# Modulation of airway inflammation in COPD

Thesis submitted for the degree of Doctor of Medicine at
the University of Leicester by

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## Contents

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## Abstract

COPD is a preventable, treatable, slowly progressive condition, associated with an abnormally amplified inflammatory response, characterised by an accelerated decline in lung function, punctuated by exacerbations. Exacerbations cause considerable mortality, morbidity, and cost. Airway inflammation in COPD is mainly neutrophilic although eosinophilic airway inflammation plays an important role, particularly during exacerbations. Inflammation in foregut derivatives outside the lung may contribute to amplification of airway inflammation. I have shown that a management strategy aiming to minimise eosinophilic airway inflammation as well as symptoms is associated with a significant 62% reduction in the frequency of severe exacerbations of COPD. This strategy was associated with no overall increase in corticosteroid treatment; there was evidence that increased corticosteroid therapy was targeted to patients with eosinophilic airway inflammation and benefit was largely confined to these patients.

I have shown an association between the sputum differential neutrophil count and airway bacterial load, and showed that a one week course of Levofloxacin significantly reduced both the % neutrophil count and bacterial load in patients with stable state COPD and bacterial load > $10^6$ cfu/ml. I have shown that the prevalence of peptic ulcer disease increases progressively with increasing severity of COPD in miners with homogeneous risk factors for development of COPD, and that peptic ulceration was a strong and independent predictor of a low $FEV_1$ % predicted and $FEV_1$/FVC ratio. In another population I showed that H.Pylori seropositivity was more common in patients with COPD compared to healthy smokers matched for age and occupation.

We have shown that TREM-1 can be measured from induced sputum and is potentially a novel marker of bacterial infection and neutrophilic airway inflammation during exacerbations.

Further work is required to ensure that measurement and modulation of airway inflammation results in improved clinical outcomes, and is made more clinically viable.

# Acknowledgements

I left my clinical training post in September 2002 to start my research post. Despite being a competent clinician, I had little insight into what would lie ahead. I had very little experience with databases, statistics software, and my word processor skills were not the best. On a personal level, I had just become single and moved house.

At the time of writing this section of thesis I am now happily married with a 15 month old son. I have presented numerous abstracts from this thesis around the world, and I am currently attempting to publish the main study findings. Overall my research has gone well, and I am still involved in various research projects.

Despite some testing times, I have enjoyed this experience and would like to take this opportunity to thank certain individuals who have joined me during this journey. Firstly I would like to thank my research supervisor, Professor Ian Pavord for all his time and effort. He has provided me with considerable support and excellent guidance over the last few years. I would also like to thank Chris Brightling and Ruth Green for their help with various technical aspects of my research. I must say a big thank-you to our research nurses: Bev, Sue, and Maria, who it has been a privilege to work with. They have provided considerable time, effort, and humour. I must thank all the laboratory personnel, in particular Will Monteiro and Debby Parker. Finally I wish to thank members of my family for their continuing support.

## Statement of work personally performed

I personally designed all the studies featured in this thesis. The study entitled "Modulation of eosinophilic airway inflammation and exacerbations of COPD: a randomised controlled trial" was co-designed by me. I obtained ethical approval for all studies. I was responsible for patient recruitment in all studies and personally obtained written informed consent from all patients. I personally attended around 95% of routine and emergency visits by patients and assessed clinical parameters in conjunction with specialist research nurses. I undertook approximately 50% of the processing of bacterial specimens, with all other laboratory work being carried out by dedicated laboratory technicians. I designed the database for each study and was responsible for all data entry. I personally analysed the data with some help from experts in statistics. I prepared all papers and abstracts for submission which have all been edited by my research supervisor.

## Publications arising from this thesis

Recent insights into the relationship between airway inflammation and asthma.

**R Siva,** M Berry, I D Pavord.

Monaldi Arch Chest Dis 2003; 59: 269-299

Multi-dimensional phenotyping: towards a new taxonomy for airway disease.

A J Wardlaw, M Silverman, **R Siva,** I.D Pavord, R Green.

Clin Exp Allergy 2005; 35:1254-1262.

Prednisolone response in patients with COPD.

I D Pavord, **R Siva,** C E Brightling.

Thorax 2004; 59(2): 179.

COPD: an inhaled corticosteroid resistant, oral corticosteroid responsive condition.

S Saha, **R Siva,** C E Brightling, I D Pavord.

Eur Respir J 2006; 27: 1-2.

H.Pylori serology in patients with COPD.

**R Siva,** K Mohan, M Berry, S S Birring, I D Pavord

Abstract Am.J Respir Crit Care Med. April 2004; vol 169, 7, A842

Peptic ulcer disease and COPD in Nottinghamshire miners.

**R Siva,** M Berry, R Green, I D Pavord

Abstract Am.J Respir Crit Care Med. April 2004; vol 169, 7, A617

Quantitative bacteriology, validation of a simple method.

**R Siva**, W Monteiro, R Free, M Barer, I D Pavord.

Abstract Eur Respir J September 2004; vol 24, supplement 48, 80s, P607

TREM-1 expression in exacerbations of COPD.

**R Siva**, W Monteiro, D Parker, C E Brightling, I D Pavord

Abstract Am.J Respir Crit Care Med. May 2005; vol 2, A406

Modulation of eosinophilic airway inflammation in COPD.

**R Siva**, R Green, C E Brightling, M J Shelley, I D Pavord

Abstract Eur Respir J September 2005; vol 26, supplement 49, 441s, P2830

Predictors of exacerbations of COPD.

**R Siva**, M Berry, C E Brightling, R Green, I D Pavord

Abstract Eur Respir J September 2005; vol 26, supplement 49, 284s, P1904

The effect of Levofloxacin on neutrophilic airway inflammation in stable state COPD; a randomised, double blind, placebo controlled trial.

**R Siva**, W Monteiro, D Parker, M Shelley, B Hargadon, S McKenna, C E Brightling, M Barer, I D Pavord.

Abstract Thorax December 2005; vol 60, supplement 2, P101

Relationship between exhaled nitric oxide and eosinophilic airway inflammation in COPD.

**R Siva,** W Monteiro, D Parker, M Shelley, B Hargadon, S McKenna, C E Brightling, I D Pavord.

Abstract Thorax December 2006

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# 1: Introduction

The Prevalence of Chronic obstructive pulmonary disease (COPD) varies between 2% and 10% depending on definition, method of diagnosis of the disease, and smoking habits of the population concerned (Pauwels et al. 2004). COPD is the fourth highest cause of mortality worldwide and over 30,000 patients die of the disease in the U.K each year (W.H.O report 2002). The mortality rate associated with COPD continues to rise in striking contrast to other common causes of mortality including ischaemic heart disease and stroke. By 2020 COPD will be the third highest cause of mortality and fifth highest cause of morbidity worldwide (Murray et al. 1997). Despite this COPD remains under-diagnosed and under-treated: 24 million Americans have evidence of impaired lung function but only about 10.5 million know they have COPD (Mannino et al. 2002) and around half of patients who have evidence of severe disease on spirometry, are not on any treatment for their airways disease (Pauwels et al. 2004). COPD also causes a huge burden on healthcare resources and the general economy: in the U.K the rate of general practice consultation for COPD is 10.3 per 1000 patients aged 75-84 per year, whilst in the U.S.A. COPD resulted in 8 million visits to doctors and outpatient departments and 1.5 million emergency department visits in 2000. The direct cost of COPD to the NHS is estimated to be around 0.8 billion pounds per year, whilst indirect costs including absence from work and reduced productivity are estimated at 1.7 billion pounds per year. In the U.S.A the total annual economic burden is estimated at 23.9 billion dollars.

COPD is a preventable and treatable, slowly progressive, chronic inflammatory condition, characterised by an accelerated decline in lung function. This decline in

lung function is not reversible and leads to the symptoms of cough, wheeze and breathlessness along with other systemic effects (Celli et al. 2004). The main aetiological agent associated with developing COPD is cigarette smoke and so far smoking cessation is the only intervention that has been proven to slow down this accelerated decline in lung function (Fletcher et al. 1977). Pharmacotherapy for COPD consists mainly of bronchodilator and anti-inflammatory medications which aim to alleviate symptoms, improve exercise capacity, improve quality of life, and reduce exacerbations.

Exacerbations are particularly important as they are responsible for considerable morbidity and mortality, as well as having a large impact on health care provision and cost (Pauwels et al. 2001). Several current therapies including inhaled corticosteroids and combination products are associated with modest reductions in exacerbation frequency. Exacerbations are associated with an increase in airway inflammation although the precise nature of this increase in airway inflammation and the extent to which it can be modulated is unclear. A better understanding of these processes should help us to develop new strategies which may result in a greater reduction in exacerbations of COPD.

Airway inflammation is at the heart of the pathophysiology of COPD, both during stable state disease and during exacerbations. Patients with COPD have been shown in numerous studies to have increased numbers of; macrophages, T-lymphocytes, neutrophils and eosinophils (Barnes 2003) with the exact pattern of inflammation in an individual patient varying according to their clinical state and the method of sampling of the airway. These cells together with their associated cytokines and

chemokines, are responsible for various different aspects of the disease, including decline in lung function and exacerbations. Consequently much interest has focussed on the ability to modulate airway inflammation and the results of doing so.

In this thesis I will describe how airway inflammation influences various aspects of COPD. Firstly, I will focus on eosinophilic airway inflammation. I will discuss the association between eosinophilic airway inflammation and exacerbations of COPD and how the presence of eosinophilic airway inflammation is associated with a response to corticosteroid therapy. After discussion of these associations, I will present a study in which we investigated the effect of a management strategy designed to reduce the sputum eosinophil count, as well as optimise symptoms, on the frequency of exacerbations of COPD. Secondly, I will focus on neutrophilic airway inflammation and discuss the association between neutrophilic airway inflammation and bacterial load of the airway, and present a randomised, double blind, placebo controlled study in which we used an antibiotic to investigate this relationship in more detail in patients with stable COPD. Thirdly, there is growing interest in the association between airway inflammation and extra pulmonary inflammation in foregut derivatives, such as the stomach, liver and thyroid gland. I will present data which supports an association between peptic ulcer disease, Helicobacter Pylori seropositivity and COPD, suggesting that there may be a theoretical benefit of suppressing extra- pulmonary inflammation.

I will also discuss how airway inflammation and airway bacterial load can be measured safely and non-invasively. I will then highlight the need for new markers of airway inflammation, particularly regarding markers of bacterial infection, which are

urgently needed to guide antibiotic therapy more objectively. This will include a pilot study looking at an interesting marker called TREM-1, a glycoprotein which shows increased expression on neutrophils during bacterial infection. I will conclude by summarising the results of my studies with the aim of demonstrating how the modulation of airway inflammation has the potential to influence both disease progression and exacerbation frequency and highlight areas where further work is needed in order to advance our management of COPD.

## 2: Role of airway inflammation in the development of airflow limitation

COPD is characterised by slowly progressive, non-reversible airway limitation which can be assessed during forced expiration. The volume of air that can be forcibly exhaled in one second ($FEV_1$) and its ratio to the total amount of air that can be forcibly exhaled ($FEV_1$/FVC ratio) are both used in clinical practice as markers for airflow limitation. The amount of limitation is dependent on two variables. The first is airway resistance which is dependant upon narrowing of the small airways. The second is an increase in lung compliance which is dependant upon emphysematous lung destruction. Both small airway narrowing and emphysema represent two distinct pathophysiological processes, both of which are caused by airway inflammation (Hogg 2004).

In the majority of patients with COPD, the stimulus that triggers the development of airway inflammation is the inhalation of cigarette smoke (Hogg 2004). Normally particulate material is dealt with by an innate immune response which includes mucociliary clearance of the airways, and tight junctions which form a physical barrier between lung tissue and airspace. However chronic exposure to cigarette smoke overwhelms these defences and causes damage to the epithelial lining of the lung, resulting in an acute inflammatory response (Hogg 2004). The early pathological manifestations of this inflammation include Squamous and Goblet-cell metaplasia, mucosal ulcers, muscle hypertrophy, fibrosis, pigment deposition and the development of an inflammatory cell infiltrate (Saetta et al. 1994). Chronic airway inflammation is present in all smokers, however only around 15% of smokers develop clinically significant COPD. There is evidence that these subjects have an abnormally

amplified inflammatory process, which in turn may result in the development of small airway narrowing, emphysema, or a combination of both.

Small airway obstruction

COPD is characterised by distal airway inflammation. In the healthy lung, these small distal airways (<2mm in diameter) account for only 10-15% of airway resistance. As a result the disease can progress significantly before manifesting itself clinically (Hogg 2004). The cause of the obstruction is likely to be a combination of mucus deposition, smooth muscle hypertrophy, and peribronchiolar fibrosis (Saetta et al. 1994). Many studies have examined the relationship between smoking and the pathological changes which result in airflow limitation. Cosio et al compared abnormalities in the small airways of smokers and non-smokers who had died accidentally. Although the mean diameter of small airways was similar in both groups, smokers had a significantly greater proportion of bronchioles smaller than 400μm than non-smokers (Cosio et al. 1980). Hale et al added to this work by adding another group of 18 lungs from patients who died with measured COPD. The amount of cellular inflammatory infiltrate, fibrosis, and muscle in the airway wall increased in line with increasing severity of COPD (Hale et al. 1984). These findings were confirmed by Hogg who assessed surgically resected lung tissue from 159 patients with COPD of varying severity. The progression of COPD according to GOLD stage was strongly associated with an increase in the volume of tissue in the wall of the airway, and an accumulation of inflammatory mucous exudates in the lumen of small airways. The percentage of airways containing neutrophils, macrophages, lymphocytes, and lymphoid aggregates increased with COPD severity. The presence

of these lymphoid aggregates suggested the presence of an adaptive inflammatory immune response in the airway (Hogg et al. 2004)

Emphysema

Emphysema is characterised by the dilatation and destruction of lung tissue beyond the terminal bronchiole. This results in an increase in lung compliance by decreasing the elastic recoil force which is needed for expiration, which results in a reduction in $FEV_1$. The presence of emphysema is strongly associated with total pack years of cigarette smoking (Hogg 2004). The role of airway inflammation in the development of emphysema is well described in a study by Retamales. This study compared a group of COPD patients with severe emphysema with smoking matched control patients with normal lung function. The patients with severe emphysema were found to have a tenfold increase in neutrophils, macrophages, T lymphocytes, and eosinophils in their lungs (Ratemales et al. 2001). The fact that only about 40% of heavy smokers will develop emphysema again suggests that only a proportion of the population are susceptible to the effects of chronic cigarette smoke inhalation and will develop an amplified inflammatory response.

Two phenotypes of emphysema have been described. Centrilobular emphysema is more closely linked to airway abnormalities and is more predominant in the upper lobes of the lung. Panlobular emphysema, which is associated with Alpha-1-antitrypsin deficiency, is associated with higher lung compliance and loss of elastic recoil, and is more prominent in the lower lobes (Hogg 2004). The two phenotypes of

emphysema affect airflow limitation in different ways. Airflow has been shown to decrease as airway abnormalities increased in centrilobular emphysema, but no relationship between airway abnormalities and airflow could be demonstrated in panlobular emphysema. On the other hand, there was a significant decrease in flow associated with a reduction in lung elasticity in panlobular emphysema but not in centrilobular emphysema. This indicates that airflow limitation in centrilobular emphysema results mainly from airway abnormalities, whilst in panlobular emphysema it results more from a reduction in elastic recoil pressure (Saetta et al. 1994). These two forms of emphysema may co-exist or occur in their pure forms although usually one form tends to be predominant. The fact that centrilobular emphysema is more associated with inflammation in the small airways suggests an airborne stimulus to the inflammatory process, whilst panlobular emphysema is more diffusely distributed suggesting that the inflammatory stimulus may be blood-borne. This raises the interesting possibility that the mechanism of development of these two phenotypes of emphysema is different, and that inflammation may cause lung pathology in a more diverse fashion using a broad variety of mechanisms. The ability to identify these mechanisms and modulate airway inflammation may result in clinically significant benefits.

# 3: Airway inflammation in COPD: The key players

It is now recognised that airway inflammation is at the heart of COPD. New definitions of COPD acknowledge the concept of an abnormal amplified inflammatory response to noxious particles or gasses (Celli et al. 2004), but the mechanism of this amplification remains unknown. Many cells and mediators are involved in the process of airway inflammation. Knowledge regarding the exact relationship between these cells and mediators, both in stable state and during exacerbations, is likely to be crucial in improving outcomes for this disease. In this chapter we will review some of the key players involved in airway inflammation in COPD.

## Neutrophils

The neutrophil is likely to play a key role in airway inflammation in COPD. It is the most predominant inflammatory cell both in stable state disease and during exacerbations. Increased numbers of neutrophils have been found in sputum and broncho-alveolar lavage (BAL) fluid of patients with COPD although less so in bronchial biopsies (Barnes 2003). Following migration to the pulmonary circulation, adhesion molecules such as ICAM-1 and E-selectin enable neutrophils to adhere to the endothelial surface. After this they are attracted towards the airway by neutrophil chemotactic factors such as interleukin-8 and leukotriene $B_4$. These chemotactic factors are produced by macrophages, epithelial cells and by the neutrophils themselves. Once in the respiratory tract neutrophils secrete serine proteases such as neutrophil elastase, cathepsin G and proteinase-3 as well as matrix metalloproteins.

These mediators are responsible for a variety of effects including airway destruction, mucus hypersecretion, and reduced mucocilliary clearance (Barnes 2003). Activation of neutrophils also results in the release of granule proteins such as myeloperoxidase which is responsible for giving sputum its greenish colour (Hill et al. 2000). Whilst the exact role of the neutrophil in COPD is not fully known, we do know that neutrophils are associated with both $FEV_1$ (Di Stefano et al. 1998) and decline in $FEV_1$ (Stanescu et al. 1996).

Macrophages

The macrophage is also likely to be crucial in the genesis of airway inflammation in COPD. There is a difference both in the number and type of macrophages seen in COPD patients compared to normal smokers. There is an increase of 5-10 fold in the number of macrophages in sputum, BAL fluid, and in bronchial biopsies in patients with COPD, and macrophage numbers have also been shown to correlate with disease severity. Macrophages from patients with COPD may also differ in size, may show prolonged survival, and have been shown to secrete more inflammatory proteins than those in normal smokers. Macrophages have been shown to release TNF-alpha, IL-8, LTB4, as well as matrix metalloproteinases, the most elastolytic of these being MMP-9. The macrophage also plays a key role as part of the innate immune system where it is responsible for phagocytosis of bacteria (Barnes 2003).

## Eosinophils

The role of the eosinophil in COPD remains controversial. Firstly there is conflicting evidence regarding the prevalence of eosinophils in stable state COPD. Whilst some researchers have found no evidence of eosinophils in BAL, induced sputum, and in bronchial biopsies (Turato et al. 2001), others including myself and others (Brightling et al. 2000) (Seatta et al. 1994) (Pizzichini et al. 1998) have shown consistent evidence for the presence of eosinophils in patients with stable COPD. The cause for this discrepancy is unknown. It is unlikely to be due to patient selection as the presence of eosinophils has been shown in patients with stable COPD in different geographical locations. Whilst the inclusion and exclusion criteria do vary between studies, they are generally quite rigorous at excluding patients with asthmatic features. More likely is that differences in eosinophil counts are related to the laboratory techniques used for processing and counting these cells. More conclusive evidence exists for the role of eosinophils during exacerbations of COPD, where increased numbers of eosinophils have been found both in induced sputum and in bronchial biopsies (Seatta et al. 1994).

## $CD8^+$ T-lymphocytes

These cells are thought to be important in the genesis of airflow obstruction and emphysema. Increased levels of $CD8^+$ T-lymphocytes have been found in resected lung tissue of patients with COPD, both in peripheral airways (Saetta et al. 1998) and in lung parenchyma (Saetta et al. 1999). Interestingly in both of these studies, the concentration of $CD8^+$ T-lymphocytes was found to inversely correlate with $FEV_1$,

and concentration of $CD8^+$ T-lymphocytes was higher in patients with COPD compared to matched smoking controls with normal lung function. $CD8^+$ T-lymphocytes are capable of producing interferon gamma and tumour necrosis factor and are thought to be involved in the resolution of acute viral infections, which are common in patients with COPD. It is plausible that recurrent viral infections might result in the excessive recruitment of $CD8^+$ T-lymphocytes resulting in excessive pulmonary damage. Finally an association between $CD8^+$ T-lymphocytes and apoptosis of alveolar cells has been shown in emphysema (Majo et al 2001).

Other cells including dendritic cells and epithelial cells are also involved in the process of airway inflammation. Whilst some of the key players have been discussed, it is now worth mentioning two homeostatic mechanisms which are at the heart of airway inflammation in COPD; the oxidant-antioxidant balance and the protease-antiprotease balance.

There is increasing interest into the roles of oxidative stress in COPD. Oxidants are generated either endogenously, for example released during phagocytosis, or generated exogenously, for example from cigarette smoke. The lungs are protected from these oxidants by enzymatic and nonenzymatic antioxidant systems. Oxidative stress occurs when the balance between oxidants and antioxidants shifts in favour of oxidants. Oxidative stress may cause direct lung injury, initiate mechanisms of airway inflammation, and may have a significant role in systemic consequences of COPD such as muscle dysfunction and weight loss (MacNee 2005). The second important mechanism is the protease-antiprotease balance, where proteases which digest structural proteins such as elastin are kept in check by antiproteases such as Alpha-1-

antitrypsin. In theory a reduction of protease activity or an increase in the level of antiprotease might result in improved clinical outcomes (Barnes 2005).

Like asthma it is now clear that COPD is a chronic inflammatory disease. However unlike asthma corticosteroid therapy appears to be less useful. The exact mechanism of this corticosteroid resistance is unknown but it is likely that levels of Histone deacetylase are important (Barnes et al. 2004). Cigarette smoke seems to have an inhibitory effect on Histone deacetylation which is required in order for corticosteroids to switch off inflammatory genes (Barnes 2005). The use of theophylline or combined long acting beta 2 agonist and steroid therapies may be important in overcoming corticosteroid resistance (Barnes 2005).

# 4: Eosinophilic airway inflammation, corticosteroids, and associated outcomes in COPD

## 4.1: Eosinophilic airway inflammation in COPD

Eosinophilic airway inflammation is common in asthma and higher levels of eosinophilic airway inflammation are found in asthma compared to COPD (Balzano et al. 1999). In COPD airway inflammation is mainly neutrophilic and the extent of eosinophilic airway inflammation in stable state disease is controversial. My own data along with previous data from our research group (Brightling et al. 2000) and other studies (Seatta et al. 1994)(Confalonieri et al. 1998) show that around a third of patients with stable COPD of various severities demonstrate a sputum eosinophilia, yet other studies have failed to demonstrate the presence of eosinophils in sputum, BAL, and bronchial biopsies during stable state disease (Turato et al. 2001). One possible reason for this may be due to different techniques used in processing the eosinophils. Differences in staining techniques and length of exposure of eosinophils to neutrophil elastase may all result in variation in cell counts and therefore there is a need for standardised processing protocols. Another reason for the discrepancy in eosinophil numbers might be due to differences in the demographics of the populations that were sampled. Some studies tend to lack an adequate breakdown of the anti-inflammatory treatment taken by patients. However in general the majority of studies have rigorous entry criteria making it less likely that patients with an asthmatic phenotype and hence greater levels of eosinophilic airway inflammation are included in COPD populations.

