UNIVERSITY OF SOUTHAMPTON

SCHOOL OF MEDICINE

The effect of smoking on the severity, and mechanisms of acute exacerbations of chronic obstructive pulmonary disease (COPD)

by

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Abstract

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DM Medicine

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COPD (Chronic Obstructive Pulmonary Disease) worldwide has a prevalence of 10% in men and 8.5% in women. Exacerbations of COPD account for approximately 10% of all acute medical admissions. Projected prevalence figures suggest that by 2020 COPD will be the third leading cause of mortality worldwide thus imposing a significant burden on healthcare resources in the future. Acute exacerbations are not only responsible for a decline in the patient's quality of life, but have a major socioeconomic impact. Following a pilot study that showed current smokers recover lung function much more slowly from their exacerbation than ex smokers, I initiated a properly powered prospective study to investigate the difference between the two groups.

A total of 58 patients admitted with acute infectious exacerbations of COPD were recruited to the study to determine the effect of smoking status on their exacerbation. Throughout the admission lung function was measured. Sputum was cultured for bacteria, and PCR used to detect viral infection. Blood and sputum cells were analyzed by flow cytometry. Serum was collected for CRP levels.

Ex-smokers recovered significantly more quickly than current smokers in all spirometric parameters ($P<0.01$), and were discharged sooner (mean 3.08 vs 5.59 days, $P<0.001$). Sputum culture was positive for more pathogenic bacteria in current smokers, especially *H. influenzae,* which was associated with a significantly higher CRP rise ($p<0.05$) than any other organism. CD8+ T cells predominated in the sputum of ex-smokers while CD4+ T cells were the dominant cell type in current smokers ($p<0.01$).

Current smoking is a risk factor for more severe exacerbations, delayed recovery and prolonged hospitalization. This may result from a variety of factors including bacterial, immune mediated responses and systemic inflammation.

Background

The idea for this research project was the result of an investigation of patients recovering from acute exacerbations of COPD who were severe enough to need admission to hospital. This pilot study was performed in Poole Hospital in Dorset in 2002. I monitored the recovery of patients during their hospital stay by performing daily pulmonary function testing until they were discharged from hospital. I analyzed the results of these investigations and found that in terms of improvement in FEV_1 and FVC the ex-smokers (defined as more than one year's abstinence from smoking) recovered much more quickly from their exacerbations than current smokers. This finding was presented at the European Respiratory Society Meeting in Stockholm in 2002. (2)

The results of this study were presented to the respiratory department at Southampton University Hospitals Trust. From this meeting a draft protocol was agreed to find the cause of the differences between the two groups, other than of course their smoking status.

DECLARATION OF AUTHORSHIP

I, Simon Charles Bourne, declare that the thesis entitled

The effect of smoking on the severity, and mechanisms of acute exacerbations of chronic obstructive pulmonary disease (COPD),

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this university

- where any part of this thesis has previously been submitted for a degree or any other qualification at this university or any other institution, this has been clearly stated;

- where I have consulted the published work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;

- I have acknowledged all main sources of help

- where the thesis is based on work done by myself or jointly with others, I have made it clear exactly what has been done by others and what I have contributed myself;

- none of this work has been published before submission

Signed:……..
Dated:……...

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Mrs Zoe Bourne

List of Contents

List of figures

Chapter 4

List of tables

List of Abbreviations

ACE	Angiotensin Converting Enzyme
AECOPD	Acute Exacerbation Of Chronic Obstructive Pulmonary Disease
ANP	Atrial Natriuretic Peptide
BNP	Brain Natriuretic Peptide
BODE	B – Body Mass Index, O – Degree of airflow Obstruction, D – MRC Dyspnoea score, and E- Exercise
BTS	British Thoracic Society
CRP	C Reactive Protein
COPD	Chronic Obstructive Pulmonary Disease
DNA	Deoxy Ribonucleic Acid
DH	Dynamic Hyperinflation
DTE	Dithioerythritol
EELV	End Expiratory Lung Volume
ECG	Electro Cardiogram
FEV_1	Forced Expiratory Volume In One Second
FVC	Forced Vital Capacity
FEV_6	Forced Expiratory Volume In Six Seconds
FBC	Full Blood Count
FRC	Functional Residual Capacity
GOLD	The Global Initiative for Chronic Obstructive Lung Disease
HDAC	Histone Deacetylase
IC	Inspiratory Capacity
IL	Interleukin

MPO	Myeloperoxidase
MPIF-1	Murine telomerase
MRC	Medical Research Council
NHANES	National Health and Nutrition Examination Survey
PAH	Pulmonary Arterial Hypertension
PEF	Peak Expiratory Flow
PBS	HEPES buffered saline
RCOP	Royal College Of Physicians
SQRQ	St. Georges Respiratory Questionnaire
SLPI	Secretary leukoproteinase inhibitor
TBP	TATA box binding proteins
TSA	Trichostatin
TORCH	**TO**wards a **R**evolution in **COPD health**
TNF	Tumour Necrosis Factor
TLC	Total Lung Capacity
UK	United Kingdom
U & E	Urea and electrolytes
VC	Vital Capacity

CHAPTER 1: INTRODUCTION

Acute Exacerbations of COPD

Chapter 1 Introduction

1.1 Introduction and definitions

The average patient with Chronic Obstructive Pulmonary Disease (COPD) will have between 1-3 exacerbations per year. These exacerbations account for at least 50% of the treatment cost and have a very high mortality. Mortality from acute exacerbations is on the rise in the U.K. and also in America (3), especially in the female and elderly population. This year in the U.K. alone, COPD will have been responsible for the premature death of 30,000 people (4).

