbial cultures and serology. These techniques are relatively cheap to perform and validated protocols are available for the isolation of organisms involved in COPD. However,

their sensitivity can be poor and they are time consuming compared to non-culture methods.

Microscopy usually requires sample preparation and staining according to validated protocols. The morphology, size and grouping pattern of the organisms help identification. The detection rate may be limited by the quality of the sample, the preparation method, the number of organisms present and the ability of the observer to recognize the organism.

Microbial cultures rely on the principle that the microbe is separated from the bacterial milieu and placed on an in vitro milieu able to selectively support its development. This in vitro milieu has to provide essential nutrients, the correct atmosphere (i.e.: aerobic, anaerobic, rich in CO2), the optimal temperature and pH necessary for the multiplication of the desired organism. The physiological state of the bacteria when separated from its milieu can influence the growth. A prior knowledge of the rate of growth of the organism is essential for its optimum identification as all organism go through a phase of adaptation to the new milieu, a logarithmic multiplication phase, a growth stabilisation state and a cell death phase when the nutrients run out which is represented in **Figure 3.**

Figure 3: Growth curve of bacteria in a petri dish



There is a defined window for the detection of each bacterium. Once growth has occurred on the culture medium, pure colonies of the organism are used to determine

individual species by their morphology and metabolic characteristics. Routine microbial culture and identification uses standardized protocols.

The sensitivity and specificity of microbial cultures can be limited due:

- quality of the sample
- culture media used
- type of organism
- rate of growth
- the narrow detection window
- prior use of antibiotics
- contamination
- presence of multiple organisms.

Studies of dental plaque have demonstrated that only about 50% of the isolated organisms can be cultured (Rogers et al 2009). In otitis media with effusion an organism can be isolated by standard cultures in only20-30% of patients (Hendolin et al 2000).
In community acquired pneumonia, the detection rate of the responsible organism from sputum samples is poor and most clinical guidelines do not recommend it for mild disease.

Serology detects the presence of antibodies specific to a certain organism in the serum of an individual exposed to that organism in the past. The need for an acute and convalescent sample is one of its main disadvantages.

1.3.5 Non-culture based methods for detection of bacteria

The detection of microbes by non-cultured based methods has been expanding in the last 20 years. The advent of polymerase chain reaction (PCR) provided an ideal basis for the development of assays to detect differences in the micro-organisms DNA sequences (Rogers et al 2009). The PCR can amplify DNA or RNA when preceded

by a reverse transcription incubation sequence. It is the most commonly used nucleic acid amplification technique for the diagnosis of infectious disease (Mackay 2004).

PCR involves 3 essential steps:

- Double-stranded DNA is separated at temperatures ≥ 90°C.
- Oligonucleotide primers anneal at 50-75 °C
- Primer extension occurs at 70-78°C

In conventional PCR the amplified product or amplicon has to be detected by agarose gel electrophoresis, Southern blot or ELISA-like systems. These post PCR handling steps are laborious and the advent of real time PCR (rt-PCR) therefore was welcome.

Real time PCR instruments combine PCR chemistry with fluorescent probe detection of the amplified products in the same reaction vessel and both steps can generally be completed in an hour or less (Espy et al 2006).

PCR can be used to detect specific organisms if the genome of the organism is known or it can be used to identify regions of phylogenetically informative genes that are conserved across the domain Bacteria. The 16S ribosomal RNA gene is one such segment present in all members of the domain Bacteria. It contains both highly conserved and variable regions (Rogers et al 2009, b). Using the 16S rRNA technique on a clinical sample containing multiple species will lead to the detection of a mix of PCR products. These products can then be separated by techniques such as 16S rRNA gene sequencing, different types of electrophoresis or terminal restriction fragment length polymorphisms (T-RFLP) profiling (Rogers 2009, b). When researchers used the 16S RNA gene diversity to study the gut micro-flora, they discovered that more than 75% of the species detected in the human large intestine did not correspond to known cultured species (Rogers 2009, b). The use of 16S rRNA and T-RFLP to assess sputum samples of cystic fibrosis (CF) patients revealed 14 or more terminal restriction fragment bands corresponding to one or more bacterial species in each sputum sample. Eighty per cent of the five most common species detected in this way were not any of the five key CF species detected by microbial cultures (P aeruginosa, Burkholderia cepacia complex, Staphylococcus aureus, H influenzae and Stenotrophomonas maltophilia) (Rogers et al 2009, a).

PCR is more sensitive, specific and less time consuming than standard microbial cultures. The bacterial species identification is based on DNA sequencing and is thus more accurate. Another advantage is that nucleic acids can be directly extracted from the clinical sample prior to identification by PCR. The increased sensitivity and specificity allow detection of difficult to grow bacteria, those contained in samples collected after starting antibiotics and those transported in sub-optimal conditions. Multiplex PCR permits the simultaneous detection of several species. However, both single target PCR assays and multiplex assays both require predetermination of which bacteria are likely to be present in a given sample.

Some of commonly cited disadvantages of PCR include detection of bacteria at low levels deemed non-pathogenic, inability to distinguish live and dead bacteria, false positives due to carryover contamination and the costs involved with the instruments and reagents. Bacteria can develop mutations which may reduce the accuracy of the PCR.