During an exacerbation of COPD the level of eosinophilic airway inflammation has been shown to increase with a 30 fold rise in eosinophils demonstrated in bronchial biopsies as well as significantly higher levels of eosinophils found in induced sputum (Seatta et al. 1994). The fact that exacerbations have a highly varied aetiology means that several exacerbations are unlikely to be associated with a significant increase in eosinophilic airway inflammation and this may account for certain conflicting results and discrepancies between studies.

The level of eosinophil activation is also controversial. One bronchial biopsy study has reported increased numbers of eosinophils in patients with COPD but lower bronchoalveolar lavage concentrations of eosinophilic cationic protein (ECP), suggesting a reduced state of activation in COPD (Lacoste et al. 1993). However, other studies (Balzano et al. 1999)(Keatings et al. 1997) using sputum induction have shown that whilst sputum eosinophil counts are lower in COPD compared to asthma, ECP levels remain similar, suggesting a higher state of eosinophil activation in COPD.

## 4.2: The relationship between eosinophilic airway inflammation and response to corticosteroids

The ability of corticosteroids to modulate eosinophilic airway inflammation in asthma has led to this treatment becoming the cornerstone of asthma therapy whilst in COPD the role of corticosteroids is more controversial. As already mentioned eosinophilic airway inflammation is less predominant in COPD but it is the different location of

this inflammation which may also be crucial. Unlike asthma, COPD is a disease of distal airway and parenchymal inflammation (Hogg et al. 2004). Whilst within the reach of oral corticosteroids, these areas are less accessible to inhaled corticosteroids and so we might expect inhaled corticosteroids to be less effective at modulating eosinophilic airway inflammation in COPD. Current smoking is also likely to influence corticosteroid resistance (Barnes et al. 2004)(Chauduri et al 2003).

There is good evidence to show that eosinophilic airway inflammation tends to respond well to treatment with oral corticosteroids (Pizzichini et al. 1998). In a study by Brightling, 67 patients were randomised into a double-blind crossover trial of placebo and 30mg of Prednisolone daily for 2 weeks. Before each treatment period patients were assessed with spirometry, visual analogue scales to assess symptoms, the chronic respiratory disease questionnaire, incremental shuttle walk test, and sputum induction. The geometric mean sputum eosinophil count fell significantly after Prednisolone (from 2.4% to 0.4%; mean difference 6 fold) but not after placebo. After stratification into tertiles by baseline sputum eosinophil count, $FEV_1$ and total quality of life scores improved progressively after Prednisolone from the lowest to the highest eosinophilic tertile, compared with placebo (Brightling et al. 2000).

In a separate study the same author has investigated the relationship between eosinophilic airway inflammation and response to inhaled corticosteroids (Brightling et al. 2005). In this randomised, double blind, crossover trial, 60 patients were randomised to treatment with either the inhaled corticosteroid Mometasone (800μg/day) or placebo. Each treatment phase lasted 6 weeks with a 4 week washout period in between. Patients underwent spirometry, assessment of symptoms and

quality of life, and sputum induction before and after each treatment phase. The results showed no significant benefit with Mometasone compared to placebo. The change in the sputum eosinophil count was not significantly different between the groups treated with inhaled corticosteroid and placebo. One interesting point was that after stratification of patients into tertiles based on their baseline sputum eosinophil count, improvement in $FEV_1$ following Mometasone compared with placebo increased progressively from the least to the most eosinophilic tertile, but the overall impact was noticeably less than that seen in an earlier study with prednisolone. The study concluded that an increased sputum eosinophil count was related to improvement in $FEV_1$ following treatment with an inhaled corticosteroid in COPD, but the improvement was not associated with a reduction in the sputum eosinophil count.

In summary both studies showed that increased benefit with corticosteroids was related to an increasing level of eosinophilic airway inflammation, but oral corticosteroids were more effective and were able to significantly reduce eosinophilic airway inflammation more than placebo in patients with COPD.

<u>4.3: Eosinophilic airway inflammation, corticosteroids, and decline in lung function.</u>

Eosinophilic airway inflammation exists in stable state COPD, and the % sputum eosinophil count has been shown to correlate with both $FEV_1$ and $FEV_1$/FVC ratio (Balzano et al. 1999). There is also further evidence to support a role for eosinophilic airway inflammation in decline in lung function. It is interesting that in a study by

Stanescu which looked at decline in lung function, the group with the fastest decline in lung function had the highest level of neutrophilic airway inflammation and eosinophilic airway inflammation (Stanescu et al. 1996). Also development of fixed airway obstruction has been described in cases of eosinophilic bronchitis (Brightling et al. 1999). As eosinophilic airway inflammation is amenable to corticosteroid therapy, we now turn our attention to how corticosteroids might influence disease progression. As COPD is a disease of progressive loss of lung function, the search for therapies which can reverse or slow down this process is a priority.

The role of oral corticosteroids in modifying disease progression has been investigated by Postma. In an observational study, she showed that the regular use of oral prednisolone in a dose of more than 10mg/day in patients with both severe and moderate disease was associated with a reduction in the rate of decline of $FEV_1$ (Postma et al. 1988). However other observational studies suggest the use of long term prednisolone is associated with considerable side effects (Decramer et al. 1992,1994), and current guidelines do not recommend the regular use of oral corticosteroids in the majority of patients with COPD (Pauwels et al. 2001).

The fact that oral corticosteroids could modulate disease progression raised optimism that inhaled corticosteroids might achieve a similar effect without the same side effect profile. Several large scale, randomised, placebo controlled trials have been carried out to assess whether inhaled corticosteroids could reduce the rate of decline in $FEV_1$ compared to placebo. The most clinically relevant of these trials, was the ISOLDE trial. In this study 751 patients with mean $FEV_1$ % predicted of 50% were randomised to treatment with either high dose inhaled Fluticasone or placebo. The primary

endpoint was decline in lung function and after 3 years the results showed no significant difference in decline in $FEV_1$ between those patients treated with inhaled corticosteroid or placebo (Burge et al. 2000). Other studies have also examined this endpoint. The Copenhagen city study which also lasted 3 years, looked at patients with relatively mild COPD who were treated with either high dose Budesonide or placebo and again found no significant difference in decline in lung function between the two groups (Vestbo et al. 1999). One study that did show a difference in decline in lung function was the Euroscop study. Out of the 912 patients who completed the study the median decline in $FEV_1$ over 3 years was 140ml in the group treated with Budesonide compared to 180ml in the group treated with placebo. However this difference could all be attributed to the first 6 months of the study during which $FEV_1$ rose by 17ml/year in the group treated with Budesonide compared to a decline in $FEV_1$ of 81ml/year in the group treated with placebo. Between 9 months and the end of the study, $FEV_1$ actually declined at a similar rate between the 2 groups (Pauwels et al. 1999).

## 4.4: Role of corticosteroids in exacerbations of COPD

We have stated that eosinophilic airway inflammation is common in stable state COPD but even more so during exacerbations. We have also described how eosinophilic airway inflammation is amenable to treatment with oral corticosteroids whilst being more resistant to inhaled corticosteroids. This therefore implies that oral and inhaled corticosteroids may have therapeutic roles in exacerbations of COPD.

Oral corticosteroids have been shown to improve outcome at exacerbation. One prospective randomised control trial compared the effect of a 2 week course of oral Prednisolone to placebo in a group of patients admitted with an exacerbation of COPD. Patients treated with Prednisolone were shown to have faster resolution of lung function and shorter hospital stay (Davies et al. 1999). A further study came to the same conclusion as well as showing a reduction in treatment failures in the group treated with Prednisolone. Generally the most benefit from oral corticosteroids occurred within the first 5 days, and there was no benefit achieved with a prolonged 8 week course compared to a 2 week course of Prednisolone (Niewoehner et al. 1999). It should be noted that in neither of these studies was there any measurement of airway inflammation. Current clinical practice makes no attempt to predict which patients are most likely to benefit from prednisolone and therefore it is recommended that all patients receive oral or intravenous corticosteroid therapy at exacerbation. The use of oral prednisolone has subsequently become standard practice in the vast majority of exacerbations of COPD, yet in theory only patients with significant eosinophilic airway inflammation should benefit from this treatment. It is therefore possible that during exacerbations of COPD, some patients are being unnecessarily treated with prednisolone.

Whilst some of the studies described above suggested no role for inhaled corticosteroids in modifying disease progression, they did however find a role for this therapy when it came to exacerbation frequency. Although the ISOLDE study showed no significant difference between the groups as regards to decline in $FEV_1$, it did show that there was a 25% reduction in median exacerbation rate in the group treated with Fluticasone compared with placebo. Although the original study did not indicate

which patients showed the most benefit, a subsequent analysis of the data revealed that Fluticasone reduced the overall exacerbation rate, compared to placebo, in moderate and severe disease but not in mild disease (Jones et al. 2003). High dose inhaled Fluticasone was also used in another study by Paggiaro where 281 patients with moderate disease were randomised to treatment with the inhaled corticosteroid or placebo. Although there was no significant difference in the number of patients having at least one exacerbation, significantly more patients had moderate and severe exacerbations in the placebo group compared to the Fluticasone group (Paggiaro et al. 1998). On the other hand another study showed that over 3 years there was no difference in exacerbation frequency between patients treated with a moderate dose of inhaled Budesonide compared to placebo. However patients in this study had only mild disease (Vestbo J et al. 1999). These studies have shaped current guidelines which recommend the use of inhaled corticosteroids in patients with moderate and severe disease who have frequent exacerbations.

Reinforcing the relationship between eosinophilic airway inflammation, corticosteroids, and exacerbations of COPD, is further data from the ISOLDE study. This data showed that the response to a 2 week trial of oral Prednisolone was able to predict future exacerbation frequency. For patients who had a significant response to Prednisolone, the mean number of exacerbations per year in patients treated with placebo compared to Fluticasone was 1.9 versus 0.85 respectively, representing a 55% reduction in exacerbation frequency. However for patients without a significant response to Prednisolone the mean number of exacerbations per year in patients treated with placebo compared to Fluticasone was 1.55 versus 1.32 respectively, representing only a 15% reduction in exacerbation frequency (Burge et al. 2003).

Previously we have discussed how the response to corticosteroids is related to eosinophilic airway inflammation. The data above suggests that corticosteroid response can predict exacerbation frequency. This therefore leads us to believe that eosinophilic airway inflammation could be a marker for exacerbation frequency and that markers of eosinophilic airway inflammation such as the sputum eosinophil count could be used as a potential tool for guiding treatment with corticosteroids. In theory modulation of eosinophilic airway inflammation should result in a change in exacerbation frequency. This theory forms a hypothesis which is tested in this thesis.

# 5: Neutrophilic airway inflammation, airway bacterial load, and associated outcomes in COPD

## 5.1: Neutrophilic airway inflammation and disease progression

We have already stated that COPD is a chronic inflammatory disease, characterised by airflow limitation that is slowly progressive. The ability to slow down the accelerated decline in lung function which characterises COPD remains the holy grail of COPD research. To date smoking cessation is the only direct intervention which has been shown to slow down disease progression although this effect seems to occur only in mild disease (Fletcher et al. 1977). Interestingly following smoking cessation, airway inflammation has been shown to persist (Turato et al. 1995, Rutgers et al. 2000), suggesting that other factors may drive airway inflammation independently of cigarette smoke. The fact that the reduced rate of decline in lung function can occur whilst airway inflammation persists may cause some doubt regarding the relationship between airway inflammation and disease progression. There is however good evidence to support the view that neutrophilic airway inflammation plays a key role in disease progression, and therefore there is an urgent need to seek out interventions which can modulate neutrophilic airway inflammation and therefore potentially influence disease progression.

Disease progression in COPD is most commonly assessed by measuring $FEV_1$, and neutrophilic airway inflammation has been shown to be associated both cross-sectionaly with $FEV_1$ and longitudinally with decline in $FEV_1$. In a study by Di Stefano, bronchial biopsies were obtained from 30 smokers with varying degrees of

COPD severity. Compared to smoking controls, smokers with severe COPD demonstrated increased numbers of neutrophils in the subepithelium and overall $FEV_1$ was inversely correlated with the number of neutrophils (Di Stefano et al. 1998). In a study by Stanescu, 46 steelworkers who were current or ex-smokers were followed up over 15 years. Lung function was assessed at the start, during, and at the end of the study. Sputum induction was also carried out at the end of the study. Subjects who developed airway obstruction were found to have significantly more % neutrophils in their sputum than those who did not develop airway obstruction. Subjects with more than 70% neutrophils in their sputum had a decline in $FEV_1$ of 27ml/year which was more than twice that observed in subjects with less than 70% neutrophils who had a decline of only 12ml/year. There was a significant correlation between decline in $FEV_1$ and the sputum % neutrophil count (Stanescu et al. 1996).

## 5.2: The association between neutrophilic airway inflammation and airway bacterial load.

We have already eluded to the fact that airway inflammation may be driven by other factors independently of cigarette smoke. One of these factors is airway bacterial infection. In the introduction I described how chronic exposure to cigarette smoke overwhelms the lungs innate defences and causes damage to the epithelial lining of the lung, resulting in an acute inflammatory response. This in turn leads to muco-cilliary dysfunction and a lesser ability of the airway to deal with bacterial flora. Subsequently an increase in airway bacterial load, and the inability to defend against

new strains of bacteria, results in an increase in airway inflammation. A self perpetuating viscous circle now forms.

Neutrophilic airway inflammation is at the heart of this process and there is good evidence to support this. The theory that bacterial infection had a causal effect on neutrophilic airway inflammation is supported by a study by Patel. In this study subjects underwent measurement of markers of neutrophilic airway inflammation and identification of bacteria during acute exacerbations of COPD. Exacerbations involving Haemophilus influenzae were associated with significantly higher sputum IL-8, TNF-alpha, and Neutrophil elastase when compared to pathogen negative exacerbations whilst exacerbations involving Moraxella catarrhalis were associated with significantly higher sputum TNF-alpha, and Neutrophil elastase (Patel et al. 2002). Further evidence for the role of bacteria in the genesis of neutrophilic airway inflammation was provided by a study by White. This study demonstrated how the resolution of airway inflammation, following treatment of an exacerbation, was related to bacterial eradication. Quantitative bacterial analysis and measurement of markers associated with neutrophilic airway inflammation (Myeloperoxidase, Neutrophil elastase, Leukotriene B4) were carried out in patients presenting with bacterial culture positive acute exacerbations of COPD. Measurements were repeated at 10 days and at 2 months. At day 10, 17 of 41 patient samples had a positive bacterial culture, and at 2 months 18 of 46 samples had a positive bacterial culture, but with a significantly lower bacterial load than at presentation. The concentration of Myeloperoxidase was lower in patients in whom bacteria were eradicated by day 10 than in those with persisting bacterial infection. The concentration of Leukotriene B4 was also lower in patients in whom bacteria were eradicated than in those with

persisting bacterial infection. At 2 months the concentrations of Myeloperoxidase and Leukotriene B4 were lower in patients in whom bacteria had been eradicated than in those with persisting bacterial infection (White et al. 2003).

In summary these previous two studies suggest that the presence of bacteria at exacerbation is associated with increased neutrophilic airway inflammation, and that eradication of bacteria following an exacerbation is associated with resolution of neutrophilic airway inflammation.

## 5.3: Modulation of bacterial load in stable state COPD.

Whilst it is clear that there is an association between neutrophilic airway inflammation and bacterial infection during exacerbations of COPD, less is known about this association in stable state disease. One study however did address this area. In an observational study, 67 patients underwent sputum induction in order to provide sputum samples which were analysed for differential cell count, quantitative bacterial analysis, and cytokine concentrations. 40% of patients had a potentially pathogenic micro-organism (PPM) in their sputum. These patients had a higher sputum % neutrophil count, and higher levels of IL-8, LTB4, neutrophil elastase, and TNF-alpha than patients without a PPM in their sputum (Banerjee et al. 2004). Despite this the extent to which bacteria are present during stable disease and what role they play in the genesis of airway inflammation remains unclear. As a consequence of this it is difficult to predict what the effect will be of modulating airway bacterial load in patients with stable state COPD.

Another study which helped our understanding of the roles of bacteria in stable disease and during exacerbations of COPD was that of Monso and Ruiz. They used the protected specimen brush to carry out microbiological sampling of the airways of patients during stable disease and at exacerbation. Positive cultures were obtained in 25% of stable patients compared to 52% of patients at exacerbation. The concentration of bacteria was also found to be increased at exacerbation. A concentration of $> 10^4$ cfu/ml of bacteria was found in 5% of patients with stable COPD whilst a concentration of $> 10^4$ cfu/ml of bacteria was found in 25% of patients at exacerbation (Monso et al. 1995). One conclusion that the authors came to was that the prevalence of lower airway bacterial colonisation in patients with stable disease was high. It is this high prevalence of bacteria which represents a therapeutic target for antibiotic treatment.

## 5.4: The potential role for antibiotics in the modulation of airway inflammation and disease outcome

Whilst the use of antibiotics in treating exacerbations of COPD remains controversial, the potential use of antibiotics in stable disease is more so. The fact that associations exist between disease progression and neutrophilic airway inflammation and between neutrophilic airway inflammation and bacterial infection, imply that the eradication of highly prevalent bacteria in stable disease using antibiotics, could impact on disease progression. However casting some doubt on this theory, is a paper by Soler which showed that a significant growth of bacteria was associated with the % neutrophil

count, but showed no association between airway bacterial colonisation and degree of airway obstruction (Soler et al. 1999).

The ability of antibiotics to reduce markers of neutrophilic airway inflammation in stable patients has already been demonstrated in patients with bronchiectasis. In an uncontrolled study by Stockley, broad spectrum antibiotics were given to patients with stable state bronchiectasis who regularly produced purulent sputum which was rich in Neutrophil elastase. Clearing of sputum and reduction of elastase activity was seen in most patients (Stockley et al. 1984).

Finally a study by Wilkinson reinforced the belief that airway bacterial load was associated with disease outcome. In this study 30 patients were followed up for 12 months. Sputum was collected at recruitment and at the end of the study, and this was analysed for cytokine concentrations and quantitative bacterial load. Bacterial load increased over the 12 months and was found to be related to decline in $FEV_1$. Higher sputum IL-8 levels and a change in colonising bacterial type were both associated with a greater decline in $FEV_1$ (Wilkinson et al. 2003).

## 6: Association between non-pulmonary inflammation and airway inflammation in COPD.

We have already described cigarette smoke as a major extrinsic risk factor in the genesis of airway inflammation in COPD. We now turn our attention to the interesting possibility that various intrinsic inflammatory processes may also contribute to airway inflammation. There is increasing evidence that conditions associated with chronic inflammation of foregut derivatives are associated with airway inflammation and dysfunction perhaps due to aberrant homing of activated lymphocytes (Birring et al. 2003,2005) (Kanazawa et al. 2003) (Brightling et al. 2002). This is most widely recognised in inflammatory bowel disease (Camus et al. 2000) but there is also evidence of increased respiratory morbidity, airway dysfunction, and airway inflammation, in patients with organ-specific autoimmune disease, particularly autoimmune hypothyroidism (Birring et al. 2003) (Brightling et al. 2002). Chronic hepatitis due to hepatitis C infection has also been associated with an accelerated decline in lung function in patients with COPD which returns towards normal following successful treatment of the hepatitis with Interferon Gamma (Kanazawa et al. 2003).

One study which investigated this concept compared twenty six non smoking women with treated hypothyroidism and 19 non smoking controls. Symptoms of cough, dyspnoea, sputum production, and wheeze were reported more commonly in the patients than controls. The patients with hypothyroidism had heightened cough reflex sensitivity and a significantly higher proportion of patients had airway hyper-responsiveness. Patients with hypothyroidism had significantly

higher induced sputum total neutrophil count, total lymphocyte count and sputum supernatant IL-8 concentrations (Birring et al. 2003).

Another study took fifty-nine patients with COPD and followed them up for five years. This cohort had been split into four groups. Group A consisted of 15 patients who were Hepatitis C negative and were ex-smokers. Group B consisted of 14 patients who were Hepatitis C negative but were current smokers. Group C consisted of 14 patients who were Hepatitis C positive and were ex-smokers. Finally group D consisted of 16 patients who were Hepatitis C positive and were current smokers. In group A the rate of annual decline in $FEV_1$ was only 33.5 ml/year. In groups B and C where patients in effect had only one inflammatory stimulus the rate of decline in $FEV_1$ was between 54.0 and 59.7 ml/year. However in group D where patients were under the influence of two inflammatory stimuli the rate of decline in $FEV_1$ was 79.5 ml/year. Interestingly the change in $FEV_1$ following Interferon therapy was assessed over a 3 year follow-up period. 8 patients were deemed to have had a response to Interferon, characterised by disappearance of Hepatitis C RNA, whilst 13 were deemed to be non-responders. Annual decline in $FEV_1$ did not significantly change in the non-responders but there was a significant reduction in the rate of decline in $FEV_1$ in the responders to Interferon (Kanazawa et al. 2003). This is preliminary but suggestive evidence that suppression of extra-pulmonary inflammatory stimuli might be able to modulate decline in lung function.

One of the commonest causes of chronic foregut inflammation worldwide is gastritis secondary to H.Pylori infection (Suerbaum et al. 2002). This gastritis

manifests clinically in the form of peptic ulcer disease. Interestingly studies carried out over 40 years ago showed evidence of an increased prevalence of peptic ulcer disease in patients with pathological evidence of emphysema compared to those without emphysema (Flint et al.1958) (Kroeker et al. 1962) (Latts et al. 1956). Most of these studies used post mortem data to diagnose peptic ulcer disease however some rely on case records and are therefore less objective, but the prevalence of peptic ulcer disease in patients with COPD was quite consistent: 21%, 28.7%, and 27% respectively, for the three studies quoted above.

This raises the intriguing prospect that eradication of H.Pylori, resulting in a reduction of a common foregut inflammatory stimulus, might potentially have an impact on airway inflammation. This in turn might have the potential to effect exacerbation frequency or decline in lung function.

## 7: The use of sputum induction in the measurement of airway inflammation in COPD.

Sputum induction is a safe, well tolerated, non-invasive procedure which is now commonly used to assess lower airway inflammation in patients with respiratory disease. The assessment of lower airway inflammation in COPD is becoming more important as there is evidence to show that neutrophilic airway inflammation is associated with $FEV_1$ and decline in $FEV_1$, whilst eosinophilic airway inflammation is associated with exacerbations of COPD. The ability to regularly assess lower airway inflammation in a safe and non-invasive way is vital in assessing the results of new therapies and management strategies designed to modulate airway inflammation. Here we assess the role of sputum induction in patients with COPD.