A recent audit from the Royal College of Physicians (RCOP) has highlighted the potentially serious problems for COPD services in the U.K. The audit was the first ever comprehensive national audit of COPD admissions. The project was undertaken by the RCOP and the British Thoracic Society (BTS) between the years 2003 and 2004. 234 hospitals in the U.K. took part in the audit, which was aimed at finding ways of improving the quality of care. This was the first time an audit of this type has covered the whole of the U.K. (5) The audit is currently being repeated in 2008, the results will be released later this year. This audit and resulting press release from the college received widespread publicity in the popular and medical press, both of which have called for an increase in funding in respiratory services. They report that COPD is the fifth most common cause of death in England and Wales, and is predicted to rise to the third leading cause worldwide by 2020. (6)

The prevalence of COPD in the community has been estimated as anywhere between 2-18%. The lower estimations come mainly from GP surgery database studies; in the United Kingdom prevalence studies based on actual spirometry put the figure at about 10%, rising with increasing age. (7-11) COPD accounts for up to 10% of all acute admissions, which makes it a priority area for all hospitals concerned with effective management of acute medical admissions

(5, 12). More than one in ten patients with COPD admitted to hospital are dead within 90 days of admission, and over 1 in 3 are readmitted to hospital during that time (5). The risk of exacerbations and hospitalisation is related to FEV1; 30% of severe exacerbations admitted to hospital are in those with an FEV1<30%. This was observed in the 2 recent large cohort exacerbation studies – the TORCH and INSPIRE studies. (13, 14) Studies on mortality after hospitalization for an acute exacerbation of COPD have shown a one-year mortality ranging from 22% to 43%, and a 2-year mortality of 36 to 49%. A cohort study looking at mortality after hospital admission has recently been published. This study recruited 416 patients who were followed up for 24 months. During the follow up 122 (29.3%) of the 416 patients died. Patients with concurrent diabetes had the highest increased mortality rate. Other risk factors were advanced age, low FEV_1 and lower health status. Current smokers in this study had a relative risk of 1.73 of dying from their COPD, compared to ex-smokers. Patients treated with inhaled corticosteroids and/or long-acting beta-2 agonists had a lower risk of death than patients using neither of these types of treatment (15). Risk factors identified in other studies are increasing age, a higher CO_2, long-term use of oral corticosteroids, reduced health status, marital status, depression, co-morbidity and prior hospital admission (16, 17).

It has been shown that median recovery times are 6 days for PEFR (Peak Expiratory Flow Rate) and 7 days for daily total symptom score; but the recovery of PEFR to baseline values can take much longer (30-90 days). It has also been shown that symptom changes during an exacerbation do not closely reflect those of lung function. (18)

To date, the only proven cost-effective therapies for COPD are the cessation or prevention of smoking, which is the single most common cause of COPD, and vaccination to prevent influenza and pneumococcal infection. Combination inhalers of corticosteroids and long acting beta agonists, (13) and long acting anticholinergics (19) reduce exacerbation burden and hospital admissions (13, 20, 21), this is important because hospitalisation and associated costs represent the greatest healthcare expenditures for people with the disease.

Long-term oxygen therapy is also among the most costly interventions in terms of total money spent on direct medical costs for COPD treatment, although it is probably cost-effective because of its positive impact on rates of mortality. (22)

In fact, oxygen therapy is the only intervention to date that has been shown to decrease death rates due to COPD, although the use of a combination inhaler containing flixotide and salmeterol in the **TO**wards a **R**evolution in **COPD** health (TORCH) study came close to showing a significant impact on mortality in those with an $FEV_1 < 60\%$ ($p=0.052$). The results of this study are backed up by the results from the INSPIRE (Investigating New Standards for Prophylaxis in Reduction of Exacerbations) study which showed a reduction in mortality versus tiotropium, although this was an additional endpoint. (23) Similarly, pulmonary rehabilitation programmes appear to benefit patients in terms of quality of life; however, long-term cost-effectiveness and effects on mortality have yet to be elucidated. (24)

COPD is a major cause of morbidity worldwide and affects 14 million patients in the United States alone. (25). The total economic cost of COPD in the US in 1993 was estimated to be over $15.5 billion, with $6.1 billion for hospitalisation, $4.4 billion for physician and other fees, $2.5 billion for drugs, $1.5 billion for nursing home care and $1.0 billion for home care. (26) Currently there are no diagnostic tests to define if a patient is suffering from an acute exacerbation, or to monitor their progress following treatment. However, several definitions have been made, these are discussed later. (27)

COPD - The Disease

COPD is a heterogeneous disease, with components of chronic bronchitis, emphysema, small airways disease, and perhaps in some patients certain features of asthma. (28) COPD is defined by the Global Initiative for Obstructive Lung Disease (GOLD) as a "disease state characterised by airflow limitation that is not fully reversible. …The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases".