1.4 ROLE OF DIFFERENT SUBSETS OF CIRCULATING LEUCOCYTES IN COPD

COPD is an inflammatory disease and the number of leucocytes present in a given sample of blood or sputum can be used to quantify the degree of inflammation present. Cigarette smoke is known to produce a 20-25% increase in circulating leucocytes compared to non-smokers (Van Eeden et al 2000). In epidemiological studies the number of circulating leucocytes has been linked to FEV_1, FEV_1 decline and respiratory and all-cause mortality (Sparrow et al 1984, Chan-Yeung et al 1988, Weiss et al 1995, James et al 1999).

Numerous studies report increased numbers of leucocytes in the airways, lungs tissue and peripheral blood of COPD patients. The neutrophils and lymphocytes play a major role in the pathogenesis and progression of COPD. The next section will be a brief overview of the role of circulating leucocytes in COPD.

1.4.1 Neutrophils

Neutrophils are part of the innate immune response and are produced in the bone marrow and released into the peripheral blood at a rate > $5\text{-}10 \times 10^{10}$ cells every day. The bone marrow can significantly increase this rate further if needed. The circulating half-life of neutrophils is short at about 6-8 hours. Cigarette smoke increases the number of circulating leucocytes but does also hasten the release of neutrophils from the bone marrow as evidence by the increased number of band cells, increased surface L-selectin expression and increased MPO levels in smokers compared to non-smokers (Van Eeden et al 2000). In the lungs neutrophils exit the bronchial circulation at capillary level (Cowburn A et al 2008). The main chemotactic agents in the COPD lungs are LTB-4 and IL-8. Other known pulmonary chemotactic agents are: C5a, CXCL1, CXCL5 and the elastase-α1-antitrypsin complex. The neutrophils are recruited from the bronchial circulation via a “rolling-tethering” process where adhesion molecules such as the selectins and integrins play

a vital role. Neutrophils contain numerous cytotoxic and immune regulatory molecules in their granules. However, they need to be primed prior to degranulation (Cowburn A et al 2008). Neutrophil elastase (NE) is a major component of the granules and is able to degrade many proteins including elastin, fibronectin, collaged, α1-antitrypsin and tissue inhibitors of matrix metalloproteinase. NE is able to produce emphysema in animal models of COPD.

Other activities of NE include:

- Activation of the airway epithelium
- Stimulation of TGF-β release from airway muscle cells
- Acting as a secretagogue for IL-8 and muc5A
- Ciliotoxic effect
- Cytotoxic effect

Neutrophils participate in the elimination of pathogens via the respiratory burst, via phagocytosis and by secreting neutrophil extracellular traps (Crowburn et al 2998).

Their broader role in the innate immunity has been discovered in recent years and includes:

- generation of CXCL10/interferon-γ-inducible protein 10 which acts as a chemo-attractant for activated natural killer and T-helper type 1 cells
- digestion and presentation of antigen fragments to dendritic cells
- trans-differentiation into longer-lived macrophage like cells

Resolution of neutrophilic inflammation is believed to occur mainly via apoptosis. A more recent theory is that there is egression of neutrophils across the bronchial epithelial layer into the airway lumen where they can be cleared through muco-ciliary clearance (Persson et al 2010).

Studies in patients with COPD have described the following changes in the circulating neutrophils:

- Down regulation of the expression of G protein subunit (Noguera et al 1998).
- Abnormal expression of adhesion molecules: L-selectin, lymphocyte function-associated antigen-1 and MAC-1 (Noguera et al 1998).
- Enhanced respiratory burst compared to smokers with normal lung function (Noguera et al 2001).

- Reduced spontaneous apoptosis during COPD exacerbations (Pletz et al 2004).

1.4.2 Lymphocytes

T lymphocytes play a strategic role in the immune response as they orchestrate the adaptive immune response. They are produced by the bone marrow and then travel to the thymus where they undergo positive and negative selection before maturation and release into the periphery as naive CD4 T lymphocytes, CD8 cytotoxic lymphocytes and γδ T lymphocytes. These cells are activated in peripheral lymphoid tissue when they encounter their corresponding bacterial/viral antigens presented by the antigen presenting cells. T cells then differentiate and proliferate as either memory T cells or effector T cells and migrate into specific organs. The activated effector T cells produce an array of cytokines that stimulate their own proliferation and orchestrate the adaptive immune response. T cells need to be stimulated to enter or home into an organ. T cells can be classified as having a type 0, I or II phenotype depending of the type of cytokines they secret. Type 1 T cells predominantly produce interferon γ, TNF β and IL-2 and type 2 T cells predominantly secret IL-4, IL-5, IL-10 and IL-13. About 5% of circulating T cells are γδ T lymphocytes, they play a key role in mucosal homeostasis and reparation after tissue damage. γδ T lymphocytes co-expressing the CD8 receptor have marked anti-inflammatory effects and exert a suppressive effects on the cell mediated immunity (Pons et al 2005).