Firstly we must address whether there is an actual need for this procedure in patients with COPD. Several patients, particularly those with chronic bronchitis, are capable of bringing up copious amounts of sputum on demand, yet there are also patients who simply cannot produce a suitable sputum sample. In a paper by the Wedzicha group, the advantages of induced sputum over spontaneous sputum were assessed. The main advantage was higher median cell viability, which ranged from 41% in spontaneous sputum, to 63% after 7 minutes of induction, and to 65% after 14 minutes of induction. The paper also demonstrated that there were no significant differences in total and differential cell counts or in levels of IL-8 between spontaneous and induced sputum (Bhowmik et al. 1998). Therefore factors such as increased cell viability, the reassurance of having a standardised technique for all patients, and ability to non-

invasively sample airway inflammation in the distal lung make sputum induction an attractive proposition.

Given that patients with COPD may have compromised lung function or oxygenation, the next aspect of sputum induction we must address is that of safety. Patients with moderate and severe COPD would in general be expected to have a lower baseline % predicted $FEV_1$ than their asthmatic counterparts. On the other hand we might expect that given the lesser extent of reversibility in lung function in patients with COPD compared to asthma, that $FEV_1$ might be relatively stable during sputum induction in COPD patients. Several similar papers address this important issue. The populations studied were composed of patients who generally had moderate and severe disease. % predicted $FEV_1$ varied between 38% to 53% and $FEV_1$ varied from just under 1 litre to almost 2 litres. Interestingly each paper used a slightly different induction protocol. In one study if the post bronchodilator $FEV_1$ was less than 1 litre then only 0.9% hypertonic saline was used and $FEV_1$ was reassessed every three minutes (Rytila et al. 2000). The second study used just 3% hypertonic saline with assessment of spirometry after seven minutes (Bhowmik et al. 1998), whilst the third study used increasing concentrations of hypertonic saline from 3% to 5% with reassessment of spirometry every five minutes (Brightling et al. 2001). Fall in mean post bronchodilator $FEV_1$ was very consistent between all three studies ranging from 10.7% to 11.7%. A fall of > 20% was rare and not associated with any adverse outcome. The change in level of oxygenation was negligible. The procedure was stopped due to symptoms on only a few occasions with the most common reasons being dyspnoea, nausea or general discomfort. Suitable sputum samples were obtained in over 90% of cases.

We have described how sputum induction in COPD patients is safe, well tolerated, successful, and provides worthwhile results. As the importance of measuring airway inflammation in COPD is acknowledged it is likely that this technique will be increasingly used. Whilst some expertise is required to process specimens, the technique is not beyond the capabilities of the average laboratory. There is also likely to be a future role for extended sputum protocols which allow for sampling of even more distal lung, which is a relatively inaccessible yet functionally important area.

## 8: Measurement of airway bacterial load using quantitative bacterial analysis.

Sputum produced by patients with COPD may contain a variety of bacterial species.

There is now considerable evidence that bacteria are involved in the genesis of airway inflammation, have an impact on symptoms, and are particularly important during exacerbations. There is therefore a growing need to be able to identify and quantify these bacteria both in stable disease and during exacerbations. Quantification of bacterial load is particularly important when assessing the effect of antibiotic therapy, where information regarding the presence or absence of bacteria alone may not provide an accurate enough assessment of the effect of therapy. The results from some techniques may be rather unreliable due to the heterogeneous nature of sputum and differences in sampling methods. Here we briefly describe a well validated simple method for quantifying bacteria in sputum.

Firstly sputum is homogenised with Sputasol (Oxoid Ltd, Basingstoke, U.K; containing 100mcg/ml dithiothreitol) for 15 minutes. A 1 ml aliquot of homogenised sputum is then diluted with sterile saline (0.9% w/v sodium chloride) to produce a dilution series from 1 in 10 to 1 in 100,000. These dilutions are then used to inoculate chocolate, blood, and MacConkey agar plates. These plates are then incubated at 36 degrees centigrade in an atmosphere of 5% $CO_2$ in air and examined at 24 and 48 hours. Plates yielding between 30 and 300 colonies are counted using a manual tally counter. Bacterial numbers are expressed as colony forming units per millilitre (cfu/ml) of original sputum (Pye et al. 1995).

The use of Sputasol was shown not to have an adverse effect on bacterial viability. The use of a precision pipette to inoculate the agar plates was associated with less variation in bacterial numbers as compared to using a calibrated disposable loop. Dispensing a larger volume (10µl) from the precision pipette resulted in the lowest variation in viable bacterial numbers. We have taken the above method and modified it slightly to be even simpler and less time consuming. Our protocol and initial validation are described later.

## 9: The search for new markers of airway inflammation: TREM-1.

We have previously described management strategies for modulating airway inflammation. These strategies are based around the ability to detect airway inflammation safely and non-invasively. For eosinophilic airway inflammation, the sputum eosinophil count acts as an excellent guide for corticosteroid therapy. In order to modulate neutrophilic airway inflammation we used antibiotic therapy, and as demonstrated later, showed that we were more successful at reducing neutrophilic airway inflammation in those patients with higher bacterial loads. However the measurement of airway bacterial load is not a practical method for guiding antibiotic therapy in clinical practice. Firstly, it requires dedicated laboratory equipment and staff. Secondly, it takes time and requires at least 24 hours before delivering any meaningful results. Thirdly, and maybe most importantly, it does not distinguish between bacteria which represent chronic colonisation and new strains of bacteria which are causing acute infection. A marker with the ability to differentiate new strains of bacteria from chronic bacterial colonisation would be useful not only for research purposes but also clinically. It might enable us to target those patients with much higher levels of neutrophilic airway inflammation caused by new bacterial strains who are likely to achieve the greatest benefit from antibiotics. It could also be used to guide antibiotic therapy more accurately during exacerbations of COPD.

The use of antibiotics in the treatment of exacerbations of COPD is controversial. Current recommendations suggest using antibiotics only when the patient complains of increased sputum purulence, or when consolidation is present on a chest

radiograph. These guidelines are highly subjective, and the evidence on which they are based is controversial. One placebo controlled study clearly showed no benefit with antibiotic treatment, however the population studied were outpatients, had relatively mild disease, and contained asthmatic patients. The level of bacterial infection as measured in sputum was also very low (Sachs et al.1995). In contrast to this, another study which looked at exacerbations of COPD where patients with relatively severe disease were admitted to an intensive care unit and subsequently ventilated, showed considerably improved outcomes with antibiotics compared to placebo. These included a reduction in mortality, a decreased need for further antibiotic therapy, reduced duration of mechanical ventilation, and reduction in length of stay in the intensive care unit. The level of bacterial infection as measured in sputum was high at around 60% (Nouira et al. 2001). One of the largest studies carried out to assess the potential benefit of antibiotic therapy was the Anthonisen study. Again this was a randomised double blind placebo controlled study mainly involving patients with exacerbations of moderate to severe COPD. Exacerbations were subdivided according to the presence of certain symptoms including increased breathlessness, increased sputum volume, or increased sputum purulence. Overall there was a modest benefit following antibiotic therapy (Anthonisen et al. 1987). On further analysis it was found that most of the benefit occurred in patients who presented with all three of these symptoms; called a type 1 exacerbation. Current guidelines are based around these findings and the presence of increased sputum purulence has become an indication for commencing antibiotic therapy. Research has shown that the appearance of purulent sputum is due to myeloperoxidase present in neutrophils (Hill et al. 2000), and neutrophil recruitment is increased during bacterial infection (Gompertz et al. 2001). However whilst sputum purulence has been shown

to be a sensitive marker for bacterial infection, it is likely to lack specificity, and hence several patients may still be receiving inappropriate antibiotic therapy.

The search for new markers of bacterial infection, which might help us to target antibiotic anti-inflammatory medication more accurately, is ongoing. Much attention has already focussed on Procalcitonin and C reactive protein (CRP). Procalcitonin, a precursor of calcitonin, has been shown to be able to differentiate between acute bacterial and viral infections (Assicot et al. 1993). Whilst Procalcitonin has proved to be a useful marker of bacterial infection in patients with pneumonia with septicaemia, its other potential use is in acute exacerbations of COPD. Here, as stated above, the presence of positive sputum cultures may not actually suggest acute bacterial infection, but rather chronic airway colonisation. The presence of positive sputum cultures has been shown not to influence both Procalcitonin and CRP, suggesting that they may be able to act as markers of acute infection without being influenced by background bacterial colonisation (Stolz et al. 2005). On the other hand, Procalcitonin may be less useful in exacerbations of COPD as localised bacterial infection without systemic manifestations results in only very small increases in Procalcitonin (Gramm et al. 1995). A recent study showed that Procalcitonin could be successfully used as a marker for bacterial infection and guide for antibiotic treatment during exacerbations of COPD. This resulted in a 56% reduction in antibiotic usage with no adverse effect on clinical outcomes. This in turn resulted in a 36% reduction in antimicrobial costs for exacerbations of COPD (Christ-Crain et al. 2004). There is contrasting evidence that CRP is able to distinguish bacterial from viral infection at exacerbations of COPD (Mygind et al. 2004, Stolz et al. 2005), and in any case CRP is likely to be less specific as it will reflect background systemic inflammation from other co-

morbidity or even severity of the underlying disease itself. Another interesting marker is Neopterin. Neopterin is produced by macrophages after induction by interferon Gamma and represents a marker of cellular immune system activation. It is detectable in urine and serum and may be able to identify cases of viral infection however levels are raised in malignancy and autoimmune diseases such as Rheumatoid arthritis and SLE (Fuchs et al. 1988)(Wachter et al. 1989). More attention is now being focussed on a relatively less well known marker called TREM-1.

TREM-1 (triggering receptor expressed on myelocytes) is a glycoprotein expressed on neutrophils and monocytes and its expression is up-regulated by exposure to extracellular bacteria and fungi. Human tissues infected with bacteria are infiltrated with neutrophils and monocytes which express high levels of TREM-1. Recently it has been shown that TREM-1 was the best independent predictor of bacterial pneumonia in ventilated ITU patients (Gibot et al. 2004). In this study patients underwent bronchoalveolar lavage. This lavage fluid was split so some was used for microbiological examination whilst the rest was used for measurement of TREM-1. Independent intensivists, who were blinded to the results of TREM-1 measurement, made a diagnosis of community acquired pneumonia, ventilator associated pneumonia, or no pneumonia based on clinical parameters and microbiological results. The level of TREM-1 was then analysed for each diagnosis. The presence of TREM-1 was shown to be highly sensitive and specific for the diagnosis of pneumonia. Of particular interest in this study was the fact that whilst TREM-1 was such a good predictor of pneumonia, the levels of procalcitonin and CRP were no different amongst the groups.

Airway inflammation in COPD is mainly neutrophilic. Neutrophilic airway inflammation increases during exacerbations and more so when these exacerbations are bacterial in aetiology. Interestingly, processes which involve neutrophilic inflammation in the absence of bacterial aetiology such as ulcerative colitis, vasculitis, and psoriasis are not associated with raised levels of TREM-1 (Bouchon et al. 2001).

A further study which improves our understanding of TREM-1 is that of Richeldi. In this study levels of TREM-1 were measured in bronchoalveolar lavage (BAL) fluid and blood samples from 3 groups of patients. The first group of patients (n=7) consisted of subjects who had a clinical and radiological diagnosis of community acquired pneumonia supported by microbiological results. The second group (n=7) consisted of subjects who had a diagnosis of tuberculosis (T.B) either confirmed on culture or following clinical improvement to anti-tuberculous therapy. The third group (n=10) consisted of patients with interstitial lung disease. This group acted as a control disease group and were included to test the hypothesis that TREM-1 expression in pneumonia was due to the presence of extracellular bacteria and not just any inflammatory lung condition. The results showed that TREM-1 expression in BAL was increased in patients with community acquired pneumonia however remained low in the 2 other groups. Interestingly this difference in expression was not seen in blood where expression remained low for all 3 groups suggesting that TREM-1 expression is localised to the site of infection. Also of interest is the fact that 2 patients with community acquired pneumonia expressed lower levels of TREM-1. The first was the only patient in that group who had received recent antibiotic therapy suggesting that measurement of TREM-1 may be influenced by antibiotic treatment.

The second patient who had low TREM-1 expression was thought to have a sub-acute presentation of pneumonia suggesting that the timing of measurement of TREM-1 during the course of disease might also be important (Richeldi et al. 2004).

These studies raise the exciting possibility that TREM-1 could be used as a marker to identify COPD exacerbations of bacterial aetiology. In order for TREM-1 to achieve this it must also be able to distinguish COPD patients with exacerbations of bacterial aetiology from COPD patients with chronic bacterial colonisation. Later we will present our pilot data looking into this new exciting marker.

# 10: Hypothesis

- Sputum eosinophilia is common in stable state COPD.
- A management strategy which aims to normalise the sputum eosinophil count will result in a reduction in exacerbations of COPD.
- The greatest reduction in exacerbations will be confined to those patients with significant eosinophilic airway inflammation.
- As COPD is a disease mainly characterised by distal airway inflammation, a number of patients will require regular oral corticosteroids in order to suppress their sputum eosinophil count.

- Quantitative bacterial analysis can be performed using a quick and simple method.
- There is a relationship between airway bacterial load and neutrophilic airway inflammation.
- Antibiotics given to patients with stable state COPD are able to reduce neutrophilic airway inflammation, and this effect is likely to be confined to those patients with high bacterial loads in their airways.
- The extent of the reduction in neutrophilic airway inflammation will be related to airway bacterial load.

- Trem-1 is detectable in induced sputum
- The proportion of patients with detectable TREM-1 in sputum will be greater in patients studied during exacerbations than whilst stable.

- Patients with COPD will have a higher prevalence of peptic ulcer disease.
- Patients with COPD will have a higher prevalence of positive H. Pylori serology.

# 11: Clinical methods

## Spirometry and lung function tests

Spirometry was performed using a Vitalograph spirometer (Vitalograph ®, Buckinghamshire U.K). Bronchodilator reversibility was assessed 15 minutes after inhalation of 400mcg Salbutamol via a large volume spacer. $FEV_1$ was recorded as the best of three successive readings within 100ml. Lung function tests were done with a benchmark (P K Morgan, Chatham, U.K) and lung volumes assessed by the Helium dilution method.

## Sputum induction

Subjects were pre-treated with 400mcg of inhaled Salbutamol via a chamber device 15 minutes before starting sputum induction in order to minimise bronchoconstriction. Sputum was induced using 3, 4, and 5% saline inhaled in sequence for 5 minutes via an ultrasonic nebuliser (Medix, Harlow, U.K; output 0.9 ml/min; mass median diameter 5.5μm). Subjects were asked to breathe tidally, taking a slightly deeper breath every minute. After each inhalation subjects blew their noses and rinsed their mouths to minimise nasal contamination and expectorated sputum into a sterile pot. $FEV_1$ was measured after each inhalation. If the $FEV_1$ fell by more than 10% but less than 20%, the same concentration of saline was administered. If the $FEV_1$ fell by more than 20% of the best post-bronchodilator value, or if significant symptoms occurred, the nebulisation was stopped and the patients were treated with repeat short acting Beta-agonist.

# 12: Laboratory methods

## Sputum processing protocol

Sputum free from salivary contamination was selected and weighed. To the selected sputum was added x 4 volume/weight of 0.1% Dithiothrietol (DDT)(Sigma, Poole, Dorset). The sputum was dispersed by gentle aspiration into a Pasteur pipette, followed by vortexing for 15 seconds, then rocking on a bench spiromix for 15 minutes. After the addition of an equal volume of Dulbeccos phosphate buffered saline (D-PBS)( Sigma Poole, Dorset), the sputum suspension was filtered through 48 μm nylon gauze and centrifuged at 2000 rpm (790g) for 10 minutes. The sputum supernatant was removed and stored at -80 degrees centigrade for future mediator assay. The cell pellet was re-suspended in a small volume of PBS. An aliquot was removed and total cell count, squamous cell count, and level of cell viability were assessed using a Neubauer haemocytometer by the Tryptophan blue exclusion method. The cell suspension was adjusted with PBS to 0.5-0.75 x $10^6$ cells/ml and cytospins were prepared from 75 μl aliquots at 450 rpm (18.1g) for 6 minutes using the Shandon 3 cytocentrifuge (Shandon,U.K). The cytospins were stained in neat Romanowski stain for 5 minutes and fixed in dilute stain for 25 minutes. A differential cell count was obtained by counting > 400 non-squamous cells on a Romanowski stained cytospin.

## Quantitative bacteriology protocol

Sputum induction (as per protocol above) took place in the mornings. The sputum sample was taken to the laboratory (containment level 2) for immediate processing (as

per protocol above) which was carried out in safety cabinet. 50 microlitres of homogenised solution was removed and frozen at – 80 degrees centigrade for future DNA analysis. 300 microlitres of homogenised sputum solution was removed and placed into an Eppendorf marked NEAT. The remaining solution was used for differential cell count as described above. Serial dilutions were now prepared by pipetting 900 microlitres of PBS into 7 Eppendorfs. These were labelled $10^{-1}$ to $10^{-7}$. 100 microlitres was then removed from the neat solution and transferred to the $10^{-1}$ solution. This was then repeated from higher to lower dilutions, changing tips between each dilution, and vortexing solutions to ensure adequate mixing. Streak plates (chocolate, blood, and CLED media) were now prepared by using a plastic loop to coat the plates with neat solution. Plates were then labelled with; date, patient number and visit number using stickers provided for this study. Plates were then sent to the microbiology department at the Leicester Royal Infirmary for identification of bacterial pathogens. Using a pen, media plates (chocolate, and CLED) were divided into quadrants. Quadrants were labelled so that they corresponded with dilutions in the dilutional series. Starting in top left quadrant with $10^{-7}$ the plates were labelled clockwise. Plates were labelled with; date, patient number and visit number (using stickers). 20 microlitres of solution was pipetted 3 times per dilution into circles in each corresponding quadrant (see next page).

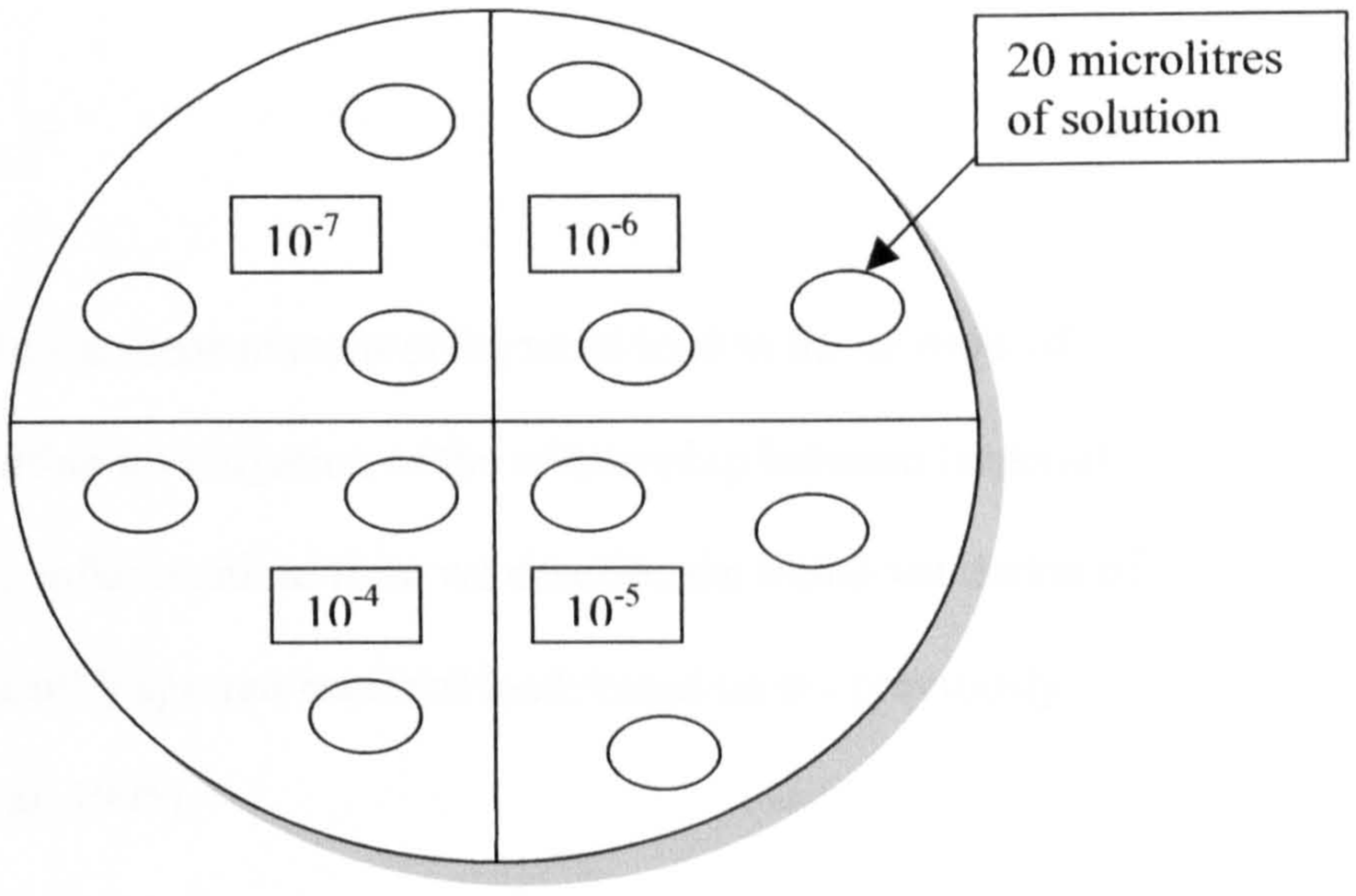



Figure 12.1: quantitative bacterial analysis; media plate

Plates were inverted and placed into an incubator for 24 hours. Incubation took place at 37 degrees centigrade with 5% $CO_2$. After 24 hours plates were inspected for bacterial growth. A quadrant with approximately 30 to 100 cfu was selected and individual colonies of bacteria were counted. The average count for the three drops in that quadrant was then calculated and multiplied by dilutional factors to obtain the final colony count. Plates were autoclaved prior to incineration.

## Validation of our quantitative bacterial analysis protocol

### Introduction

Well validated techniques for the measurement of bacterial load in the airways of patients are important for further investigation of the relationship between bacterial load and neutrophilic airway inflammation. Here we describe the initial validation of our simplified method to quantify sputum bacterial load, based on the previously mentioned protocol (Pye et al. 1995).