The absence of reversibility tends to exclude asthma by definition, although asthma can coexist with COPD, with asthma being a relatively common disease in the general population. The GOLD workshop report explicitly defines airflow limitation using simple spirometric indices. Specifically, airflow limitation is defined as an FEV_1/forced vital capacity (FVC)<70% and a post bronchodilator FEV_1<80% of predicted. A good quality test is a necessary precondition for adequate interpretation, but it is not sufficient. One significant unsolved problem

is that the interpretation of spirometry as "normal" or "abnormal" is based on comparisons with reference values derived from healthy persons. Lower limits of the healthy subject range (normal range) are treated as absolute demarcations of normal or abnormal. This is particularly problematic for COPD in that it is a condition defined by spirometry with no other independent means of making the diagnosis. The true sensitivity and specificity of current categorisations is unknown.

COPD Pathogenesis

The clinical impact of cigarette smoking, which ranges from negligible to end-stage lung disease, is determined to some extent by individual susceptibility factors. (29) Understanding the factors that lead to emphysema is useful for predicting which individuals are at risk. α1 antitrypsin deficiency is one factor that results in a predisposition to smoking-induced emphysema, but despite intensive study, few other susceptibility factors are as well defined. (30)

COPD is a more of a syndrome than a distinct disease; comprising a combination of emphysematous changes and chronic bronchitis of varying proportions within the lung parenchyma; combined with a systemic inflammatory response. The terms ‘pink puffer’ to denote the cachectic emphysematous patient with a barrel shaped hyperinflated chest and the ‘blue bloater’ describing the patient with chronic bronchitis with cyanosis and cor pulmonale are striking in their imagery, but represent the end stages of the 2 extremes of the disease. We do come across such patients in our clinical practice but for the most part this patient group is heterogeneous.

The key inflammatory cells involved in the pathogenesis of COPD are thought to be the CD8+ T cells and the neutrophil. (31) We do not fully understand what initiates this inflammatory and destructive process in certain individuals, but spares the majority with an extensive smoking history.

There is also increasing interest in autoimmunity as the process that drives/initiates the disease; a recent paper has shown that emphysema is possibly an autoimmune disease characterized by the presence of antielastin antibodies and T-helper type 1 (T_H1) responses, which correlate with emphysema severity. (32)

The central airways in COPD exhibit a sub epithelial infiltration of mononuclear cells, mucous gland hypertrophy and inflammation. In severe COPD there is also a sub epithelial infiltration of neutrophils. In the smaller peripheral airways there is pronounced goblet cell hyperplasia and sub epithelial infiltration of CD8+ T lymphocytes, smooth muscle hypertrophy, wall fibrosis, and destruction of the alveolar attachments. (33, 34)

More details of the various cells and inflammatory mechanisms are provided under separate headings which focus on individual biomarkers of COPD.

Definition of acute exacerbation of COPD

Exacerbations of COPD are thought to be caused by interactions between host factors, bacteria, viruses, and changes in air quality to produce increased inflammation in the lower airway. Most do not take into account the role of co-morbidities in the symptomology.

In 1987, Anthonisen and colleagues investigated whether antibiotic therapy could have a beneficial effect in the management of COPD exacerbations. (35) They defined exacerbations specifically in terms of increased dyspnoea, sputum production, and sputum purulence: signs indicative of an infectious aetiology. However, exacerbations of COPD comprise a range of symptoms, and the presence or absence of increased sputum production or purulence provides only part of the picture. In a study of exacerbations, some patients showing other hallmark signs of an exacerbation would be excluded by using this definition; which serves to demonstrate how an all-encompassing, standardized definition is essential to assess the impact of clinical research.

Following the 1999 Aspen Lung Conference dedicated to COPD, a working group of respiratory physicians from the US and Europe was convened to discuss a common operational definition that could be presented to international health-care providers and all groups involved in respiratory care. The definition agreed was that an exacerbation of COPD is:

`a sustained worsening of the patient's 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`. (36). This definition has also been adopted in the latest GOLD Guideline. (37)
When defining an exacerbation, it is necessary to pinpoint when the decline in any of the continuous measures is worse than expected, taking into account day-to-day variability (figure 1.1 below).

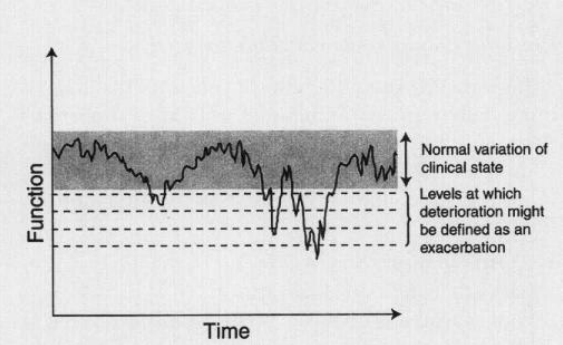


FIGURE 1. Day-to-day variability of a patient with COPD

Figure 1.1. Day to day variability in FEV1 in a patient with COPD taken from (36)

We must however be alert enough to take into account that other co-morbidities may play a role when a patient with COPD presents with an acute exacerbation e.g. heart failure.

The working group also tried to further define an exacerbation according to respiratory and systemic symptoms and signs (table 1.1), and also to stage the exacerbation in terms of use of healthcare services (table 1.2). The problem with the use of health care utilization is the fact that it adds unpredictable variables to an already complex cocktail of pathological and physiological variables.