Numerous studies have described the role of lymphocytes, in particular T cells, in the COPD airways and lung parenchyma. However, studies describing the circulating lymphocytes have included very different groups of COPD patients and the results are thus difficult to compare.

The following section is a summary of the results of a literature search using the terms COPD and T cells:

- Higher percentage of CD8+ T lymphocytes in non-smoking COPD patients
    - (De Jong et al 1997)
- Higher CD4:CD8 ratio is associated with higher %FEV_1 in non-smoking

  - COPD patients (De Jong et al 1997).
- In non-smoking COPD patients a higher percentage of B cells was associated with a higher %FEV_1 (De Jong et al 1997)
- Increased production of interferon γ and decreased production of IL-4 by circulating CD4+ T cells in COPD patients compared to control subjects, no difference in cytokines produced by CD8+ T cells (Majori et al 1999)
- Correlation between CD4+/CD8+ ratio and FEV1/FVC ratio (Kim et al 2002)
- Higher CD8+ T cells in patients with COPD and normal DLCO/VA (Kim et al 2002).
- Increased apoptosis of circulating T cells of COPD patients compared to controls (Hodge et al 2003).
- Increased apoptosis of circulating T cells and decrease in Bcl-2 and IL-7 (=anti-apoptotic proteins) in COPD patients treated with inhaled corticosteroids (Hodge et al 2005).
- Increased γδ T lymphocytes in smokers with preserved lung function and blunted γδ T lymphocytes in COPD patients (Pons et al 2005).
- Increased proportion of circulating CD4 and CD8 cells expressing FAS receptor in COPD patients compared to healthy smokers and non-smokers (Domagata-Kulawik et al 2006). FAS bearing cells can to undergo apoptosis.
- BAL T cells in COPD patients produce more TH2 type cytokines than healthy controls, no such relationship is observed in peripheral blood (Barcelo et al 2006).
- COPD patients have higher absolute numbers of circulating CD8 cells than healthy non-smokers (Hodge et al 2007).
- Increased percentage of circulating CD8 cells producing TNF-α and INF-γ in COPD patients compared to healthy non-smokers (Hodge et al 2007).
- Increased CD8 cells in blood, BAL and bronchial brush of COPD patients compared to healthy non-smokers (Hodge et al 2007).
- Increased production of TNF-α by CD8 cells in blood, BAL and bronchial brush of COPD patients compared to non-smokers (Hodge et al 2007).
- Decreased percentage of circulating CD4 cells producing TGF-β in COPD patients and active healthy smokers compared to healthy non-smokers (Hodge et al 2007).

- Active smokers with COPD have increased %CD4 cells, increased CD4:CD8 ratio and decreased % CD8 cells in peripheral blood compared to smokers without COPD and healthy controls (Koch et al 2007).
- Active smokers with COPD have increased expression of $CXCR_3$ receptor on their circulating CD8 cells compared to smokers without COPD and healthy controls (Koch et al 2007). Activation of $CXCR_3$ increases the migration of CD8 cells into the lung.
- Smokers with and without COPD have more activated and cytotoxic effector CD8 cells in peripheral blood compared to non-smokers (Koch et al 2007).
- Increased recruitment of memory T cells into the lungs of smokers with and without COPD compared to non-smokers (Smyth et al 2007).
- Increased regulatory T cells in the lungs compared to peripheral blood in smokers with and without COPD compared to non-smokers (Smyth et al 2007).
- There was no difference in the circulating numbers of naïve and memory CD4 and CD8 cells between COPD patients, smokers with normal lung function and non-smokers. This was in contrast to the BAL fluid where COPD patients had higher naïve and memory CD8 cells compared to smokers without COPD (Barcelo et al 2008).
- Smokers with and without COPD had significantly lower percentages of regulatory CD8 cells in BAL than never smokers. No such relationship was seen in peripheral blood (Barcelo et al 2008).
- Decreased circulating CD4 cells in COPD patients compared with healthy non-smoking controls (Zhu et al 2009).
- Circulating CD8 cells of COPD patients produced more IL-4 and IFN-γ than those of healthy non-smoking controls (Zhu et al 2009).
- The percentage of circulating CD4 cells was related to $\%FEV_1$ (Zhu et al).
- The circulating CD4 cells of COPD patients predominantly produced TH1 type cytokines and the circulating CD8 cells produced IL-4.

These different studies evaluated different aspects of circulating T cells and they generally included small numbers of patients. The largest study included only 81 patients (Zhu et al 2009). Therefor they are difficult to compare.

1.5 CONCLUSION

COPD is an inflammatory disease with considerable morbidity and mortality. The disease imposes a high burden on society. The main risk factor for developing COPD is cigarette smoking. The disease is characterized by progressive airflow limitation and acute exacerbations which are responsible for most of the burden of the disease. Exacerbations are characterized by increases in airway and systemic inflammation that are heterogeneous. The aetiology of exacerbations is variable and may account for some of the heterogeneity observed. Infection plays a major role in COPD, and microbial cultures are currently the gold standard for detection of bacteria. The use of non-culture based detection of bacteria in COPD is uncommon in COPD and warrants further study.

The introductory chapter was a general overview of COPD and a literature review on the role of bacteria and leucocytes in COPD.