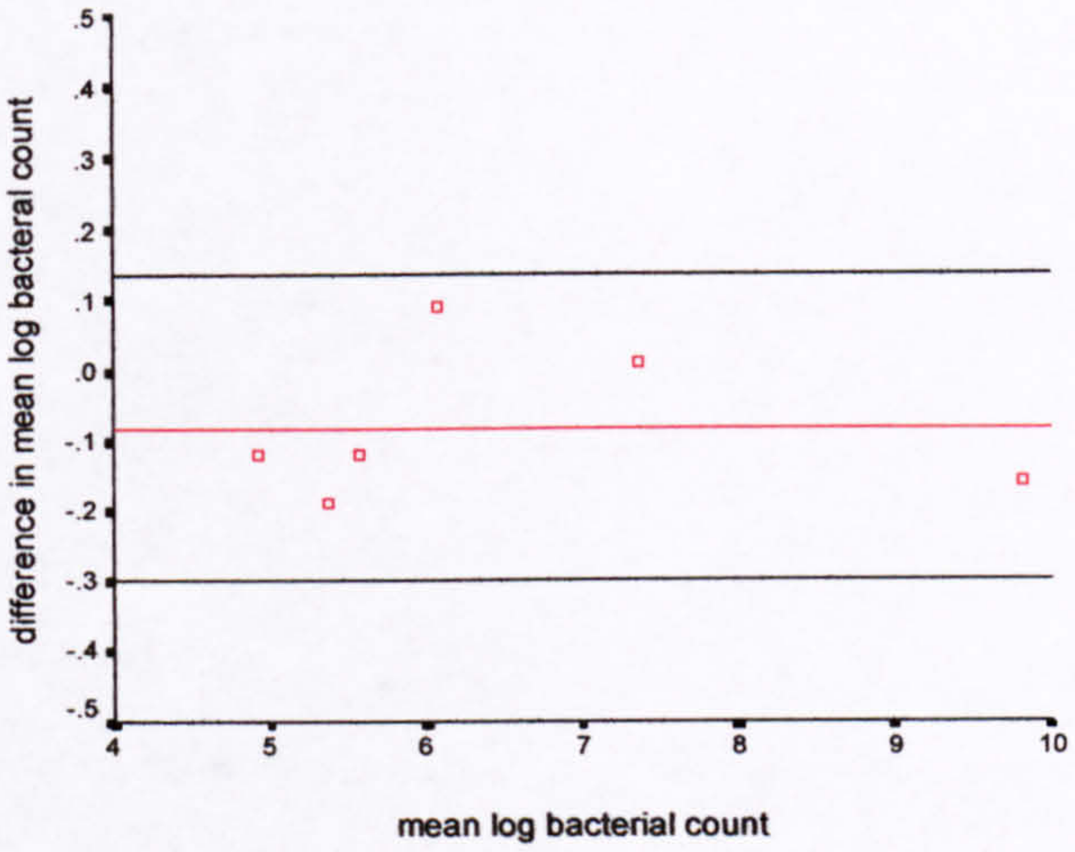
### Method

6 subjects (3 COPD, 3 normal; mean age 63, mean $FEV_1$/FVC 58.9%, mean % predicted $FEV_1$ 50) underwent sputum induction using standard methods. Sputum was homogenised using DTT and differential cell count was performed. The homogenised sputum was diluted with PBS to make 2 serial dilutions ranging from $10^{-1}$ to $10^{-7}$. The neat solution was used to inoculate streak plates for bacterial identification. Chocolate and CLED agar plates were divided into quadrants and 3 drops of each dilution were inoculated individually into each quadrant. Plates were then incubated for 24 hours with 5% $CO_2$. Plates were inspected and the quadrant where the number of colonies ranged between 30 and 100 was used to count the bacterial colonies. The average number of colonies per quadrant was used to calculate total bacterial load (see laboratory methods for full protocol).

Results

The geometric mean (range) of bacterial load (cfu/ml) was $2.5x10^6$ ($3.2 x10^4$ to $5.37 x10^9$). Within sample variation expressed as the intraclass correlation coefficient was 0.9632; 95% ci 0.8721 to 0.9894, $p<0.0001$, and log within subject standard deviation was 0.1074. There was a non-significant correlation between log bacterial load and sputum % neutrophil count; $r =0.71$, $p=0.116$. The following pathogens were cultured: Haemophilus Influenza, Streptococcus Pneumonia, Moraxhella Cataralis, Pseudomonas Aeroginosa, Candida, and coliform/bacillus species.

## Chocolate media




## CLED media

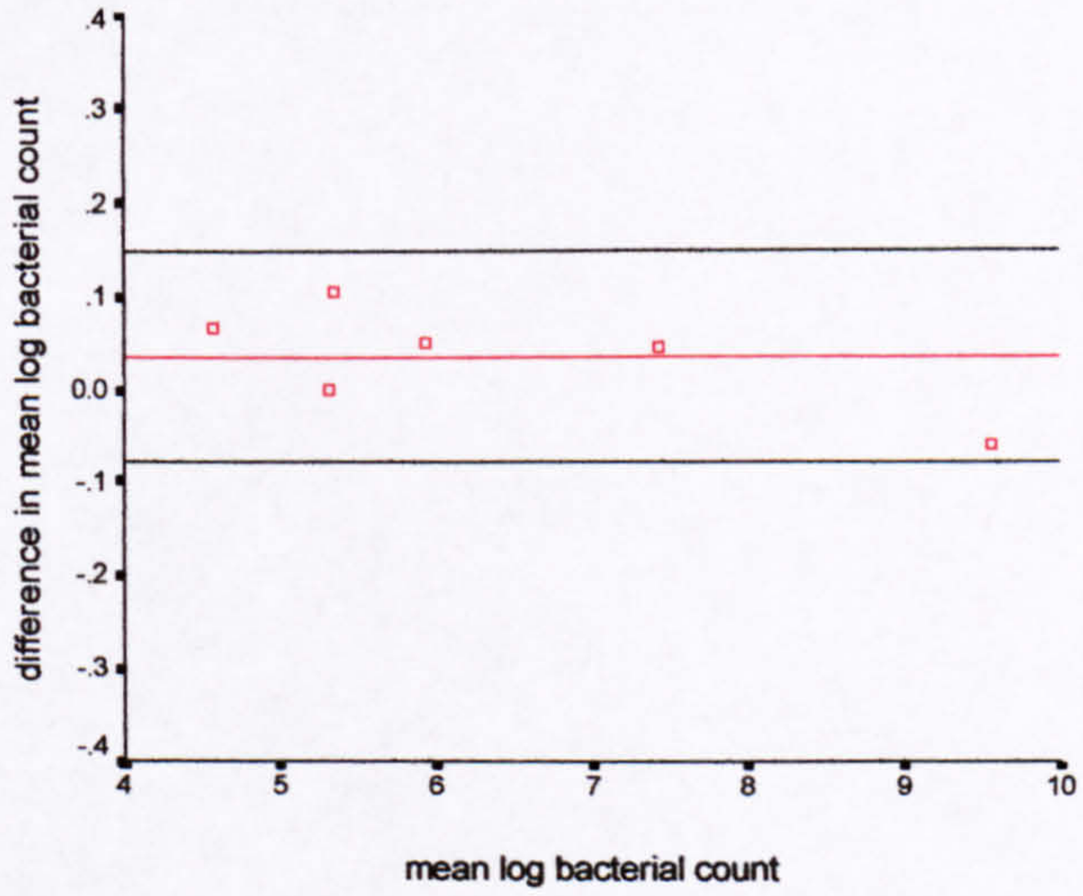



Figure 12.2: Bland-Altman plots to show range of variation in bacterial load measurements in paired samples

## Discussion

We have presented a quick and simple method for quantitative bacterial analysis and shown that within sample variability is minimal. All well known potentially pathogenic micro-organisms associated with bacterial infection in COPD were identified at sputum culture.

Our protocol was designed to be as rapid and as simple as possible and differs slightly from current validated methodology. Firstly we only used two different agar plates; chocolate and CLED. Despite this we were able to culture all the well known respiratory pathogens in adequate quantities. A second difference is that we counted bacteria only after 24 hours. There is therefore a theoretical possibility that we may be underestimating the number of any more slow growing bacteria.

Further validation of our protocol is required to show comparable efficacy to standard methods. We hope the introduction of simpler techniques will help us in our investigation of the relationship between bacterial load, airway inflammation, and response to therapies.

TREM-1 ELISA Protocol

Products required:

- Plates: COSTAR High Binding EIA plates with lids (Code 3361)
- Bovine Serum Albumin (Fraction V) from Fisher Scientific (Code BP1600-100)
- Phosphate Buffered Saline for assay diluent and blocking buffer from Sigma (Code D8537)
- Capture antibody: Monoclonal anti-human TREM-1 from R&D Systems (Code MAB1278)
- Recombinant human TREM-1/Fc Chimera from R&D Systems (Code 1278-TR)
- Detection antibody: Biotinylated anti-human TREM-1 from R&D Systems (Code BAF1278)
- Wash Buffer: PBS + 0.05% Tween 20
- Block Buffer: PBS + 1% BSA + 5% Sucrose
- Assay diluent: PBS + 1% BSA

Step by step guide:

1. Coat plates overnight at room temperature with 2.5ug/ml capture antibody in PBS

   100ul/well.

   50ul @ 500ug/ml Stock (50ul+ 9950ul)
2. Wash x 3
3. Add 300ul/well of block buffer and incubate for 1 hour at room temperature.
4. Wash x 3
5. Make up standards 200, 100, 50, 25, 12.5, 6.25 & 3.125 pg/ml in assay diluent with 0.01% DTT. 20ul 1% DTT + 1980ul diluent (equivalent to the concentration of DTT in a 1 in 4 dilution of sputum supernatant). Use assay diluent to dilute samples

to 1 in 4. Add 100ul/well of assay diluent with 0.01% DTT to the first row of wells on the plate for blank values then add 100ul/well of standards and samples and incubate for 2 hours at room temperature.

15ul @ 100ug/ml Stock

1in100=1000ng/ml (5ul + 495ul)

1in100=10000pg/ml (5ul + 495ul)

1in50=200pg/ml (20ul + 980ul)

6. Wash x 3
7. Add 100ul/well of 200ng/ml biotinylated detection antibody in assay diluent and incubate for 2 hours at room temperature.

   50ul @ 50ug/ml Stock (50ul + 12450ul)

8. Wash x 3
9. Add 100ul/well of a working dilution of Streptavidin-HRP (usually 1 in 200) in assay buffer and incubate for 20 minutes at room temperature.
10. Wash x 3
11. Add 100ul/well of TMB substrate solution and incubate for 30 minutes in the dark at room temperature.
12. Stop the reaction by adding 50ul/well of 2 N $H_2SO_4$.
13. Read plate at 450nm
14. Construct standard curve and use the equation for the linear part of the curve to calculate the unknowns.

## Study 1

# Modulation of eosinophilic airway inflammation and exacerbations of COPD: a randomised controlled trial

**Abstract**

Background

There is evidence to suggest that eosinophilic airway inflammation is important in the pathogenesis of severe COPD exacerbations. We hypothesised that a management strategy which aims to reduce the sputum eosinophil count is associated with a reduction in exacerbations of COPD.

Methods

82 patients with COPD aged 45-82, with a mean (s.d) $FEV_1$ % predicted of 38.2(15.3), and a 49.1(28.8) pack years smoking history, were randomised into two groups. One group was treated according to traditional guidelines (BTS group, n=40), and the other with the additional aim of minimising eosinophilic airway inflammation, assessed using the induced sputum eosinophil count (sputum group, n=42). Patients were seen and sputum eosinophils assessed nine times over 12 months. Primary outcome was exacerbations categorised as mild (self managed), moderate (resulting in unscheduled visit to clinic or G.P), or severe (resulting in hospitalisation).

Findings

The frequency of severe exacerbations/patient/year was 0.5 in the BTS group and 0.2 in the sputum group (mean reduction 62%; 95% C.I 5 to 72; p=0.037). There was no difference in the frequency of mild and moderate exacerbations. The average daily dose of inhaled or oral corticosteroid during the trial did not differ between the two groups. Out of 42 patients in the sputum group, 17 required regular oral corticosteroids to minimise eosinophilic airway inflammation; in 5 corticosteroids were stopped.

Interpretation

A management strategy that aims to minimise eosinophilic airway inflammation as well as symptoms is associated with a reduction in severe exacerbations of COPD.

Key words: Chronic obstructive pulmonary disease, corticosteroids, exacerbations.

## Introduction

Within 15 years COPD will be the fifth highest cause of morbidity and third highest cause of mortality worldwide (Pauwels et al. 2001). The clinical course of the disease is that of an accelerated decline in lung function, usually caused by cigarette smoking, resulting in increasing symptoms and disability, punctuated by exacerbations of the disease. These exacerbations contribute heavily to levels of morbidity and mortality and are responsible for significant reductions in quality of life (Seemungal et al. 1998). Whilst milder exacerbations account for a large part of the workload in primary care, it is severe exacerbations, particularly those which result in hospital admissions, which cause most morbidity and mortality, and are more expensive, costing European hospitals an estimated 3.4 billion Euros per year (ERS/ELF 2002). Exacerbations may be associated in the increased rate of progression of disease since studies show $FEV_1$ does not return to the pre-exacerbation level (Donaldson et al. 2002). Therefore, management strategies that are associated with a reduction in exacerbations of COPD are important since they are likely to be associated with a reduction in morbidity, mortality, cost and perhaps the rate of progression of the disease. Several therapeutic interventions including long acting Beta-agonists (Mahler et al. 1999), long acting anticholinergics (Casaburi et al. 2002), and combination inhalers (Calverley et al. 2003) have been shown to result in a modest reduction in exacerbation frequency.

Exacerbations are associated with an increase in airway inflammation and a decline in lung function. Although traditionally viewed as a neutrophil predominant inflammatory response, eosinophilic airway inflammation may play a role (Brightling

et al. 2000), particularly in more severe exacerbations of COPD. During exacerbations increased numbers of eosinophils have been detected both in induced sputum and in bronchial biopsies (Saetta et al. 1994), whilst a blood eosinophilia has been associated with increased mortality in COPD (Hospers et al. 1999). Moreover, corticosteroid treatment, which modulates eosinophilic airway inflammation but not neutrophilic airway inflammation (Brightling et al. 2000) is effective in the treatment (Davies et al. 1999) and prevention (Burge et al. 2000)(Paggiaro et al. 1998) of exacerbations of COPD. We have already shown that a management strategy, which minimised eosinophilic airway inflammation, resulted in a significant reduction in exacerbations and hospitalisations due to asthma (Green et al. 2002). We have tested the hypothesis that a similar management strategy is helpful in patients with COPD.

## Method

### Subjects

We invited consecutive patients who met the entry criteria at Glenfield hospital, between February 2003 and January 2004 to participate in the study. The diagnosis of COPD was based on a compatible history, and spirometry (Vitalograph ®, Buckinghamshire U.K) showing a post bronchodilator $FEV_1$/ FVC ratio of < 70%, and % predicted $FEV_1$ of < 80%. All patients had fixed airway obstruction as suggested by an $FEV_1$ increase of < 15% or if $FEV_1$ < 1.2L, <200ml 15 minutes after 400mcg of inhaled Salbutamol via a large volume spacer. We excluded patients who were aged under 45, those with a clinical history of asthma or acute wheeze, breathlessness or deterioration associated with allergens, and patients with clinically important co-morbidity such as heart failure, bronchiectasis or lung cancer. The study

was approved by the local research ethics committee and all patients gave written informed consent.

Measurements

The following baseline characteristics were recorded: age, sex, detailed smoking history, pulmonary rehabilitation status, BMI, serum IgE, Alpha-1-antitrypsin level, and blood eosinophil count. The use of corticosteroids, antibiotics, and number of hospitalisations due to COPD in the past year, validated by case note review, was also noted. Patients then had a chest X-ray, and measurement of exhaled Nitric Oxide, calculated from the best of three attempts at an exhalation flow rate of 250ml/s, with a chemiluminescence analyser (Logan Research, Rochester, U.K). Full pulmonary function tests were performed on all patients. Spirometry was performed using a Vitalograph® and taking the best of three readings. Gas transfer and total lung volumes were measured using the single breath hold carbon monoxide and helium dilution techniques respectively. Sputum induction was performed according to a standard protocol (Pavord et al. 1992). The patients filled in symptom diary cards and recorded morning peak flow everyday. Prior to each visit short acting Beta-agonists or anti-cholinergics and long acting bronchodilators were withheld for 6 and 12 hours respectively. At each visit patients underwent measurement of Nitric Oxide, spirometry before and fifteen minutes after 400mcg of inhaled Salbutamol via a large volume spacer device, and sputum induction. Quality of life was also assessed using the Chronic Respiratory Questionnaire (CRQ) (Guyatt et al. 1987). Patients symptoms were assessed with the aid of visual analogue scales (VAS). At each visit patients marked 3 lines each measuring 100mm which represented the symptoms of breathlessness, cough, and sputum production. We assumed a significant change in

symptoms and therefore a need to step up or step down treatment if the total VAS score differed by more than 34mm from the score at the baseline visit, since this is more than two standard deviations outside the limits of repeatability of this measure in patients with COPD (Brightling et al. 2001).

Protocol

In order to ensure optimal matching of groups, the process of minimisation (Treasure et al. 1998) was used by a third party (CEB) to randomise the patients into two groups stratifying patients according to % predicted $FEV_1$, baseline sputum eosinophil count, and whether patients had had a hospital admission for COPD in the previous year. Patients were followed up monthly for the first six months then every two months for the next six months. One group was treated according to a protocol designed to optimise symptoms (BTS management group), and the other according to a protocol designed to minimise eosinophilic airway inflammation as well as optimising symptoms (Sputum management group)(table 13.1.2A). Patients in both groups were informed of changes to treatment by telephone within 5 days of the previous visit. For the BTS management group the hierarchy of treatment was: short acting Beta-agonist PRN, regular anticholinergic, long acting Beta-agonist, long acting anticholinergic, Theophylline, and finally a trial with a nebuliser (table 13.1.2B). In this group, inhaled corticosteroids were continued if patients were already taking them. In other patients inhaled corticosteroids were commenced at visit 1 if there was a > 15% or 200ml improvement in $FEV_1$ following a 2 week course of 30mg of Prednisolone (as per guidelines at the time) (BTS guidelines for the management of COPD. 1997). For the sputum management group, patients followed the same hierarchy for symptom control as above but also followed an additional protocol designed to minimise the

sputum eosinophil count, by using the smallest appropriate dose of anti-inflammatory treatment. Our aim was to keep the sputum eosinophil count at < 3%, as there is little evidence of benefit of corticosteroids at below this level (Brightling et al. 2000) (Pavord et al. 1999). Where the sputum eosinophil count was > 3% anti-inflammatory treatment was increased. Where the count was between 1% and 3% anti-inflammatory treatment was not changed and where the count was < 1% anti-inflammatory treatment was reduced. The hierarchy for anti-inflammatory treatment (inhaled corticosteroid doses quoted as mcg Beclomethasone equivalent/day) was as follows: no corticosteroid, inhaled corticosteroid upto 400mcg, inhaled corticosteroid upto 800 mcg, inhaled corticosteroid upto 2000 mcg, oral Prednisolone 5mg daily, oral Prednisolone 10mg daily, and oral Prednisolone 30mg daily (table 13.1.2B). When the patient could not produce an adequate sputum sample, we used the concentration of Nitric Oxide in exhaled air as a surrogate marker of eosinophilc airway inflammation, and our aim was to achieve a Nitric Oxide level between 3 and 8 parts per billion using the same anti-inflammatory hierarchy of medication (Maziak et al 1998). Bias was avoided by ensuring that the investigators responsible for identifying exacerbations and determining clinical control, as well as the clinician responsible for changing treatment, were not aware of the randomisation status of the patients.

Exacerbations as defined below were monitored using symptom diary cards. Treatment was not stepped down if a severe exacerbation had occurred in the past month irrespective of symptoms. For this study the definition of an exacerbation of COPD was " a sustained worsening of the patients condition from the stable state and beyond normal day to day variations that is acute in onset and necessitates a change in regular medication in a patient with underlying COPD", characterised by either two or

more of three major symptoms: increasing breathlessness, sputum volume or sputum purulence , or any one major symptom together with any two minor symptoms: increase in nasal discharge, wheeze , sore throat, cough or fever. Exacerbations were recorded as; mild, moderate, or severe (Paggiaro et al. 1998) as follows:

Mild : confirmed by change in major and/or minor symptoms (see above) as noted in the diary card but successfully self managed at home.
Moderate : patient treated by G.P or made an unscheduled clinic attendance.
Severe : patient admitted to hospital.

Analysis

The primary endpoints were mild, moderate, and severe exacerbations of COPD as defined previously. Secondary endpoints were mean daily use of inhaled and oral corticosteroids, change in lung function, symptom visual analogue scores, and quality of life scores. The demographics of the two groups were compared by using simple descriptive statistics. Mean sputum eosinophil counts for the 12 months, expressed as total area under the curve, were compared using independent T tests. Frequency of severe exacerbations was analysed using Poisson regression with adjustment for the slight difference of baseline frequency of severe exacerbations in the previous year. Frequency of mild and moderate exacerbations did not fit a Poisson distribution; they were compared using negative binomial regression. Change in $FEV_1$, symptoms, quality of life, and total oral corticosteroid and inhaled corticosteroid usage was analysed by repeated measures ANOVA. Doses of inhaled corticosteroids have been expressed as Beclamethasone dose equivalents with Fluticasone considered to be twice as potent and Budesonide equipotent. The study was powered to have a > 80% chance at the 5% level of detecting a 25% reduction in moderate and a 66% reduction in severe exacerbations, based on a mean (s.d) estimated exacerbation frequency of 1.9 (2.6) per year (as per ISOLDE study) and our own audit data showing an

estimated severe exacerbation frequency of 0.6/patient/year. Data from patients who did not complete the study was analysed by intention to treat and extrapolated for the 12 month period. All data were analysed with SPSS for Windows (version 10.0) with the exception of data used for Poisson regression and negative binomial regression which were analysed using STATA for Windows (version 7.0).

**Results**

112 patients were approached of whom 90 agreed to participate in the study. The majority of patients who declined study entry did so for logistical reasons. Prior to randomisation, 3 patients withdrew without giving a reason, and 1 withdrew as he was started on dialysis. 1 patient died due to an exacerbation of COPD and 1 patient died of myocardial infarction. 2 patients were excluded as their lung function did not meet the entry criteria with 1 patient having a % predicted $FEV_1$ of > 80% and the other patient had significant bronchodilator reversibility. 82 patients were randomised into the two groups (figure 13.1.1). Patients were well matched for baseline characteristics (table 13.1.1). Sputum was successfully obtained at 603 out of 746 attempts (80.8 % success rate). There were no complications following any of the sputum induction procedures. Overall the mean sputum eosinophil count, expressed as total area under the curve, was not statistically significantly lower in the sputum management group (mean reduction 11%, 95% C.I -58 to 50, p=0.70). However when we stratified each group according to the baseline sputum eosinophil count, the sputum management was associated with a significant 63% reduction (95% C.I 21 to 83, p=0.01) in the sputum eosinophil count over 12 months in the subgroup with a baseline eosinophil

count of > 3% (n=23), (figure 13.1.2). The cut off of >3% was prospectively chosen to identify patients with a higher level of eosinophilic airway inflammation.

The total number of severe exacerbations was 20 in the BTS group and 8 in the sputum group. The route of admission was via general practitioner in 75% of cases and 999 emergency phone call in 25% of cases. The estimated mean (95% C.I) frequency of severe exacerbations/year in the BTS group was 0.5 (0.3 to 0.8) and 0.2 (0.1 to 0.4) in the sputum group. There was a significant reduction of 62% (95% C.I 5 to 72, p=0.037) in severe exacerbations between the BTS management group and the sputum management group (figure 13.1.3). Post hoc analysis identified that most of the benefit occurred in the subgroup who had a baseline sputum eosinophil count of >3%. In this subgroup (n=23), the frequency of severe exacerbations was 0.08 in the sputum group (n=12, 1 exacerbation in 1 patient) and 0.7 in the control group (n=11, 8 exacerbations in 3 patients). The estimated mean (95% C.I) frequency of mild exacerbations was 10.2 (9.2 to 11.2) and 7.9 (7.0 to 8.8) in the BTS group and sputum group respectively (mean reduction 23%, 95% C.I -14% to 49%; p=0.22). The estimated mean (95% C.I) frequency of moderate exacerbations was 2.8 (2.3 to 3.4) and 2.5 (2.1 to 3.1) in the BTS group and sputum group respectively (mean reduction 10%, 95% C.I -30% to 43%; p=0.66).