Category	Descriptor
Respiratory	Increase shortness of breath
	Increase volume and purulence of sputum
	Increased Cough
	Shallow rapid breathing
Systemic	Increased body temperature
	Tachycardia

Table 1.1. Clinical descriptors used to characterize acute COPD exacerbations (36)

Severity	Level of health care utilization
Mild	Increased need for medication, can manage at home
Moderate	Increased need for medication, needs medical assistance
Severe	Patient recognises rapid deterioration and requires hospitalisation

Table 1.2. Staging of a COPD exacerbation – health care utilization (36)

A staging system has also been devised by the East London COPD research group to take into account upper and lower airway symptoms. A patient is defined as having an exacerbation if the following symptom patterns are experienced for at least two consecutive days: either two or more of three major symptoms (increase in dyspnoea, sputum purulence or sputum volume) or any one major symptom together with any one of the flowing minor symptoms: increase in nasal discharge, wheeze, sore throat, cough or fever. (38)

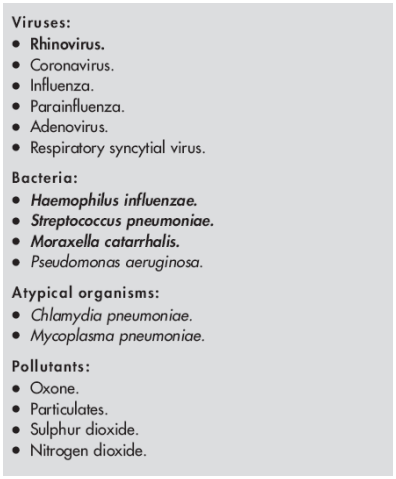


Figure 1.2. Aetiologies of exacerbations of COPD (39)

The common causes of exacerbations are presented in Figure 1.2, and will be discussed in more detail individually.

A combination of these definitions is probably more useful than each one individually. For the purposes of my study I used the following definition 'An acute exacerbation of COPD is defined as a sustained worsening of the patient's condition which could be attributable to their COPD, 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. It often presents with the combination of increasing dyspnoea, and an alteration in sputum production'.

1.2 Spirometry

At the heart of this study lies spirometry. Forced Expiratory Volume in one second (FEV_1) is the most commonly employed measurement used in COPD. The tight link between FEV_1 and COPD reflects the fact that spirometry is the standard for defining the presence of airway obstruction. (25, 37, 40) The progressive loss of FEV_1, the physiological variable that characterises COPD severity also predicts mortality.

FEV_1 is also used because of its consistency, reproducibility, and because it is also discriminatory. (37) The problem with analysing the research in this area is that FEV_1 can be reported in many different ways:

1. As a percentage of baseline
2. As a percentage of predicted
3. In units of volume
4. As a percentage of achievable

Many experts in the field do not recognise percentage change as being wholly clinically relevant, and prefer to see absolute change in millilitres (ml) recorded and charted. The natural average variability in FEV_1 for patients with moderate COPD is approximately 160ml.

There are also problems with measuring FEV_1. COPD is a diverse disease primarily of the small airways and FEV_1, besides being effort dependent, is not a very sensitive measure of small airways function. FEV_1 also correlates very poorly with dyspnoea, quality of life and some other long-term outcome measures. (41)

An often used measurement in respiratory physiology is Inspiratory Capacity (IC). Increases in IC and Vital Capacity (VC) reasonably reflect the lung volume response to a bronchodilator and can be used to infer the decrease in end-expiratory lung volume. (42-45)

IC can also measure the reduction in lung volume secondary to treatment; a higher IC infers a patient is able to perform spirometry from lung volumes lower than before treatment. Because the spirogram is performed at lower lung volumes, flow rates, being dependent on absolute lung volume, may not appear to be increased. (46) This can be inferred (rather than impractically measuring lung volumes) from an increase in their IC, due to a reduction in Functional Residual Capacity (FRC).

Given the emerging physiological and clinical rationale for pharmacological lung-volume reduction, assessment of volume responses to bronchodilators is likely to be highly relevant in COPD. It has been shown that patients with COPD develop dynamic hyperinflation (DH), which contributes to dyspnoea and poor

exercise tolerance. (44) DH has been found to correlate well with more sophisticated measurements of lung volume, even during exercise. (47) IC has been found to predict functionality during exercise in patients with COPD, and is responsible for the blunting of the usual increase in tidal volume during exercise. (48)

Progressive DH leads to an increasing sensation of breathlessness that makes an important contribution to the limitation of exercise in most patients with COPD. In these patients changes in end-expiratory lung volume (EELV) constitute an important outcome in assessing the effects of therapeutic interventions on the development of dynamic hyperinflation during exercise. (48) The assessment of dynamic changes in EELV is routinely carried out by serial IC manoeuvres assuming that, in patients with COPD, total lung capacity (TLC) does not change appreciably during exercise.