Overall there was no difference in the use of oral corticosteroids between the 2 groups. The mean (s.d) dose of oral Prednisolone per patient per day was 1.9mg (0.9) in the BTS group compared to 2.0mg (0.6) in the sputum group, (p=0.22). The mean (s.d) dose of inhaled corticosteroid (Beclomethasone equivalent dose per patient per day) was 1248mcg (25) in the BTS group compared to 976mcg (51) in the sputum

group. Although less inhaled corticosteroid was used in the sputum group throughout the study (p=0.001), the change in dose of inhaled corticosteroid between the 2 groups from baseline did not differ (p=0.22). Out of the 42 patients in our sputum management group, 17 required oral corticosteroids to reduce sputum eosinophils at some stage. 11 out of the 42 finished the study on less inhaled corticosteroid than they had started with and out of these 5 patients were completely weaned off their inhaled corticosteroid. There was no significant difference in change in post bronchodilator $FEV_1$, quality of life, or symptoms between the 2 groups (figure 13.1.4). In order to measure the success of our blinding procedures, patients were asked to guess which group they thought they had been allocated to. 28 patients guessed correctly, 28 patients guessed incorrectly, and 16 patients were unsure.

**Discussion**

We have shown that a management strategy that aims to minimise eosinophilic airway inflammation as well as symptoms is associated with a significant reduction in the frequency of exacerbations of COPD requiring hospital admission. The management strategy was associated with no overall increase in the use of inhaled or oral corticosteroids although there was evidence that increased corticosteroid therapy was targeted to patients with eosinophilic airway inflammation in the intervention group. We saw no difference in the frequency of mild, self-managed exacerbations or in the frequency of moderate exacerbations requiring G.P or unscheduled clinic review.

Our findings suggest an association between eosinophilic airway inflammation and severe exacerbations of COPD. This interpretation is consistent with earlier work identifying increased eosinophilic airway inflammation at the time of a COPD exacerbation (Saetta et al. 1994) and epidemiological evidence of an association between the peripheral blood eosinophil count and death from exacerbations of COPD (Hospers et al. 1999). Corticosteroid therapy appears to have a selective inhibitory effect on eosinophilic airway inflammation in COPD (Brightling et al. 2000, 2005) and further support for a role of eosinophilic airway inflammation in the genesis of exacerbations of COPD is provided by consistent evidence that corticosteroid treatment increases the rate of recovery from severe exacerbations (Davies et al. 1999) and prevents the occurrence of severe exacerbations (Paggiaro et al. 1998). Whether eosinophilic airway inflammation is causally associated with the exacerbation or indicates the presence of another corticosteroid responsive mechanism is unclear. Further studies with a more selective inhibitor of eosinophilic airway inflammation such as antibodies to anti-IL-5 (Leckie et al. 2000) may help answer this question.

The absence of an effect of our management strategy on mild and moderate exacerbations suggests that the mechanism of these events might differ from the mechanism of severe exacerbations of COPD. There is some support for this view since treatment with inhaled fluticasone is associated with a greater reduction in severe exacerbations of COPD than less severe events (Paggiaro et al 1998). Inhaled corticosteroid therapy are also more effective in patients with more severe airflow obstruction (Jones et al. 2003) and it remains possible that the underlying pathology

and corticosteroid responsiveness of COPD exacerbations differ with increasing disease severity. Future studies should investigate this possibility.

There are parallels between our findings in patients with predominantly severe COPD and our earlier findings in a study of patients with severe asthma (Green et al. 2002), where a similar management strategy was associated with a 68% reduction in severe exacerbations and a marked reduction in the number of exacerbations requiring hospital admissions. This suggests that there might be similarities in the mechanism of exacerbations of COPD and severe asthma. However, one important difference in the findings of the studies is that we were less successful in reducing the sputum eosinophil count in our intervention group in patients with COPD than we were in asthma. This is likely to be because a sputum eosinophilia was a less consistent feature in patients with COPD than in asthma. it appeared that around a third of patients had persistent sputum eosinophilia, a third of patients never showed sputum eosinophilia and the remaining third showed variable levels of sputum eosinophils. Another possibility is that COPD is associated with a degree of inhaled corticosteroid resistance, perhaps because the functionally important eosinophilic airway inflammation is confined to the distal airways (Hogg et al. 2004) (Berry et al. 2005). It is notable that when we confined our analysis to patients with a baseline sputum eosinophil count of > 3%, we saw a significant decrease in the sputum eosinophil count over the 12 months of the study and much of the benefit was confined to this subgroup. Interestingly, it was often necessary to use long-term oral corticosteroid therapy to achieve this.

Another potential limitation of our study is that sputum differential cell counts were not available on around 20% of visits. We elected to use exhaled nitric oxide as a surrogate marker of eosinophilic airway inflammation. We acknowledge that it may be an imperfect marker, particularly in current smokers (Maziak et al. 1998)(Berry et al. 2005). However our approach is supported by evidence that exhaled nitric oxide is more closely related to a positive response to corticosteroid therapy than other clinical markers in patients with airways disease in general (Smith et al. 2005) and COPD in particular (Papi et al. 2000).

It was not possible to conduct our study in a truly double blind fashion. However, we were careful to ensure that the clinician making decisions about clinical control was blind to the patients' randomisation status and that management decisions were protocol driven. Moreover, decisions about hospitalisation and management of exacerbations were largely made by the patient or their primary care physician, who were both blind to the randomisation status. We based our decisions about clinical control on the patients' response to a previously validated symptom visual analogue score (Brightling et al. 2001). We believe that this strategy, and our management hierarchy, is in keeping with the management strategy advocated by guidelines at the time although the possibility that tighter control of symptoms might have been associated with better control of exacerbations cannot be excluded.

Our study aim was to investigate the effects of modulation of eosinophilic airway inflammation on outcome in COPD; we did not set out to evaluate the clinical utility of our management strategy and we have not performed a cost-benefit analysis. Although sputum induction appears to be safe in patients with severe COPD

(Brightling et al. 2001), processing of induced sputum is a relatively complex procedure associated with significant technician time and expense. The clinical utility of a management strategy incorporating measurements of eosinophilic airway inflammation is likely to be greater if the technique can be simplified and made to provide more immediate results. Our findings should stimulate the development of such techniques.

| | Group 1: BTS | Group 2: Sputum |
|---|---|---|
| N | 40 | 42 |
| Age (range) | 70 (49-80) | 68 (45-82) |
| Sex M:F | 30:10 | 25:17 |
| Smoking; Current : ex : never | 8 : 31 : 1 | 12 : 30 : 0 |
| Pack years | 47.5 (27.8) | 50.6 (30.0) |
| $FEV_1$ (L) | 1.07 (0.44) | 0.96 (0.47) |
| FVC (L) | 2.28 (0.84) | 2.11 (0.67) |
| Post B.D $FEV_1$ (L) | 1.14 (0.48) | 1.04 (0.50) |
| % Predicted $FEV_1$ | 38.4 (15.5) | 38.1 (15.4) |
| TLC (% predicted) | 98.6 (15.3) | 99.7 (15.4) |
| RV (% predicted) | 123.6 (38.1) | 133.1 (47.1) |
| KCO (% predicted) | 75.0 (17.9) | 65.5 (22.9) |
| BMI | 26.1 (3.5) | 26.0 (0.2) |
| Baseline sputum eosinophil (%)* | 1.5 (0.6) | 1.7 (0.6) |
| Blood eosinophils x $10^9$/ L | 0.24 (0.24) | 0.20 (0.15) |
| Baseline inhaled steroid dose – BDP equivalent (mcg/day) | 1200 (1007.6) | 1024 (902.2) |
| Number of patients admitted in previous year | 12 | 12 |
| Rate of admission/patient/year | 0.55 (0.7) | 0.43 (0.6) |
| Attended pulmonary rehab within 1 year prior to study | 10 | 9 |

Table 13.1.1: patient demographics, mean (s.d) except where stated

*geometric mean

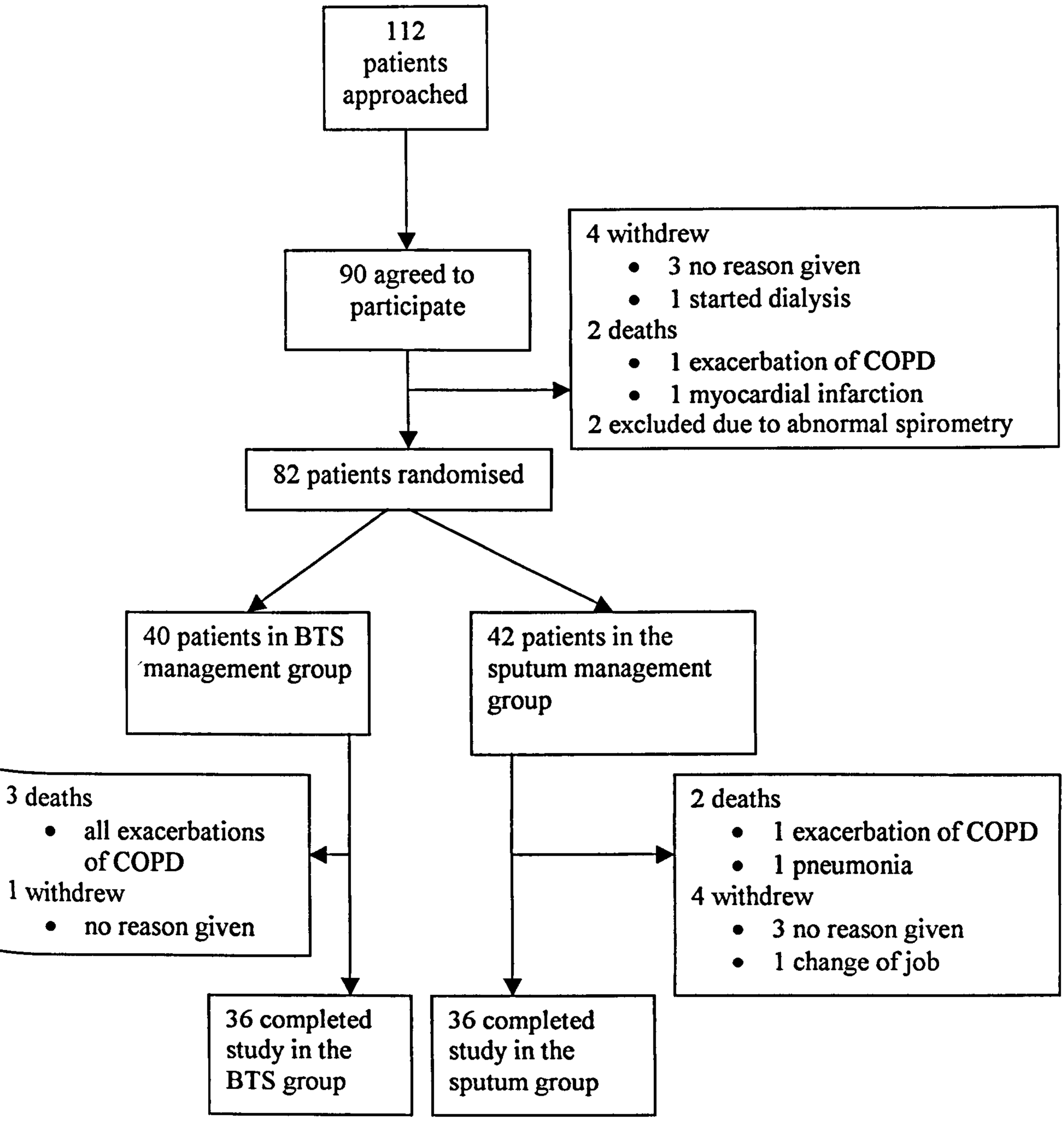



Figure 13.1.1: trial profile

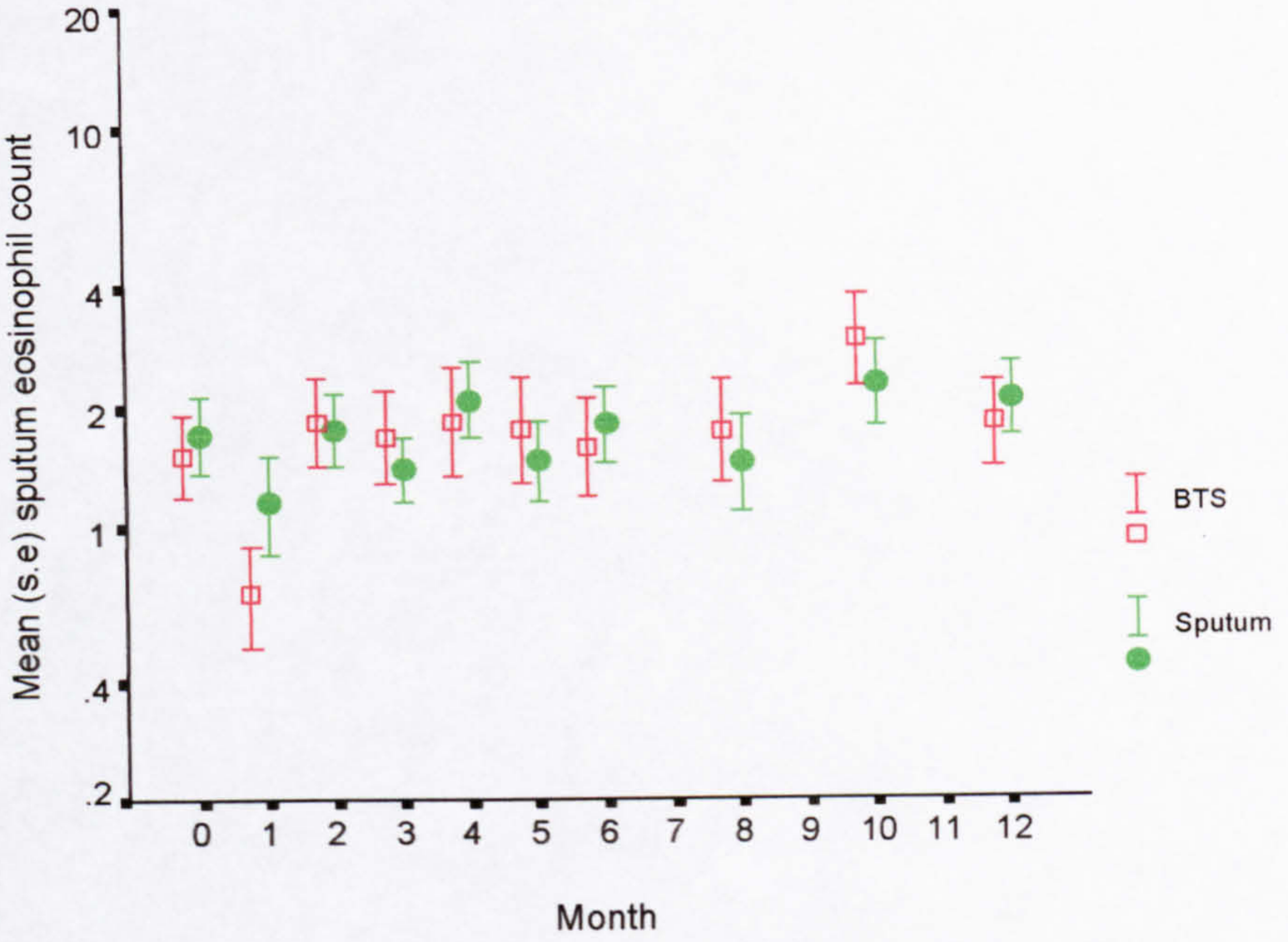
| Symptoms | Sputum eosinophils < 1% | Sputum eosinophils 1% - 3% | Sputum eosinophils > 3% |
|---|---|---|---|
| Improved | Decrease bronchodilator<br>Decrease anti-inflammatory | Decrease bronchodilator<br>No change anti-inflammatory | Decrease bronchodilator<br>Increase anti-inflammatory |
| No change | No change in bronchodilator<br>Decrease anti-inflammatory | No change in bronchodilator<br>No change anti-inflammatory | No change in bronchodilator<br>Increase anti-inflammatory |
| Worse | Increase bronchodilator<br>Decrease anti-inflammatory | Increase bronchodilator<br>No change anti-inflammatory | Increase bronchodilator<br>Increase anti-inflammatory |

Table 13.1.2A: treatment algorithm

| | Bronchodilator | Anti-inflammatory |
|---|---|---|
| Step 1 | No treatment | No treatment |
| Step 2 | Short acting beta-agonist as required | ICS upto 400 mcg |
| Step 3 | Regular short acting anti-cholinergic | ICS upto 800 mcg |
| Step 4 | Long acting beta-agonist | ICS upto 2000 mcg |
| Step 5 | Long acting anti-cholinergic | Prednisolone 5 mg |
| Step 6 | Theophylline | Prednisolone 10 mg |
| Step 7 | Nebuliser trial | Prednisolone 30 mg |

Table 13.1.2B: bronchodilator and anti-inflammatory treatment hierarchy

(A)




(B)

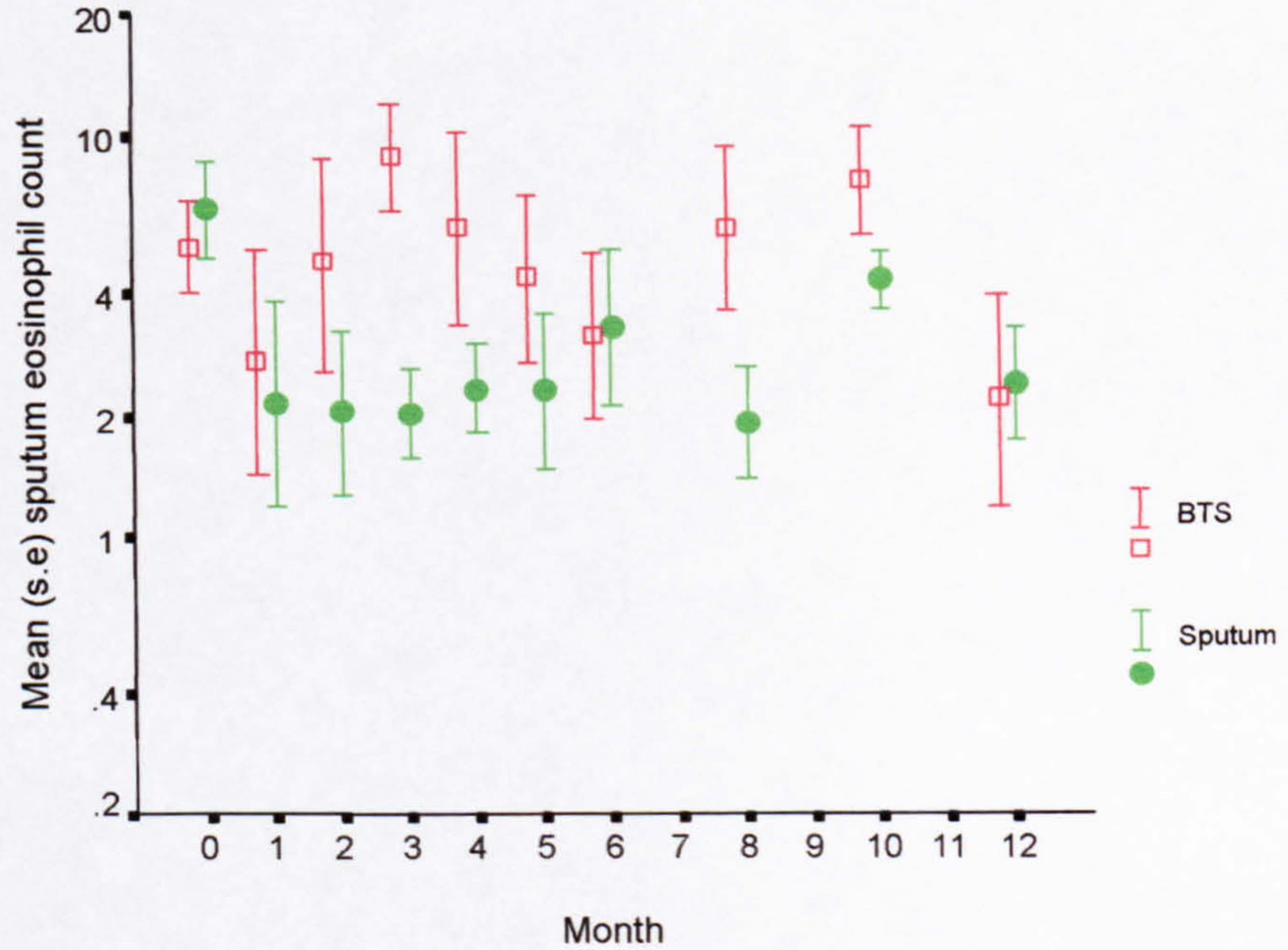



Figure 13.1.2: geometric mean (s.e) sputum eosinophil counts per month for (A): all patients, and (B): patients with baseline sputum eosinophil count > 3%.

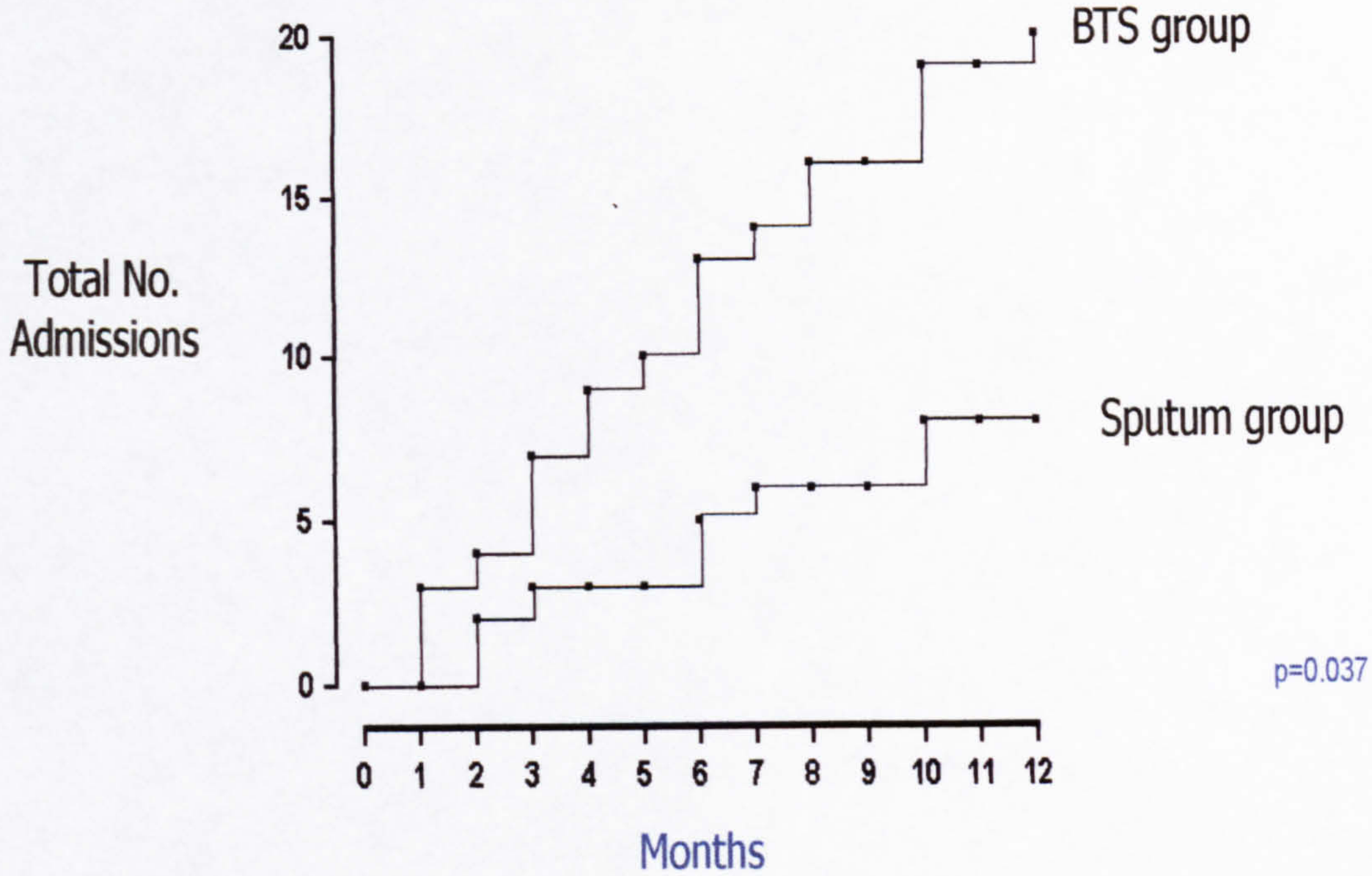



Figure 13.1.3: hospital admissions in each group

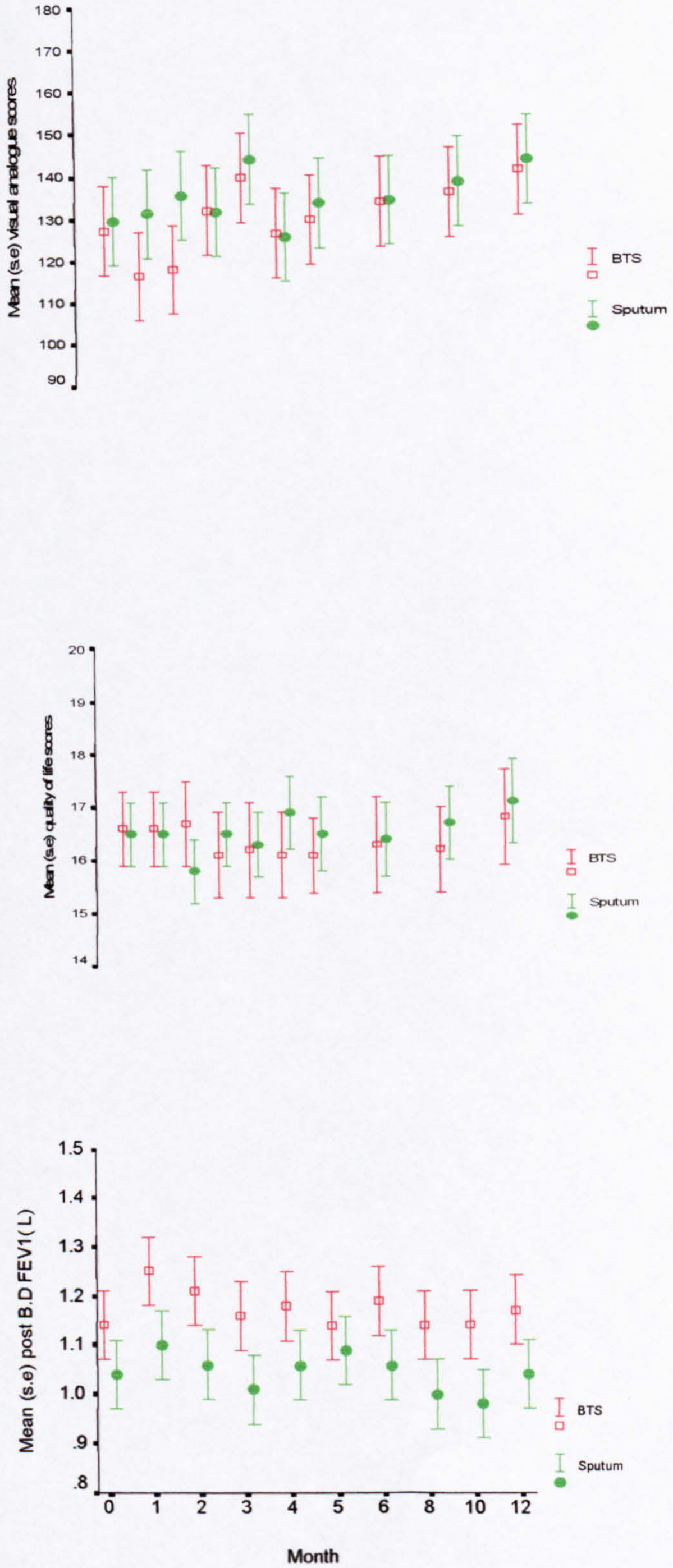



Figure 13.1.4 mean (s.e) symptom scores, quality of life scores and $FEV_1$ over 12 months

# Study 2

# The effect of Levofloxacin on neutrophilic airway inflammation in stable COPD: a randomised, double blind, placebo controlled trial.

## Abstract

Rationale

Airway inflammation persists following smoking cessation in established COPD, suggesting that other factors drive airway inflammatory response.

Objectives

We have tested the hypothesis that high levels of bacterial colonisation are associated with increased levels of neutrophilic airway inflammation in stable COPD by examining the cross-sectional relationship between these measurements and by conducting a randomised, double blind, placebo controlled study of the effect of Levofloxacin in patients with stable state COPD.

Methods

Patients were randomised to either Levofloxacin 500mg daily or placebo for 7 days and underwent spirometry, and sputum induction for a differential cell count and quantitative bacterial analysis at baseline and at days 7, 14, and 28.

Results

Sputum % neutrophil count correlated with airway bacterial load at baseline ($r = 0.56$, $p=0.003$). Levofloxacin reduced bacterial load compared to placebo by 4.9 fold (95% C.I 1.4:25.7, $p=0.02$) at day 7 but had no effect at any time point on any marker of neutrophilic airway inflammation. In subjects with a baseline bacterial load of $> 10^6$ cfu/ml, Levofloxacin treatment was associated with a 26.5% (95% C.I; 1.8: 51.3, $p=0.04$) greater reduction in the % neutrophil count compared to placebo at day 7. Change in % neutrophil count correlated significantly with baseline airway bacterial load and change in airway bacterial load.

Conclusions

In stable COPD, Levofloxacin treatment causes a short term reduction in bacterial load. This is associated with a reduction in neutrophilic airway inflammation in patients with high bacterial loads. Further studies are required to investigate whether this effect is clinically advantageous.



Key words: bacteria, antibiotics, sputum

## Introduction

COPD is a slowly progressive chronic inflammatory disease affecting mainly the airways, characterised by an accelerated decline in lung function (Pauwels et al. 2001). The main aetiological agent associated with developing COPD is cigarette smoke and so far smoking cessation is the only intervention that has been proven to slow down this accelerated decline in lung function (Fletcher et al. 1977). However, the rate of decline of lung function is variable amongst patients and other sources of local (Wilkinson et al. 2003) and systemic (Kanazawa et al 2003) inflammation, may contribute to decline in lung function. In keeping with this view, airway inflammation has been shown to persist following smoking cessation in patients with established COPD (Turato et al. 1995).

Neutrophilic airway inflammation has been shown to be associated both cross-sectionaly with $FEV_1$ (Di Stefano et al. 1998) and longitudinally with decline in $FEV_1$ (Stanescu et al. 1996) suggesting a role in the genesis of the small airway inflammation and fibrosis that leads to the progressive airflow obstruction seen in COPD (Hogg et al. 2004). One potentially modifiable cause of neutrophilic airway inflammation is bacterial infection of the airway. The presence of potentially pathogenic micro-organisms (PPM) has been associated with higher levels of markers of neutrophilic airway inflammation during stable disease (Banerjee et al. 2004) and at exacerbation (Patel et al. 2002). Moreover, uncontrolled studies have shown that reduction of bacterial load with antibiotic therapy is associated with resolution of neutrophilic airway inflammation in patients studied during an exacerbation (White et al. 2003). Less is known about the relationship between bacterial colonisation of the

airway and neutrophilic airway inflammation in stable COPD. We have tested the hypothesis that high levels of bacterial colonisation are associated with increased levels of neutrophilic airway inflammation in stable COPD by examining the cross-sectional relationship between these measurements and by conducting a randomised, double blind, placebo controlled study of the effect of a one week course of Levofloxacin on bacterial load and markers of neutrophilic airway inflammation in patients with stable state COPD.

## Method

### Subjects

We recruited 27 consecutive patients who met the entry criteria at Glenfield hospital, between February 2004 and March 2005. The diagnosis of COPD was based on a compatible history, and spirometry (Vitalograph ®, Buckinghamshire U.K) showing a post bronchodilator $FEV_1$/ FVC ratio of < 70%, and % predicted $FEV_1$ of < 80%. All patients had fixed airway obstruction as suggested by an $FEV_1$ increase of < 15% or if $FEV_1$ < 1.2 l, <200ml, 15 minutes after inhaled Salbutamol 400mcg via a large volume spacer. All patients had filled in diary cards to ensure they were free of exacerbation as defined using Anthonisen criteria (Anthonisen et al. 1987) for the 6 weeks preceding the study. We excluded patients who were aged under 45, if they had a clinical history of asthma or acute wheeze, breathlessness or deterioration associated with allergens, or clinically important co-morbidity such as heart failure, bronchiectasis or lung cancer. The study conformed to the Declaration of Helsinki and was approved by the local research ethics committee. All patients gave written informed consent.

Measurements

The following baseline characteristics were recorded: age, sex, BMI, serum IgE, alpha-1-antitrypsin level, smoking history, validated by review of the medical records, and co-morbid history. All patients underwent a chest x-ray and full pulmonary function testing. Prior to spirometry, short acting Beta-agonists or anti-cholinergics and long acting bronchodilators were withheld for 6 and 12 hours respectively. Spirometry was always carried out in the morning and performed by taking the best of three readings using a Vitalograph® before and fifteen minutes after inhalation of Salbutamol 400mcg via a large volume spacer device. Gas transfer and total lung volumes were measured using the single breath hold carbon monoxide technique and helium dilution technique respectively. At each visit patients underwent spirometry, sputum induction according to a standard protocol (Pavord et al. 1992), diary card review, and assessment of symptoms using visual analogue scales where patients marked 3 lines each measuring 100mm which represented the symptoms of breathlessness, cough, and sputum production (Brightling et al. 2001). Sputum was processed according to a standard protocol (Pavord et al. 1992) and analysed for differential cell count by counting > 400 non-squamous cells on a Romanowski stained cytospin. A quantitative bacterial count was performed by using homogenised sputum to create a dilutional series which was then pipetted onto media plates and incubated for 24 hours. Bacterial colonies were identified and counted in order to calculate airway bacterial load; expressed as colony forming units per millilitre of sputum (cfu/ml) (Pye et al. 1995). Sputum supernatants were frozen at –80 degrees centigrade for future analysis of IL-8 concentrations which were measured by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (R&D Systems Europe, Abingdon, U.K). Previous validation of our assay showed a

limit of detection of IL-8 of 1.5μg/ml of sputum and between-assay and within-assay variabilities were between 5% and 10% (Brightling et al. 2000)

Protocol

At the baseline visit patients were randomised to receive either a 7 day course of Levofloxacin 500mg once daily or matched placebo in a randomised, double blind fashion. The randomisation process was carried out by the Royal Hallamshire Hospital who supplied the medication. The study codes were retained by our pharmacy who distributed the medications. The patients were followed up in the morning, at least 2 hours after taking their study medication, on days 7, 14, and 28 and measurements were repeated as stated above. Patients were asked to complete symptom diary score cards from the baseline visit to day 28.

**Analysis**

The primary endpoints were change in sputum % neutrophil count, change in sputum IL-8 concentration, and the relationship between airway bacterial load and sputum % neutrophil count. Secondary endpoints were change in sputum total neutrophil count, symptoms, and post bronchodilator $FEV_1$. The demographics of the two groups were compared using simple descriptive statistics. Changes in % neutrophil count, total neutrophil count, bacterial load, and IL-8 between each group were compared using unpaired T tests. The relationships between change in % neutrophil count and baseline bacterial load, as well as change in % neutrophil count and change in

bacterial load were analysed using Spearmans Rank correlation. The study was powered to have a greater than 80% chance of detecting a 20% difference in the % neutrophil count or a 2 fold difference in IL-8 with Levofloxacin compared to placebo at the 5% significance level (Brightling et al. 2001). All data was analysed with SPSS for Windows (version 10.0). All data was analysed with intention to treat; missing data was assigned using measurements extrapolated from the last available visit.

**Results**

The patients were well matched at baseline (table 13.2.1). 2 patients in the Levofloxacin group withdrew from the study prior to day 7 due to Achilles tendonitis. Otherwise sputum was successfully obtained at all visits. At baseline there was a correlation between the sputum % neutrophil count and log airway bacterial load ($r = 0.56$, $p = 0.003$) (figure 13.2.1). At day 7, the bacterial count increased from $1.1 \times 10^6$ cfu/ml to $1.5 \times 10^6$ cfu/ml with placebo and decreased from $0.7 \times 10^6$ cfu/ml to $0.2 \times 10^6$ cfu/ml with Levofloxacin. There was a 4.9 (95% C.I 1.4:25.7, p=0.02) fold difference in the change in bacterial load between the groups. Overall there was no effect of Levofloxacin at any time point on any markers of neutrophilic airway inflammation (table 13.2.2). By day 7 there was a 1.3% reduction (95% C.I -11.7:14.3) in % neutrophil count with placebo, and a 12% reduction (-5.9:30.1) in % neutrophil count with Levofloxacin (mean difference 10.8%; 95% C.I; -9.7: 31.2; p=0.29). However, we then confined our analysis to patients with a baseline bacterial load > $x 10^6$ cfu/ml, chosen prospectively as bacterial load above this level has been shown to be associated with increased neutrophilic airway inflammation (Stockley et

al. 2004). In this group Levofloxacin treatment was associated with a 26.5% (95% C.I; 1.8: 51.3, p=0.04, n=12) greater reduction in the % neutrophil count compared to placebo, and a 46.8 ng/ml (95% C.I -11.2:104.8, p=0.11, n=12) reduction in sputum IL-8 concentration. There was no evidence of a treatment effect at any other time (table 13.2.2).

Following Levofloxacin treatment there was a strong and significant correlation between change in % neutrophil count and baseline bacterial load (r = -0.78, p=0.003) and between change in % neutrophil count and change in bacterial load (r = 0.85, p=0.001). No such relationships were seen following placebo (figure 13.2.3).

When sputum culture at baseline revealed the presence of a potentially pathogenic micro-organism (PPM), the reduction in % neutrophil count following Levofloxacin at day 7 was 45.0 % (n=3) compared to 2.6% when there was non specific growth (n=9), (mean difference 42.3%, 95% C.I 15.5:69.0, p=0.006).

There was no difference between the groups in change in mean post bronchodilator $FEV_1$ or mean visual analogue score from baseline to day 7 (28ml; 95% C.I -70:127; p=0.56) and (2.6; 95% C.I -12.7: 17.3; p=0.71) respectively, or at any other time.

**Discussion**

We have shown a strong correlation between sputum % neutrophil count and baseline airway bacterial load and evidence that a reduction in bacterial load is associated with

a reduction in neutrophilic airway inflammation in patients with high airway bacterial loads. This effect was only evident at day 7. There were correlations between change in % neutrophil count and baseline bacterial load and change in % neutrophil count and change in bacterial load. These effects were not associated with improvements in symptoms or $FEV_1$.

Our findings are consistent with previous studies showing an increase in markers of neutrophilic airway inflammation associated with airway bacterial loads of > $10^6$ cfu/ml (Stockley et al. 2000) and with work showing that levels of neutrophilic airway inflammation are only suppressed for a short period of time following a course of antibiotics in patients with COPD (White et al. 2003). Our findings in stable COPD are consistent with an earlier uncontrolled trial in patients with stable bronchiectasis, where there was evidence that neutrophil elastase concentration was decreased following treatment with Amoxycillin (Stockley et al. 1984). The similar time course of effect of Levofloxacin on bacterial load and sputum neutrophils, and the correlation between change in sputum neutrophils and baseline bacterial load and change in bacterial load strongly supports a causal link between airway bacterial colonisation and neutrophilic airway inflammation. These findings are important as they represent the first demonstration in a placebo controlled trial, that antibiotic therapy is capable of modulating neutrophilic airway inflammation in stable COPD.

We did not see any obvious clinical benefit with Levofloxacin therapy during the study although our study was not designed to show this. The long term consequences of the effect of Levofloxacin on neutrophilic airway inflammation and important clinical outcomes such as decline in lung function, quality of life, and symptoms,

remain unclear. However, a relationship between neutrophilic airway inflammation, decline in $FEV_1$, and airway bacterial load is supported by a study which showed that an increase in bacterial load over 12 months was related to a greater decline in $FEV_1$ (Wilkinson et al. 2003). Higher levels of sputum IL-8 and a change in colonising bacterial type were also associated with a greater decline in $FEV_1$. These findings raise the possibility that reduction of airway bacterial load and neutrophilic airway inflammation with antibiotic therapy might be associated with a reduction in disease progression.

Our study was also not designed to show whether the effects of Levofloxacin were related to infection with a potentially pathogenic micro-organism. Potentially pathogenic micro-organisms tended to be present in high concentrations and reduction in sputum neutrophils with Levofloxacin was particularly marked in these patients. So it is possible that the effect of Levofloxacin was due to a reduction in potentially pathogenic micro-organisms only. Further, larger, studies are needed to investigate this.

We chose to use Levofloxacin because it has a broad spectrum of activity against common respiratory pathogens, it is given only once daily, it can be given to patients who are allergic to penicillin, and it is less likely to cause antibiotic resistance (Lode et al. 2002). Whilst the use of antibiotics in the treatment of exacerbations of COPD remains controversial, even more controversial is the idea that antibiotics could play a significant role in stable disease. Any potential benefits achieved by the increased use of antibiotics would have to be balanced against growing levels of bacterial antibiotic resistance and potential side effects, such as Achilles tendonitis and Clostridium

Difficile diarrhoea which may potentiate the considerable morbidity which already exists amongst these patients. However our findings do provide a strong basis for trials investigating the effect of long term antibiotic therapy on clinically relevant outcomes in selected patients with stable COPD.

| | Placebo | Levofloxacin |
|---|---|---|
| N | 13 | 14 |
| Age (range) | 68 (45-80) | 65 (52-74) |
| Sex M:F | 13:0 | 10:4 |
| Smoking status; Current : ex | 3 : 10 | 7 : 7 |
| Smoking pack years | 49.0 (29.7) | 49.0 (29.0) |
| $FEV_1$ /L | 1.2 (0.4) | 1.2 (0.4) |
| FVC /L | 2.5 (0.6) | 2.3 (0.6) |
| Post bronchodilator $FEV_1$ /L | 1.3 (0.4) | 1.3 (0.4) |
| Post bronchodilator % predicted $FEV_1$ | 43.8 (13.7) | 48.0 (16.6) |
| TLC (% predicted) | 98.0 (18.7) | 97.7 (15.1) |
| RV (% predicted) | 126.9 (60.4) | 133.8 (45.7) |
| KCO (% predicted) | 66.0 (26.5) | 74.1 (18.6) |
| BMI | 25.7 (4.3) | 24.4 (3.5) |
| Sputum total cell count (x $10^6$/g) | 2.62 (1.8) | 3.8 (2.3) |
| Sputum neutrophil count (%) | 66.2 (19.8) | 70.4 (21.4) |
| Sputum total neutrophil count (x $10^6$/g*) | 1.3 (0.4) | 2.0 (0.5) |
| Airway bacterial load (x $10^6$ cfu/ml*) | 1.1 (0.8) | 0.7 (0.9) |

Table 13.2.1: patient demographics at baseline. Mean (S.D) unless stated

* geometric mean and log S.D

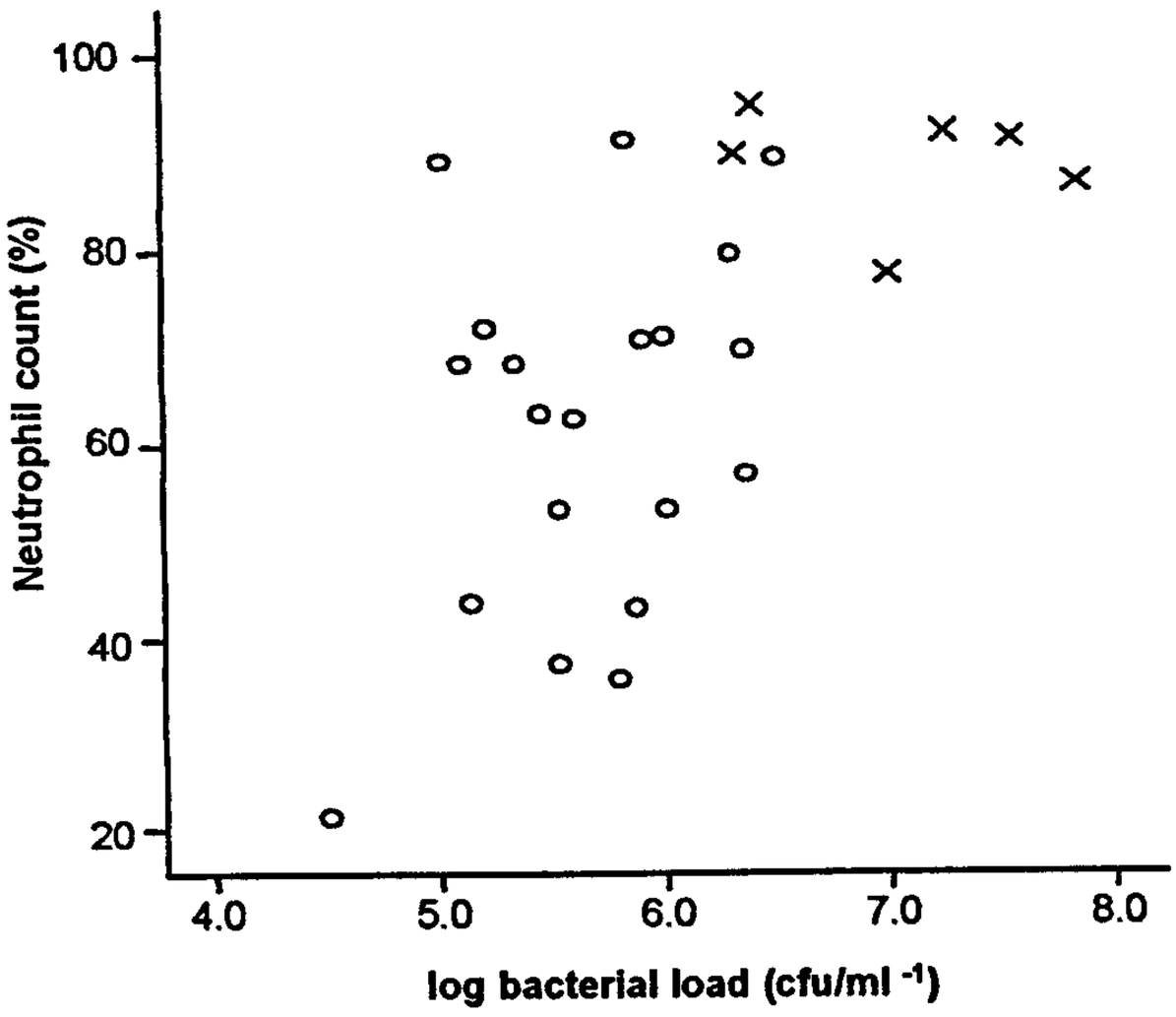



Figure 13.2.1: relationship between airway bacterial load and % neutrophil count at baseline. (**X** = PPM, **O** = Non specific growth)

$r = 0.56$, $p = 0.003$.

| | Treatment | Baseline | Day 7 | Day 14 | Day 28 |
|---|---|---|---|---|---|
| neutrophil count (%) | Placebo | 66.2 (19.8) | 64.9 (17.7) | 58.7 (20.9) | 59.1 (19.4) |
| | Levofloxacin | 70.4 (21.4) | 58.2 (16.7) | 68.9 (16.4) | 70.1 (15.6) |
| IL-8 (ng/ml) | Placebo | 81.9 (45.5) | 113.6 (82.3) | 120.8 (62.1) | 95.4 (75.0) |
| | Levofloxacin | 95.1 (67.7) | 85.0 (56.3) | 124.8 (69.7) | 91.7 (74.5) |
| Total neutrophil count (x $10^6$/g) | Placebo | 1.3 (0.4) | 1.0 (0.5) | 0.9 (0.5) | 1.3 (0.4) |
| | Levofloxacin | 2.0 (0.5) | 1.2 (0.5) | 1.3 (0.4) | 2.0 (0.4) |
| Log bacterial load (cfu/$ml^{-1}$) | Placebo | 6.05 (0.2) | 6.18 (0.3) | 6.28 (0.3) | 6.15 (0.3) |
| | Levofloxacin | 5.87 (0.2) | 5.33 (0.2) | 5.86 (0.1) | 5.69 (0.2) |

Table 13.2.2: mean (S.D) % neutrophil count, sputum IL-8, geometric mean (log S.D) total neutrophil count, and mean log (S.E) airway bacterial load.