There are a significant number of patients with COPD who do not develop progressive hyperinflation during exercise but still report dyspnoea as the main cause of exercise limitation. (48) The results reported for this category of COPD patients are, however, discrepant as EELV has either been reported to remain constant with increasing intensity or actually to fall, as is commonly seen in healthy subjects. Accordingly, exercise limitation in these patients is not associated with end expiratory dynamic hyperinflation. This implies that simply tracking changes in EELV during exercise is not informative of all the factors that intensify dyspnoea and reduce exercise capacity in these patients. (49)

IC is determined by a slow manoeuvre (slow inspiration until maximum volume after regular tidal breathing) and is easy to measure with standard equipment available in any hospital and on some spirometers in general practice. Using standard spirometers the patient is asked to perform at least 3 tidal breathes before performing a full inspiratory manoeuvre, followed by a forced expiratory manoeuvre. Changes in IC from quiet breathing measured by the spirometer show a good relationship with optoelectronic plethysmography during exercise and recovery. (49)

Some studies have also shown an increase in IC after bronchodilator administration, which correlates closely with the improvement of dyspnoea sensation at rest. (50) Other studies have also shown that an improvement in IC

of 0.3l appears to correspond to a clinically meaningful improvement in dyspnoea scores and exercise tolerance. (42) The explanation for these findings is that lung deflation allows the patient to breathe at a lower, more comfortable lung volume, providing relief from dyspnoea.

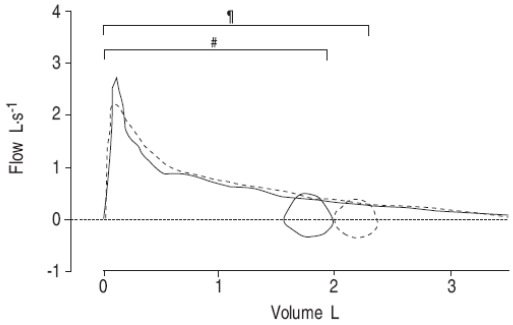


Fig. 2.–Flow/volume tracings for a male aged 40 yrs with severe chronic obstructive pulmonary disease. Pre- (solid line) and post- (dashed line) albuterol (bronchodilator) tracings show that inspiratory capacity (IC) increased 19% (from 1.97 to 2.35 L) after albuterol treatment. Illustrations for pre- (solid) and postalbuterol (dashed) tidal breaths are represented by the circular tracings on the figure. Forced expiratory volume in one second increased by only 9%. #: prealbuterol IC; ¶: postalbuterol IC.

Figure 1.3 Flow volume tracing in COPD – This figure shows changes are observed in inspiratory capacity after inhalation of a short acting β agonist; however no change in FEV1 is observed – improvements in symptoms may be secondary to a reduction in hyperinflation. Taken from reference (44)

In a study by Pellegrino (44), one-third of patients with chronic airflow obstruction treated with salbutamol demonstrated a bronchodilator response based on FEV_1. Two-thirds of the patients responded with changes in IC. Of the two, IC appears to be the best available choice. (figure 1.3).

As may be expected by their infrequent use, IC and Peak Expiratory Flow (PEF) parameters involve some unresolved issues. Their measurement accuracy and precision are not well known and current pulmonary function testing guidelines do not yet address the details of their measurement.

The last measurement is Forced Vital Capacity (FVC). FVC has to be carefully standardised, because in extremely well motivated patients it can be prolonged by as much as 20 seconds. (51) It is however one of the worst measurements in terms of reproducibility, with patients terminating their breath before a plateau is reached or being too unwell to perform a FVC manoeuvre. Forced Expiratory

Volume in six seconds (FEV_6) has been found to be a good surrogate for FVC and in the healthy lung study FEV_1/FEV_6 was found to be equivalent to FEV_1/FVC in predicting decline at 5 years. Reference equations based on the third National Health and Nutrition Examination Survey (NHANES III) study include both FEV_1/FVC and FEV_1/FEV_6 ratios. (52). A study in 502 consecutive patients in Christchurch, New Zealand, demonstrated that the FEV_1/FEV_6 ratio performed as well as FEV_1/FVC in diagnosing airflow obstruction. (51). The sensitivity and specificity of FEV_1/FEV_6 for obstruction defined by FEV_1/FVC were 99.5 and 100%, respectively, after allowing for a possible 100 ml error in FEV_1 and FEV_6. The FEV_6 was 25% less variable than FVC.

1.3 Biomarkers in COPD

A marker of an exacerbation has been the Holy Grail in chest medicine for a number of years. There are currently no specific diagnostic tests on sputum, blood, or airway biopsies that can be measured to tell us if a patient with COPD is having an exacerbation. However, this field has been extensively studied.

For several years physicians have been using the Anthonisen definition of an infective exacerbation, (35) but we know that not all exacerbations conform to this definition. (38) Although it is presumed that exacerbations are associated with increased airway inflammation, as in patients with asthma, there is little information available on the nature of inflammatory markers at exacerbation, especially when studied close to the onset of symptoms. Concentrations of inflammatory cytokines in induced sputum have been shown to be increased in patients with COPD when stable, but changes in induced sputum at exacerbation have not been extensively studied. (53) It certainly has rarely been studied in patients admitted with their COPD exacerbation.

Induced sputum has provided considerable information about the inflammatory process, including proximal airways but may not closely reflect distal inflammatory processes. (54) Exhaled gases and breath condensate are non-invasive procedures, so repeated measurements are possible, but for some assays the variability is relatively high. (55) There is relatively little information about how any of these biomarkers relate to other clinical outcomes, such as progression of the disease, severity of disease, clinical subtypes, or response to therapy. More information is also needed about the variability in these measurements. In the future, pulmonary biomarkers may be useful in predicting

disease progression, indicating disease instability, and in predicting response to current therapies and novel therapies, many of which are now in development. (54)

Only sputum neutrophils, (54) and IL-8, (56, 57) as well as serum TNFα, (56) and C-reactive protein (CRP), (58) show any trend toward separating different stages of COPD. In some studies changes in inflammatory markers have been associated with the type of infection at exacerbation. In a study of 30 patients admitted with their COPD, patients with bacterial growth in their sputum had significantly higher levels of MPO and IL-8 in sputum compared to patients with evidence of a viral insult. (59)

Even among non-current smokers there is good evidence for low grade systemic inflammation in those with chronic airflow limitation. This suggests that, once COPD develops, cessation of smoking may not fully attenuate the inflammatory process associated with this condition. (60, 61)

Secretary leukoproteinase inhibitor (SLPI) is the main proteinase inhibitor found in the larger airways and submucosal glands and also has antiviral and antibacterial properties (62). During the stable clinical state the level of SLPI in the sputum has been found to be significantly lower in patients with frequent exacerbations (three or more exacerbations in the preceding 12 months) than in those with infrequent exacerbations. (63)

CRP is raised in most studies of patients with exacerbations of COPD, however the standardised CRP usually used in hospitals is not always sensitive enough, therefore, most studies rely on high sensitivity assays that give results of hsCRP. (60) CRP has also been shown to be linked to worse pulmonary function even in those subjects with no pulmonary disease, in a large cohort of `healthy subjects` those with FEV1 falling in the lowest quartile was associated with a non attributable rise in CRP. (64) In the last couple of years there has been an explosion of papers linking COPD with CRP. The relationship between ventilatory function and markers of systemic inflammation, such as CRP, is affected by several factors that are associated with subclinical systemic inflammation, including smoking, obesity, and reduced cardiorespiratory fitness. (65)

CRP is an acute-phase protein produced by the liver in response to IL-6 stimulation. CRP is raised in most conditions associated with infection, inflammation, or tissue damage, for which it is a sensitive marker. It has also been shown in a recent large study of 90 patients looking for biomarkers of acute COPD to be the most sensitive indicator of exacerbation, along with MPIF-1 and IL-6, its predictive value is increased significantly when used alongside symptom scores. (58) (figure 1.4) In this study there were no significant relationships between biomarker concentrations and clinical indices of exacerbation severity**.**

The most commonly studied biomarkers in plasma and sputum are endothelin 1 levels, IL6, IL8, TNF-α and its soluble receptors sTNF-R55 and –R75, CRP, and fibrinogen. The problem with these measurements is that they require a certain degree of technical expertise that is not present in all hospitals.

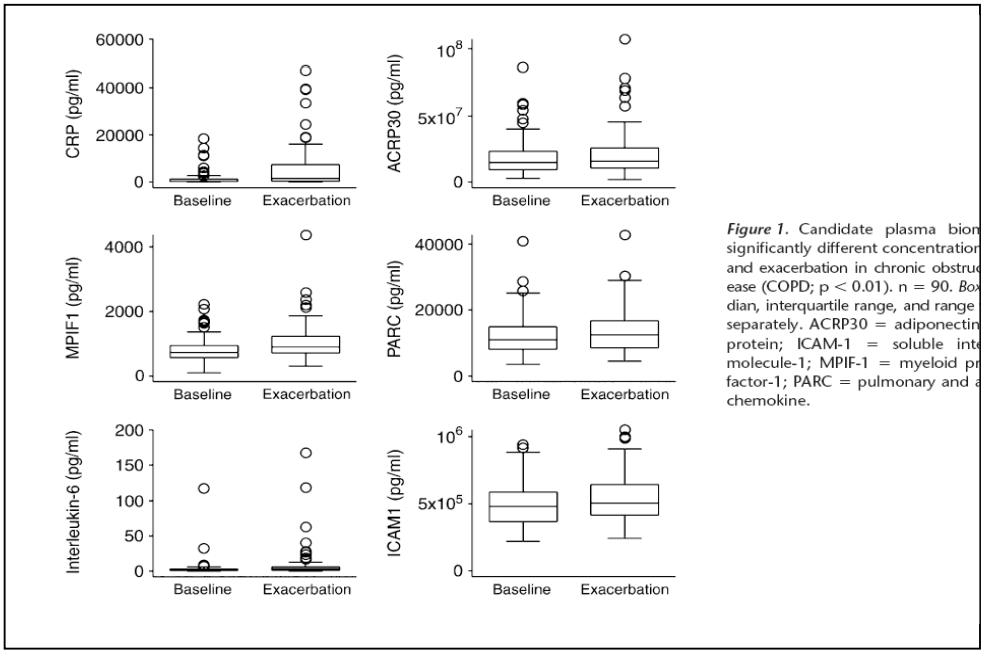


Figure 1.4: from Hurst, J. R.(2006). 'Use of Plasma Biomarkers at Exacerbation of Chronic Obstructive Pulmonary Disease' (58). Plasma CRP is as good as IL6 and other more difficult to measure cytokines during an exacerbation.