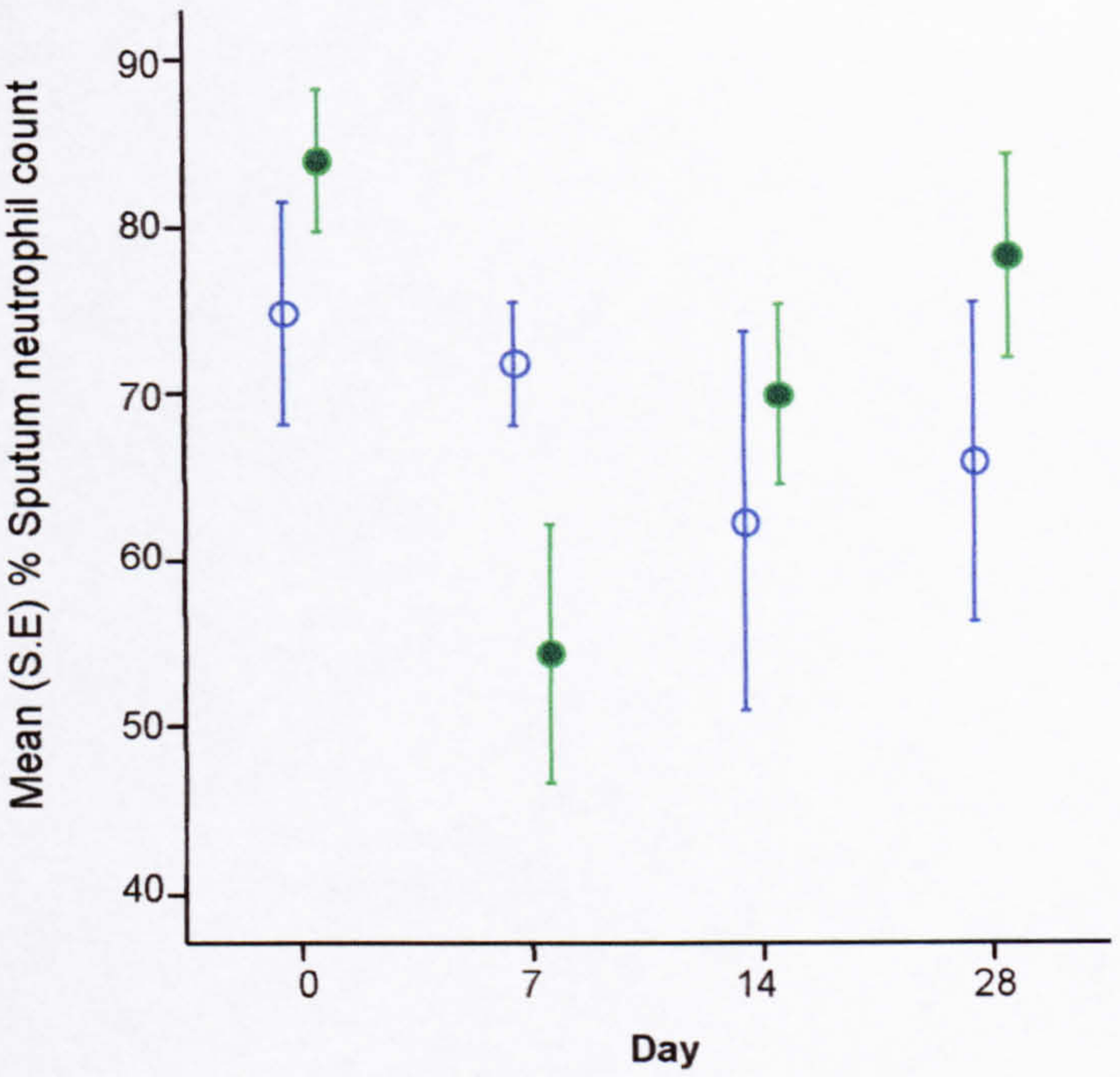
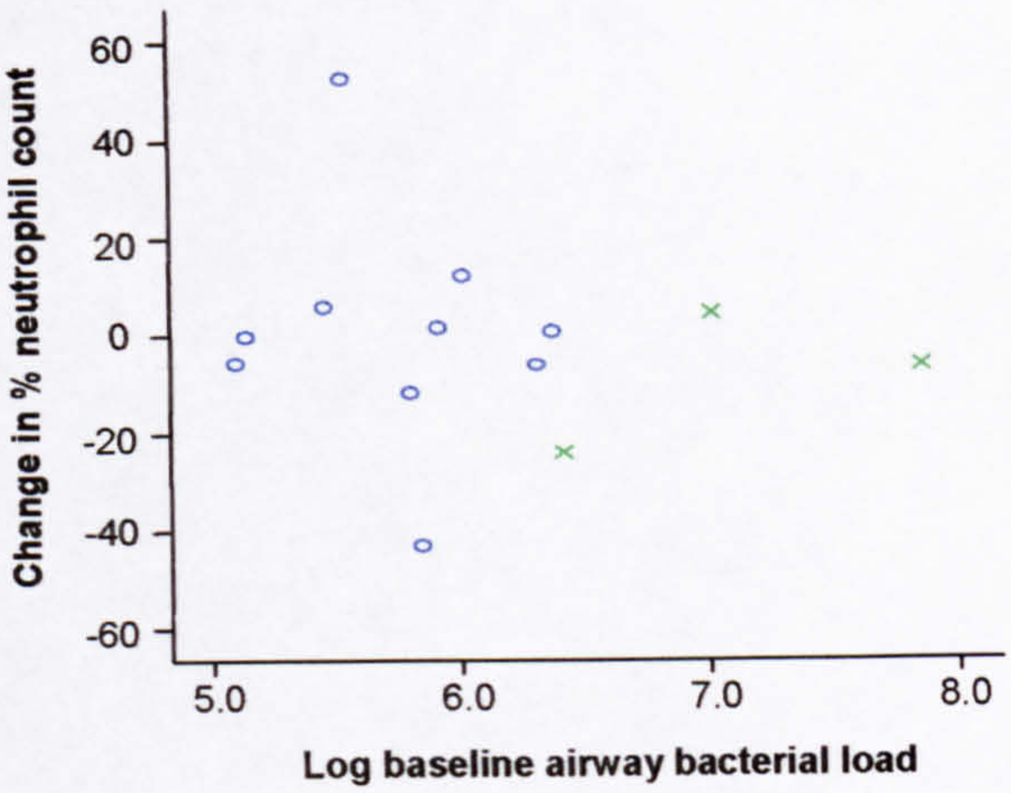



Figure 13.2.2: mean (s.e) change in % neutrophil count in patients with airway bacterial load of $> 10^6$ cfu/ml.

(Open circles = placebo, closed circles = Levofloxacin)

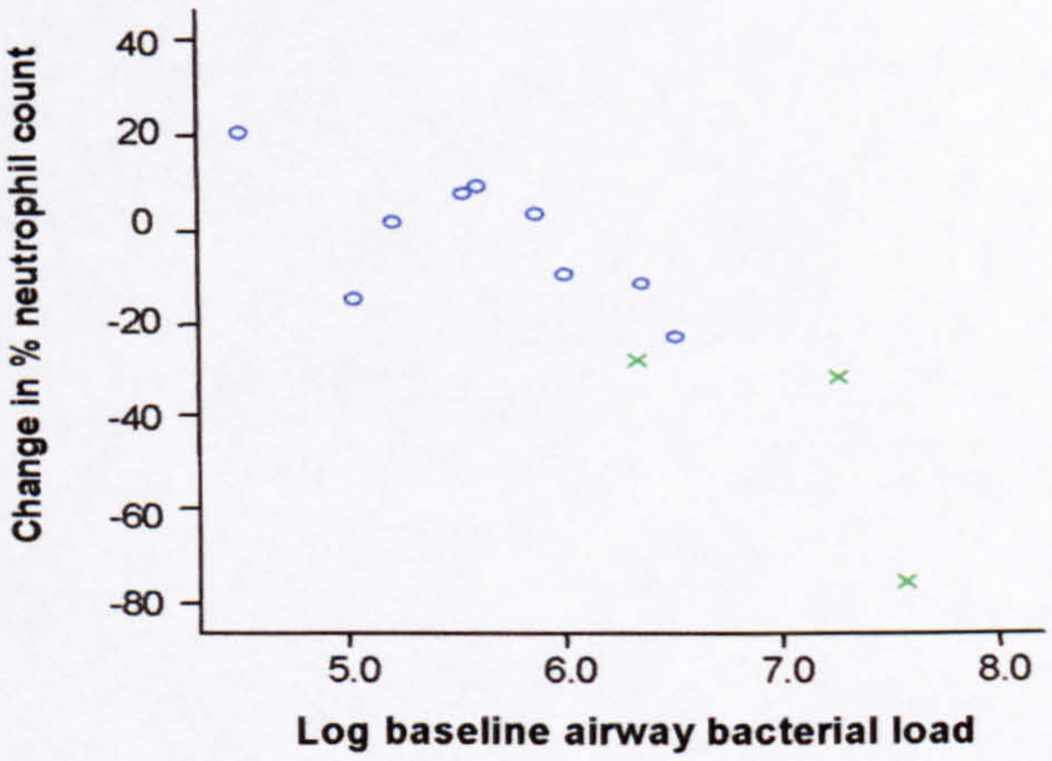
(A)




r = - 0.18

p = 0.55

(B)




r = - 0.78

p = 0.003

Figure 13.2.3 : mean change in % neutrophil count following (A) placebo and (B) Levofloxacin according to log baseline airway bacterial load. (X = Potentially pathogenic micro-organism, O = Non specific growth).

# Study 3

# Peptic ulceration, Helicobacter Pylori seropositivity and Chronic Obstructive Pulmonary Disease

## Abstract

Background

We have suggested that chronic inflammation of other foregut derivatives might be associated with airway inflammation and amplification of the response to inhaled stimuli such as cigarette smoke leading to COPD. One of the commonest causes of chronic foregut inflammation is gastritis secondary to H. Pylori infection. We tested the hypothesis that peptic ulceration and H. Pylori seropositivity are associated with COPD independently of other shared risk factors.

Methods

We reviewed primary care medical records and performed full lung function tests on 329 miners seen over 2 years as part of the DTI miners compensation scheme. We also carried out a case control study investigating the prevalence of H.Pylori seropositivity in patients with varying degrees of severity of COPD compared to an age, smoking history, and social class matched control population.

Findings

The prevalence of proven peptic ulcer disease in miners classified as: normal, chronic bronchitis, mild, moderate, and severe COPD was 3.2%, 16.2%, 21.4%, 42.4%, and 56.2% respectively. Multivariate analysis with age, pack years of smoking, years underground, and peptic ulcer disease as covariates showed that the presence of peptic ulcer disease was independently associated with a 13.3% ( 95% ci 6.7:19.9 ,$p<0.001$) lower % predicted $FEV_1$ and a 5.06% ( 95% ci 1.8:8.2, $p<0.001$) lower $FEV_1/FVC$ ratio. In the case control study the prevalence of positive H. Pylori serology was significantly higher in patients with COPD (54.7%) compared to controls (23.5%), ($p=0.026$).

Interpretation

Our findings are consistent with a strong and independent relationship between peptic ulceration, H Pylori infection and COPD and suggest that the relationship between peptic ulcer disease and COPD is more that just a shared susceptibility to infective and environmental stimuli.

## Introduction

Chronic Obstructive Pulmonary Disease (COPD) is a leading cause of morbidity and mortality worldwide. It is associated with a destructive inflammatory response in the distal airways in response to inhaled noxious particles such as cigarette smoke (Pauwels et al. 2001). Only around 15% of smokers will develop clinically significant COPD. There is evidence to suggest that susceptible smokers have an abnormally amplified inflammatory response to inhaled stimuli (Barnes 2003). Attention has focussed on genetic (Silverman 2002) dietary, and environmental factors (Anto et al. 2001) that might explain this. Less attention has been paid to the possibility that other endogenous chronic inflammatory conditions may contribute to the amplification process in susceptible subjects.

There is growing evidence that chronic inflammation of structures that are embryologically related to the lungs such as the intestines, liver, and thyroid gland is associated with airway inflammation (Birring et al. 2005), respiratory symptoms (Birring et al. 2005, 2003) airway dysfunction (Birring et al. 2005), and accelerated decline in lung function (Kanazawa et al. 2003). One of the commonest causes of chronic foregut inflammation worldwide is gastritis secondary to H.Pylori infection (Suerbaum et al. 2002) This infection is often acquired early in life and causes an intense cytokine mediated infiltration of the gastric epithelium by neutrophils and mononuclear cells (Craig et al. 1992) which most commonly presents clinically in the form of peptic ulceration (Suerbaum et al. 2002). Previous studies have shown that COPD and peptic ulcer disease coexist more commonly than expected. (Flint et al. 1958) (Kroeker et al. 1962) (Latts et al. 1956) (Monson 1970). However, many of these studies are limited by the absence of objective confirmation of the diagnosis of

peptic ulcer disease and COPD, limited information on the chronology of the development of these conditions, and a failure to account for shared risk factors such as smoking and social class. These deficiencies make it difficult to form firm conclusions about the mechanism of the association.

Here we present an audit of the prevalence of peptic ulcer disease and COPD defined using objective criteria in a well defined population of miners whose other risk factors for the development of COPD were relatively homogeneous. We also present a case control study investigating the prevalence of H.Pylori seropositivity in patients with varying degrees of COPD compared to an age, smoking history, and social class matched population with normal spirometric values.

## Method

### Subjects

We reviewed all primary care medical records and full lung function data of 329 miners who were assessed over 32 months from January 2001 by a single physician as part of the Department of Trade and Industry miners compensation scheme. COPD was diagnosed on the basis of a consistent clinical history and post bronchodilator $FEV_1$/ FVC ratio of < 70%. Severity of the disease was classified according to former BTS guidelines; % predicted $FEV_1$ of >60%, 40%-60%, and <40% indicating mild, moderate, and severe disease respectively. Patients with a post bronchodilator $FEV_1$/ FVC ratio of < 70%, but with % predicted $FEV_1$ of >80% were classified as having mild disease (as per GOLD guidelines- Pauwels et al. 2001). Chronic bronchitis was diagnosed when miners who did not meet the diagnostic criteria for COPD, gave a

history of cough and sputum production for at least 3 months for more than 1 year, and this history was supported by two or more primary care entries in the notes referring to a productive cough. The presence of peptic ulcer was accepted in miners who had a consistent clinical picture and had objective evidence of peptic ulceration as indicated by typical findings on barium meal, endoscopic examination, or at surgery. Self reported smoking history was validated by review of primary care records and by measurement of exhaled carbon monoxide at the time of pulmonary function testing.

For the case control study we recruited patients with a prior diagnosis of COPD from a database of research volunteers and from outpatient and inpatient settings. COPD was classified as in the audit above. Controls, matched for age, smoking history and socio-economic status but with normal spirometric values were recruited using newspaper and hospital advertisements. Socio-economic class was classified according to the British Registrar General (categories ranked 1 to 5 for professional, intermediate, skilled manual, partly skilled manual and unskilled manual workers respectively). The study was approved by the local research ethics committee (Directorate of research and development, university hospitals of Leicester) and all patients gave written informed consent.

Measurements

Spirometry was performed using a Vitalogrraph ® spirometer. For the case-control study, patients and controls completed a questionnaire relating to; occupation, pack year smoking history, past history of peptic ulceration or gastrooesophageal reflux disease, and use of ant-acid or acid suppression medication. A blood test was

performed in both groups to test for H.Pylori sero-prevalence (IgG Elisa: Enzygnost anti Helicobacter Pylori 2 IgG , Dade Behring, Germany).

**Results**

Table 13.3.1 shows the prevalence of peptic ulcer in miners classified according to the presence and severity of COPD. The mean age at diagnosis of peptic ulcer was 43, and the mean age at diagnosis for COPD and chronic bronchitis were 58 and 48 respectively. Multivariate analysis with age, pack years of smoking, years underground, and peptic ulcer disease as covariates showed that the presence of peptic ulcer was independently associated with a 13.3% (95% ci 6.7 : 19.9, $p<0.001$) lower % predicted post bronchodilator $FEV_1$ and a 5.1% (95% ci 1.8 : 8.2, $p<0.001$) lower post bronchodilator $FEV_1/FVC$ percentage.

Table 13.3.2 shows the prevalence of positive H.Pylori serology in a group of COPD patients stratified according to disease severity and in a control group matched for age, socioeconomic class, and pack year smoking history. The prevalence of positive H.Pylori serology was significantly higher in patients with COPD compared to the control group ($p=0.026$). The prevalence of self reported peptic ulcer disease was higher in the COPD group (12 out of 64) compared to the control group (2 out of 17), $p = 0.498$. Use of a proton pump inhibitor was also higher in the COPD group (10 out of 64) compared to the control group (0 out of 17), $p = 0.08$.

## Discussion

We have shown that the prevalence of peptic ulcer disease increases progressively with increasing severity of COPD in a cohort of Nottinghamshire miners. This observation was made in a population where other risk factors for the development of COPD such as socioeconomic class, smoking history, occupational exposure and early life experiences are homogeneous. Furthermore, peptic ulceration was a strong and independent predictor of a low post bronchodilator $FEV_1$ % predicted and $FEV_1$/FVC ratio. We found a similar although less striking association between H.Pylori seropositivity and COPD in the case control study.

These findings are consistent with studies carried out over 40 years ago by Flint (Flint et al. 1958) who showed evidence of peptic ulcer at autopsy in 21% of patients with pathological evidence of emphysema and in 1.6% of those without emphysema, and the work of others (Latts et al. 1956), including Kroeker (Kroeker et al. 1962) who reported upto a 14 fold increase in peptic ulcer prevalence in patients with COPD compared to a general population without COPD. We also found an increased prevalence of positive H.Pylori serology and a trend for increased markers of peptic ulcer disease such as self reported history and use of PPI in our case control study. These findings are based on small numbers and need to be confirmed in larger population based studies. However, they are consistent with other studies showing an increased incidence of positive H Pylori serology in patients with chronic bronchitis and bronchiectasis (Roussos et al. 2002) (Tsang et al. 1998). These studies suggest a more general association between H.Pylori infection and airway disease. The background rate of positive H.Pylori serology was less in our study than in these others (Suerbaum et al. 2002), (Roussos et al. 2002), (Tsang et al.1998). This may be

due to geographical factors or may reflect the rapid reduction in H.Pylori seropositivity that is known to have occurred in recent years (Vyse et al. 2002). Another factor might be H.Pylori eradication secondary to the co-prescribing of proton pump inhibitors for dyspepsia and antibiotics for lower respiratory tract infections, an effect that might be expected to particularly affect H.Pylori seropositivity in patients with COPD.

Early hypotheses on the mechanism of the association between peptic ulcer and COPD focussed on the presence of shared risk factors such as socioeconomic class and smoking, the possibility of reporting bias, or the effects of medications needed to treat COPD or the gastric mucosa. Several key findings of our audit suggest a more fundamental and perhaps causal relationship. Firstly, we showed that the association between peptic ulcer disease and COPD remains strong when these conditions are diagnosed objectively. Secondly, the prevalence of peptic ulcer disease increases progressively with increasing severity of COPD. Thirdly, the association was seen in a homogeneous population with many shared risk factors and remained strong and essentially unchanged when potentially confounding factors were accounted for. Finally, we found a strong tendency for peptic ulcer disease to be diagnosed well before the onset of respiratory symptoms making it very unlikely that the relationship we have seen is due to confounding by the effects of treatment.

Since the earlier description of the association between peptic ulcer and COPD there has been a major increase in our understanding of the pathogenesis of peptic ulcer disease. Peptic ulceration is now seen as a complication of H.Pylori infection often acquired in early life and is responsible for chronic gastric inflammation (Suerbaum et

al. 2002). Smoking (Barnes et al. 2003) and H.Pylori infection (Craig et al. 1992) are associated with lymphocytic and neutrophil mediated inflammation centred around the small airways and stomach respectively. It is plausible that shared genetic factors result in abnormal amplification of the inflammatory response in both sites in response to different stimuli resulting in more tissue damage and disease. However, our finding of an increased prevalence of positive H. Pylori serology in patients with COPD suggests that there might be more to the association between peptic ulcer disease and COPD than just a shared susceptibility to mount an exaggerated and damaging immune response to infection and irritant stimuli and raises the possibility of a direct causal association between H.Pylori infection and COPD.