1. IL-6/IL-8

IL-6 is produced by airway macrophages and bronchial epithelium, while IL-8 is largely produced by neutrophils and macrophages. Correlations examined in stable sputum samples suggest that those patients with a longer history of smoking have higher IL-6 levels. (53). In the same study there was no relationship between cytokine levels and the current smoking status, and exacerbation frequency was found to be correlated with stable IL-6 levels, and IL-8 levels. (figure 1.5). The median IL-6 level was significantly higher at the time of an exacerbation than during stable conditions. The differences in IL-8 levels and the cell counts between exacerbation and baseline values did not reach statistical significance.

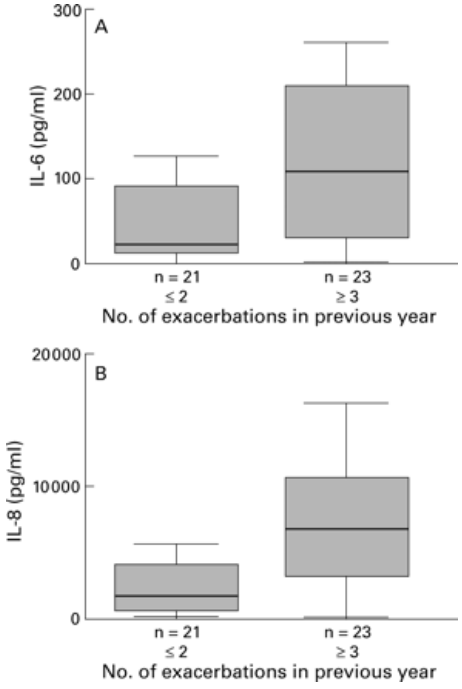


Figure 1.5: Comparison of sputum IL-6 and IL-8 levels according to exacerbation frequency. From (53)

The total cell count, neutrophil count, and macrophage count in the sputum, have not always been shown to be increased in patients with more frequent exacerbations. This suggests that increased IL-6 and IL-8 expression in the

sputum may be due to increased cytokine production by the bronchial epithelium.

IL-8 levels are related to cell counts, both neutrophils and macrophages, (38) but it is likely that a component of the increased sputum IL-8 level in patients with frequent exacerbations is produced by bronchial epithelial cells. It is likely that the increase in IL-6 levels during an exacerbation might partly be due to production by the epithelial cells, and is not necessarily proportional to the rise in total non-squamous cell count. There is debate as to the exact role of the interaction between the airway cells, and bacteria as an inflammatory stimulus in COPD. Some studies have observed that IL-6 is produced in response to *H. influenzae* endotoxin. (66)

Levels of IL-8 are related to the sputum neutrophil and total cell counts, (38) suggesting that neutrophil recruitment is a major source of IL-8 during an exacerbation. The observation that plasma fibrinogen levels are also associated with increased rates of hospital admission with exacerbation, as well as with reduced lung function and risk of COPD (67), may support a role for IL-6 which is the primary cytokine regulating fibrinogen expression.

2. TNFα

TNF-α has been described as being loosely correlated with resting energy expenditure and weight loss in COPD. (68) Interleukin-8, but not TNFα, was found to be significantly higher in the COPD patients when compared to asthmatics. (69)

An additional study has suggested that monocytes from underweight patients with COPD produce increased amounts of TNF-α in response to endotoxin challenge. (70) In this context, TNF-α may contribute to the muscle weakness, muscle loss, and cachexia, which limit exercise performance.

Unfortunately a recent clinical study has also failed to demonstrate a clinical effect of anti-TNF α antibody (infliximab) in patients with moderate to severe COPD. (71)

3. Endothelin 1

Endothelin-1 (ET-1) is a potent vasoconstrictive and bronchoconstrictive peptide that has been implicated in the pathophysiology of asthma. (72). It is thought to have important pro-inflammatory effects in the airways, being both a chemoattractant and an upregulator of other inflammatory mediators such as the IL-6 and IL-8. (73) There are several sites of ET-1 production within the human lung, including the bronchial epithelium, pulmonary endothelium, and alveolar macrophages. Rises in both plasma and sputum ET-1 levels have been found during exacerbations, with the sputum showing relatively larger changes. (74)

4. Fibrinogen

The mature fibrinogen molecule is composed of three pairs of polypeptide chains: two α chains, two β chains, and two γ chains. *In vitro* functional studies have shown that synthesis of the β chain is rate-limiting. Fibrinogen is expressed by the liver and levels can increase in response to intense acute phase stimuli such as trauma, surgery, or strenuous exercise, and fibrinogen levels are chronically elevated in the presence of mild inflammatory stimuli such as smoking. IL6 is the main mediator of acute phase-induced fibrinogen synthesis, and sequences responsive to IL6 are present in the promoter regions of the genes coding for the three fibrinogen chains. (75) Fibrinogen levels rise non-specifically and are a general measure of inflammation.