There is increasing evidence that conditions associated with chronic inflammation of foregut derivatives are associated with airway inflammation and dysfunction perhaps due to aberrant homing of activated lymphocytes. This is most widely recognised in inflammatory bowel disease (Camus et al. 2000) but there is also evidence of increased respiratory morbidity, airway dysfunction, and airway inflammation in patients with organ-specific autoimmune disease, particularly autoimmune hypothyroidism (Birring et al. 2005) (Brightling et al. 2002). Chronic hepatitis due to hepatitis C infection has been associated with an accelerated decline in lung function in smokers which returns towards normal following successful treatment of the hepatitis with Interferon $\gamma$ (Kanazawa et al. 2003). A plausible explanation for the association between peptic ulcer disease and COPD is that chronic gastritis secondary to H.Pylori infection results in airway inflammation and amplification of the response to inhaled stimuli. If this is correct then it raises the possibility that the abnormally

amplified immune response in the airways of patients with COPD can be reversed by successful eradication of H.Pylori infection.

| Stage (n) | Normal (125) | C.Bronchitis (99) | Mild (56) | Moderate (33) | Severe (16) |
|---|---|---|---|---|---|
| Age (range) | 52 (33-86) | 61 (32-91) | 65 (38-90) | 75 (57-86) | 74 (60-89) |
| $FEV_1$%pred* (SD) | 106.2 (16.1) | 97.5 (18.3) | 85.7 (17.1) | 51.8 (5.8) | 33.1 (6.0) |
| $FEV_1$/FVC*% (SD) | 80.6 (5.7) | 79.2 (6.1) | 63.6 (4.5) | 54.8 (8.0) | 47.3 (10.6) |
| Smoking pack years (SD) | 12.9 (14.2) | 17.6 (15.4) | 24.5 (17.1) | 29.5 (15.9) | 36.0 (15.3) |
| Years underground (SD) | 22.2 (10.3) | 29.9 (11.8) | 31.6 (12.9) | 33.7 (10.5) | 32.3 (10.3) |
| **Ulcer** | 4 **(3.2%)** | 16 **(16.2%)** | 12 **(21.4%)** | 14 **(42.4%)** | 9 **(56.2%)** |

Table 13.3.1: prevalence of peptic ulcer disease according to BTS stage
(* post bronchodilator)

| Stage | Normal | Mild | Moderate | Severe |
|---|---|---|---|---|
| n (male) | 17 (13) | 9 (7) | 19 (12) | 36 (26) |
| Age (range) | 65 (56-77) | 71 (64-80) | 67 (45-81) | 69 (58-79) |
| %$FEV_1$* (SD) | 101.5 (14.8) | 65.6 (2.8) | 49.6 (5.0) | 28.6 (7.5) |
| $FEV_1$/FVC %* (SD) | 76.4 (5.0) | 56.4 (8.0) | 58.0 (11.0) | 39.7 (8.7) |
| Smoking pack years (SD) | 39.0 (34.0) | 49.4 (21.6) | 51.1 (30.5) | 45.8 (26.0) |
| Social class score | 2.8 | 3.5 | 3 | 3.1 |
| **Positive H.Pylori serology** | **23.5%** | **44.4%** | **52.6%** | **57.0%** |

Table 13.3.2: prevalence of positive H.Pylori serology by BTS stage
(* post bronchodilator)

# Study 4

## TREM-1 in exacerbations of COPD

## Introduction

Airway inflammation in COPD is mainly neutrophilic and neutrophilic airway inflammation increases during exacerbations and more so when these exacerbations are bacterial in aetiology. Markers for stratifying exacerbations of COPD into different aetiological categories may be useful in treating these exacerbations more appropriately. Current methods for differentiating bacterial from non-bacterial causes of an exacerbation are either too subjective or not suitable for near patient use. The identification of a marker which could detect these raised levels of inflammation and is specific for bacterial infection would be extremely useful in guiding antibiotic therapy.

TREM-1 is a glycoprotein expressed on neutrophils and monocytes. Expression of TREM-1 is up-regulated on contact with extracellular bacteria during acute infections. TREM-1 has been detected in broncho-alveolar lavage (BAL), and has already been shown to be a sensitive and specific marker for the diagnosis of pneumonia (Gibot et al. 2004). This raises the exciting possibility that TREM-1 could be used as a marker to identify COPD exacerbations of bacterial aetiology. In order for TREM-1 to achieve this it must also be able to distinguish COPD patients with exacerbations of bacterial aetiology from COPD patients with chronic bacterial colonisation of their airways. Here we hypothesise that TREM-1 will be expressed in some exacerbations of COPD but not in patients with stable state disease.

## Method

Subjects

Firstly we recruited 15 patients with stable COPD. The diagnosis of COPD was based on a compatible history, and spirometry (Vitalograph ®, Buckinghamshire U.K) showing a post bronchodilator $FEV_1$/ FVC ratio of < 70%, and % predicted $FEV_1$ of < 80%. All patients had fixed airway obstruction as suggested by an $FEV_1$ increase of < 15% or if $FEV_1$ < 1.2 l, <200ml, 15 minutes after 400mcg of inhaled Salbutamol via a large volume spacer. All patients were free of exacerbation defined using Anthonisen criteria (Anthonisen et al.1987) for the 6 weeks preceding the study. We excluded patients who were aged under 45, if they possessed severe physical handicap, a clinical history of asthma or acute wheeze, breathlessness or deterioration associated with allergens, or clinically important co-morbidity such as heart failure, bronchiectasis or lung cancer.

For our exacerbation group we recruited 18 patients retrospectively from a previous study (study 1). These patients had all been diagnosed as having COPD according to the criteria above. They had presented to us voluntarily as an unscheduled clinic visit due to a clinical worsening of their respiratory symptoms.

Measurements

All patients underwent spirometry and sputum induction according to fixed protocols (Pavord et al. 1992). In patients with stable COPD, the processed sputum was analysed for differential cell count and quantitative bacterial analysis. In patients who had suffered from an exacerbation, sputum was analysed for differential cell count,

however we were not able to perform quantitative bacterial analysis at that point in time as we had not learned the required techniques. The concentration of TREM-1 in the sputum supernatants was measured using an ELISA.

Protocol

Spirometry and sputum induction were performed according to standard protocols (see clinical methods). Supernatants from the processed sputum of both groups were frozen for future TREM-1 analysis.

Sputum differential cell count, quantitative bacterial analysis, and TREM-1 analysis of frozen supernatants using ELISA were also carried out according to fixed protocols (see laboratory methods).

**Results**

| Patient status | Age (range) | Pack years (s.d) | FEV1/FVC ratio (s.d) | % predicted $FEV_1$ (s.d) | Total cell count x $10^6$/g (s.d) | % Neuts (s.d) | Patients expressing TREM-1 | Median TREM-1 Pg/g (range) |
|---|---|---|---|---|---|---|---|---|
| Stable | 65 (68-80) | 51.9 (34.4) | 56.3 (15.2) | 52.0 (15.3) | 3.49 (2.1) | 66.6 (23.6) | 0/15 | 0 (0) |
| Exacerbation | 68 (65-76) | 45.9 (33.1) | 47.2 (12.7) | 31.2 (11.0) | 14.88 (18.9) | 82.3 (18.9) | 10/18 | 63.87 (0-2508) |

Table 13.4.1: demographic data, differential cell count data, and TREM-1 data for patients with stable state and exacerbation state COPD.

Recovery from the assay was 100%, with a coefficient of variation of 1.7%. The inter-assay and intra-assay variability were both < 5%. None of the 15 patients with stable COPD expressed TREM-1, whilst TREM-1 was detected in 10 out of 18 patients at exacerbation. Significantly more patients expressed TREM-1 at exacerbation compared to stable state disease (p=0.008). Mean (range) bacterial load in the group with stable COPD was $4.48x10^6$ ($1.23x10^4$ - $3.7x10^7$) cfu/ml of sputum

## Discussion

We were successful at detecting TREM-1 in sputum using an ELISA.TREM-1 was not detected in the sputum supernatants of any stable state patients despite a broad range of airway bacterial colonisation. TREM-1 was expressed in around half of the sputum supernatants from patients with an exacerbation of COPD. The aetiology of these exacerbations was unknown.

This is only a small pilot study and further prospective studies using larger numbers of patients are now required. The fact that TREM-1 was not expressed in patients with stable disease even with a high bacterial load is important as this may imply that TREM-1 is able to distinguish chronic bacterial colonisation, associated with a relatively low level of inflammatory response, from new bacterial infection, which could be associated with a greater inflammatory response and hence need for antibiotic anti-inflammatory therapy. The future detection of TREM-1 in stable state disease would not be surprising as this marker may act as more of a guide as to which patients may achieve potential anti-inflammatory benefit with antibiotic therapy

irrespective of clinical status rather than just a marker which suggests bacterial aetiology at exacerbation.

Further work is needed to establish whether TREM-1 is a marker for bacterial infection and whether its measurement is clinically useful, and whether measurement of TREM-1 is feasible and cost effective on an individual basis.

# 14: Conclusions

Whilst the morbidity and mortality of COPD continue to increase, this disease remains under-diagnosed, under-treated, and its impact underestimated. Despite recent measures to discourage cigarette smoking in developed countries, smoking cessation rates remain very poor, and with increasing smoking in developing countries, and with improved survival from other causes of mortality, the problems posed by this disease are set to escalate further.

At the heart of COPD lies a complex inflammatory picture about which we know relatively little in comparison to the relatively similar disease of asthma. Much effort is currently being focussed on trying to improve our understanding of the mechanisms of inflammation in COPD. Key questions, such as why do only a small proportion of smokers go on to develop COPD, and why is COPD relatively resistant to corticosteroid therapy, have yet to be answered fully. Whilst the pharmaceutical industry makes progress in developing new inhibitors to various parts of the inflammatory process, there remains a lot of basic clinical work to be carried out. Antibiotics and corticosteroids are just some of the tools currently at our disposal with which we may be able to further our understanding of airway inflammation in COPD. In this thesis I have attempted to answer basic clinical questions regarding the modulation of airway inflammation in COPD.

We have shown that a management strategy that aims to minimise eosinophilic airway inflammation as well as symptoms is associated with a significant reduction in the frequency of exacerbations of COPD requiring hospital admission. It is these severe

exacerbations which are responsible for a large proportion of expenditure in COPD as well as considerable morbidity and mortality. The management strategy was associated with no overall increase in the use of inhaled or oral corticosteroids. We saw no difference in the frequency of mild, self-managed exacerbations or in the frequency of moderate exacerbations requiring G.P or unscheduled clinic review.

We must now focus on how we can bring this strategy into clinical practice, and whether its use is feasible, cost effective, and continues to result in improvements in clinical outcomes. Sputum induction has been shown to be relatively quick and safe, but does require dedicated laboratory facilities and staff. Whilst we carried out repeated measurements of the sputum eosinophil count, we have shown that the group most likely to benefit from this type of strategy could be identified from just one sputum induction at the baseline visit. The management strategy was associated with no overall increase in the use of inhaled or oral corticosteroids and there was evidence that increased corticosteroid therapy was targeted to patients with eosinophilic airway inflammation in the intervention group. In the same way patients in the intervention group who did not demonstrate eosinophilic airway inflammation were successfully weaned off corticosteroids. Blind withdrawal of inhaled corticosteroids in patients with COPD is associated with an increase in exacerbation frequency as highlighted in the COPE study (Van der Valk et al. 2002). Therefore an additional benefit of this strategy is that corticosteroid therapy is only targeted to those patients who require it and even then it is given at the lowest possible dose in order to suppress the sputum eosinophil count. Whilst it was clear that some patients required only modest doses of inhaled corticosteroids to achieve this, it was also clear that the sputum eosinophil count was resistant to inhaled corticosteroids in a significant proportion of patients in

whom oral corticosteroids had to be used. The mechanism for this resistance is yet unknown but is likely to be due to either the amount of eosinophilic airway inflammation, the site of eosinophilic airway inflammation, or differences in smoking habit. In the next few years, the measurement of eosinophilic airway inflammation and its response to corticosteroid therapy, should become incorporated into the routine clinical management of patients with COPD.

Whilst a lot of attention has been given to the aetiological role of cigarette smoke in the pathogenesis of airway inflammation, it is clear that other aetiological factors are involved. It is important to identify these factors, firstly because the rate of smoking cessation is poor, secondly because even after smoking cessation, airway inflammation has been shown to remain, and thirdly because some of these factors may be treatable. It is likely that bacteria play a considerable role in the genesis of airway inflammation. If this is the case then we might expect antibiotics to have some impact on airway inflammation in certain circumstances.

We have demonstrated a relationship between airway bacterial load and % sputum neutrophil count. We have shown that overall, a one week course of Levofloxacin had no significant effect on markers of neutrophilic airway inflammation in patients with stable state COPD. However when we confined our analysis to patients with high airway bacterial load, Levofloxacin significantly reduced the % neutrophil count in these patients. The effect of Levofloxacin was only short term, which is consistent with previous data, and could have been confined to those patients with high bacterial airway load or patients from whom we cultured PPMs, suggesting that only a proportion of patients with stable COPD may benefit from a strategy which aims to

reduce bacterial infection in the airways. There was a trend towards a reduction in sputum supernatant IL-8 in the patients treated with Levofloxacin and again this was confined to patients who demonstrated high bacterial loads. We also demonstrated correlations between change in % neutrophil count and baseline airway bacterial load, and % neutrophil count and change in airway bacterial load following treatment with Levofloxacin. Finally we demonstrated that a significantly greater reduction in % neutrophil count following treatment with Levofloxacin was associated with the culture of potentially pathogenic micro-organisms (PPM) rather than non-specific growth. There was no effect on symptoms or lung function.

We have shown that the prevalence of peptic ulcer disease increases progressively with increasing severity of COPD in a cohort of Nottinghamshire miners. This observation was made in a population where other risk factors for the development of COPD such as socioeconomic class, smoking history, occupational exposure and early life experiences are likely to be homogeneous. Furthermore peptic ulceration was a strong and independent predictor of a low $FEV_1$ % predicted and $FEV_1$/FVC ratio. We found a similar although less striking association between H.Pylori seropositivity, and COPD in our case control study. These findings raise the possibility that the abnormally amplified immune response in the airways of patients with COPD could be modulated by successful eradication of H.Pylori infection and effective treatment of the chronic gastric inflammation associated with peptic ulcer disease. The presence of systemic manifestations has now been accepted as part of the definition of COPD. It is possible that systemic pathology may have the capacity to effect the disease of COPD rather than just be influenced by it. This raises the

intriguing possibility that modulation of systemic pathophysiology may play a role in modulating airway pathophysiology and hence altering outcomes in COPD.

There is a need for new markers of airway inflammation which may enable us to target anti-inflammatory treatment more accurately. Ideally these should be suitable for near patient use and provide rapid, reliable results which will aid the clinician in prescribing optimum therapy as quickly as possible. We have shown that TREM-1 can be measured from induced sputum and has the potential to be a marker of bacterial infection and neutrophilic inflammation. At present measurement of TREM-1 requires dedicated laboratory staff and facilities, and is unlikely to prove cost effective when measured on an individual basis. Measurement of Procalcitonin has been carried out in a clinical study and has been shown to be able to significantly reduce the use of antibiotic therapy without adverse outcome, and reduce antibiotic prescribing costs. A similar interventional study using TREM-1 is also likely to show similar benefits. Further work will be needed to prove overall cost-effectiveness and whether strategies which incorporate measurement of these novel markers can be adapted for wider use in primary and secondary care settings.

In the next few years much effort will be needed to ensure that the measurement of airway inflammation becomes easier, cheaper, and more accessible. Much work has already been done using exhaled nitric oxide (eNO) as a surrogate marker for eosinophilic airway inflammation. Further interventional studies will be required to see whether management strategies based on the measurement of eNO result in improved clinical outcomes.

More work will be needed to demonstrate that strategies which involve the modulation of airway inflammation are feasible, cost effective, and associated with improved clinical outcomes. At present such strategies are likely to be adopted into secondary and tertiary care settings, for example in the form of specialist clinics, where there is access to specialist facilities and personnel. However over time the challenge will be to incorporate strategies which involve the modulation of airway inflammation into routine clinical practice and clinical guidelines.

In summary we have shown how modulation of both eosinophilc and neutrophilic airway inflammation, using corticosteroids and antibiotics respectively, has the potential to alter key clinical outcomes in COPD. There is a growing emphasis to consider COPD as more of a systemic disease and we have also demonstrated that there may be a role for the modulation of non-pulmonary inflammation, in this case gastric inflammation, in trying to alter disease outcome. We have shown how airway inflammation can be measured accurately, safely, and non-invasively and presented our initial pilot data looking at a possible new marker of airway inflammation; TREM-1.

# 15: Future work

In the first study we demonstrated that a management strategy that aimed to minimise eosinophilic airway inflammation as well as symptoms was associated with a significant reduction in the frequency of exacerbations of COPD requiring hospital admission. Of particular interest was the fact that the greatest reduction in hospital admissions seemed to be in those patients with a high baseline sputum eosinophil count. Also despite the use of high dose inhaled corticosteroids, several patients required low doses of oral corticosteroids in order to reduce eosinophilic airway inflammation. In retrospect a more aggressive use of oral corticosteroids in order to reduce eosinophilic airway inflammation even further may have resulted in an even greater reduction in hospital admissions. Although long term use of oral corticosteroids is associated with considerable morbidity due to side-effects, there may be certain patients who would benefit from a strategy involving the long term use of low dose oral corticosteroids. Further work needs to assess if this strategy could result in an even greater reduction in exacerbation frequency, or as previously shown, reduce the rate of decline in lung function. Ethical issues such as the use of prophylactic bisphosphonates and the regular monitoring of blood pressure and blood glucose would need to be addressed.

We have shown that a one week course of an antibiotic significantly reduced the % neutrophil count in patients with stable state COPD. This effect was only short term and was confined to those patients with high bacterial loads. Larger long term interventional studies are now required to analyse the effect of antibiotics on airway

inflammation and important clinical outcomes such as symptoms, quality of life, exacerbation frequency, and decline in lung function. This research however, would have to take place on a background of growing antibiotic resistance, increasing incidence of so-called superbugs, and other potential antibiotic related side-effects. If antibiotic therapy is associated with improved clinical outcome, then further questions will need to be answered. Firstly we need to know which antibiotics could be potentially used. Whilst broad spectrum antibiotics are likely to have a greater impact on a greater variety of bacteria and probably result in a greater reduction in overall bacterial load, they are also likely to be associated with a greater side effect profile. There is also growing evidence that it is the acquisition of new bacterial strains rather than just a rise in bacterial load that may influence neutrophilic airway inflammation and exacerbation frequency. Therefore identification of specific new strains of bacteria and the use of only the appropriate antibiotic based on culture and sensitivities may be a more appropriate management strategy. Further work would therefore be required in order to develop rapid, cheap, and non-invasive methods of bacterial identification. In addition we would need to know the optimum dose of antibiotic to use and how often it should be administered. A current study using pulsed Moxifloxacin is ongoing and will address some of these issues.

The findings that peptic ulceration was a strong and independent predictor of a low post bronchodilator $FEV_1$ % predicted and $FEV_1$/FVC ratio, and that there was an association between H.Pylori seropositivity, and severity of COPD, pave the way for a long term intervention study. Diagnosis and eradication of H.Pylori should be straightforward but this study would be likely to require a large number of patients being followed up for a long period of time. Confounding factors such as smoking

status, exacerbation frequency, co-morbidity and medical treatment would all have to be taken into account.

Much more work is needed in trying to validate TREM-1 as a marker for bacterial infection in exacerbations of COPD. In our small pilot study TREM-1 was not expressed in any patients with stable COPD despite a wide range of airway bacterial colonisation. Around half of the patients who had had an exacerbation expressed TREM-1. A prospective study of patients at exacerbation is now required to fully characterise which patients express TREM-1 and whether TREM-1 expression is related to bacterial infection. If successful an interventional study using TREM-1 to guide antibiotic therapy would be required to see if antibiotic therapy could be prescribed more accurately, and whether this would be cost effective and associated with improvement in clinical outcomes. Measurement of TREM-1 may also have implications for other respiratory diseases. It might be able to guide us in prescribing antibiotic therapy more accurately in bronchiectasis and theoretically it might be able to help in the exclusion of a diagnosis of Tuberculosis (T.B). T.B is an intracellular bacteria usually found in the phagasomes of macrophages, whereas bacteria responsible for the majority of pneumonias are extracellular. Infection with intracellular bacteria is not associated with increased levels of TREM-1, unlike extracellular bacteria. Therefore TREM-1 could be used to help differentiate between common community acquired pneumonias and T.B by acting as a negative predictor of T.B.

Finally further work is required to classify and characterise exacerbations of COPD more accurately and objectively. Exacerbations of COPD are associated with high

levels of morbidity and mortality. They are extremely heterogeneous both in aetiology and in physiological parameters, yet in general exacerbations are treated in a stereotypical manner usually comprising of a course of oral Prednisolone and a course of antibiotics without real confirmation as to the aetiology of the exacerbation. It is clear that the pattern of airway inflammation and changes in lung function vary considerably amongst patients at exacerbation. Whether this heterogeneity of presentation is due to the aetiology of the exacerbation or whether it is due to the patient themselves is unknown. A greater understanding of this heterogeneity might enable us to treat exacerbations in a more accurate fashion.

Exacerbations of COPD are currently defined in one of two ways. Firstly there is an operational definition as used by GOLD and NICE which relates to the patient not only having a worsening of symptoms, but also seeking medical intervention and usually requiring extra medication. Exacerbations can be graded as mild, moderate, or severe according to whether patients self medicate or seek intervention from primary or secondary care. Secondly there is the definition based around the Anthonisen criteria, where the presence of certain symptoms identifies a group of patients who are likely to benefit from antibiotic treatment. Neither of these definitions are particularly objective. They do not take into account changes in lung function and airway inflammation and they overlook the important psychological component of the exacerbation.

A new classification for exacerbations of COPD is needed. I propose an "ABC" classification which will identify the following three important disease components; **A**irway inflammation, **B**acterial infection, and **C**o-morbidity. It is clear that

exacerbations may involve any of these components, either individually or in combination. At present the last component of co-morbidity which includes factors such as anxiety, depression, and poor coping skills, is often overlooked. Each of the components is associated with a very different therapeutic option. These are corticosteroids, antibiotics, and non pharmacological therapies such as counselling and social support. Identification of these key components will enable clinicians to target therapy much more accurately and appropriately, avoiding the use of costly unnecessary interventions. Obviously further work is needed to demonstrate that the identification of these components is practical and worthwhile, and that the use of such a classification is associated with improved clinical practice and outcomes.

In conclusion we have shown that modulation of airway inflammation has the potential to transform our management of COPD. The development of new strategies and technologies ensures that the future for exacerbations of COPD is exciting but for the time being I suggest it may be as simple as ABC.

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