There also seems to be a relationship between the changes in fibrinogen, and IL-6 levels in stable COPD patients. Analysis of data in stable COPD patients has revealed significantly greater rises in fibrinogen when exacerbations were associated with purulent sputum, increased cough, and symptomatic colds. (76). A meta-analysis has found than fibrinogen levels are higher in COPD patients when compared to controls (60). Data from Dahl *et al* (67) suggest that individuals with increased systemic inflammatory markers such as fibrinogen experience an accelerated decline in lung function and are at increased risk of COPD hospitalisations in the future. (67) Moreover, during periods of exacerbation, plasma levels of fibrinogen and serum levels of IL-6 increase significantly. This may further contribute to increased cardiovascular morbidity, and mortality in patients with COPD. (76)

There is now a large body of evidence to indicate that systemic inflammation is present in patients with stable COPD. This finding may explain, at least in part,

the high prevalence of systemic complications such as cachexia, osteoporosis, and cardiovascular diseases among patients with COPD. Future studies are needed to determine whether attenuation of the systemic inflammatory process can modify the risk of these complications in COPD.

5. Bronchial Biopsies

Although the inflammation in COPD involves predominately lung parenchyma and small airways (bronchioles), (77, 78) bronchial biopsies reflect the cellular abnormalities seen in the peripheral lung (33, 79). Bronchial biopsies have been useful for documenting the structural changes, cellular patterns, and expression of inflammatory proteins in patients with COPD. In stable COPD, there is increased infiltration of macrophages and activated T lymphocytes, particularly of CD8 T lymphocytes (79), which express IFN-γ, CXCL10 (IFN-γ inducible 10-kD protein (IP-10), and IL-9. (80) Moreover, these lymphocytes express chemokine receptors associated with a Th1 type response, such as CXCR3, in contrast to lymphocytes in asthma which express chemokine receptors typical of a Th2 type response (CCR4). (81) . Although a prominent neutrophilia is present in the airway lumen of patients with stable COPD, it is not observed at the tissue level, except in patients with severe airflow limitation. (82) Finally, during exacerbations of the disease, an increased recruitment of eosinophils and neutrophils has been described, which is associated with upregulation of specific chemoattractants, such as CCL5, and CXCL5 (epithelial neutrophil-activating peptide-78). (83) There is also a reduction in histone deacetylase (HDAC) activity and HDAC2 expression in bronchial biopsies of patients with COPD compared with normal smokers and nonsmokers, and this is correlated with a reduction in NF-κB activity and increased expression of inflammatory genes. (84) These changes in bronchial biopsies may reflect the changes in NF-κB and HDAC found in lung parenchyma. The main problem with using biopsies is that we are not sure how representative they are of what is happening in the whole lung.

6. Leptin

Weight loss is common among patients with COPD and has a significant negative impact on mortality in this condition (figure 1.6). (85) The cause of this

weight loss is far from completely understood but has been associated with an increase in inflammatory cytokines, especially TNF α. (86) Although the increased work of breathing could account for some of this loss, the fairly new view that COPD is systemic inflammatory disease is also gaining credence.

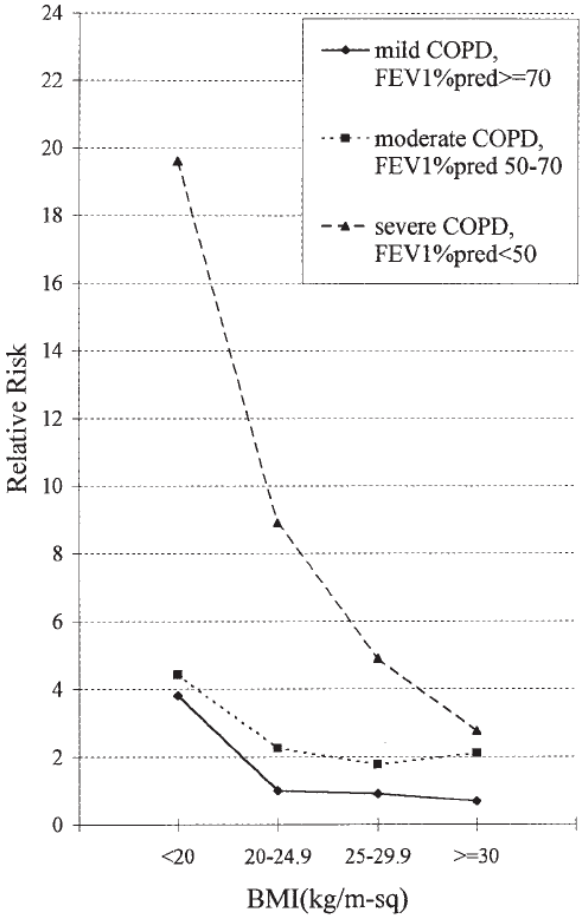


Figure 2. COPD-related mortality by BMI in subjects with mild, moderate and severe COPD. Normal-weight subjects (BMI 20 to 24.9 kg/m^2) with mild COPD were used for reference.

Figure 1.6. BMI and mortality according to stage of COPD using FEV1 percentage predicted. Taken from (85).

Leptin is an adipocyte-derived hormone coded by the *ob* gene which plays an important role in energy homeostasis by signaling the brain about the amount of adipose tissue stored in the body. After interaction with specific receptors located in the central nervous system and in peripheral tissues, leptin induces a complex response, including control of body weight and energy expenditure. Improvements in lipid metabolism and glucose homoeostasis, and increased thermogenesis, are considered to be some of the important metabolic effects of leptin. Circulating leptin levels alter according to the body mass index (BMI) of a patient.

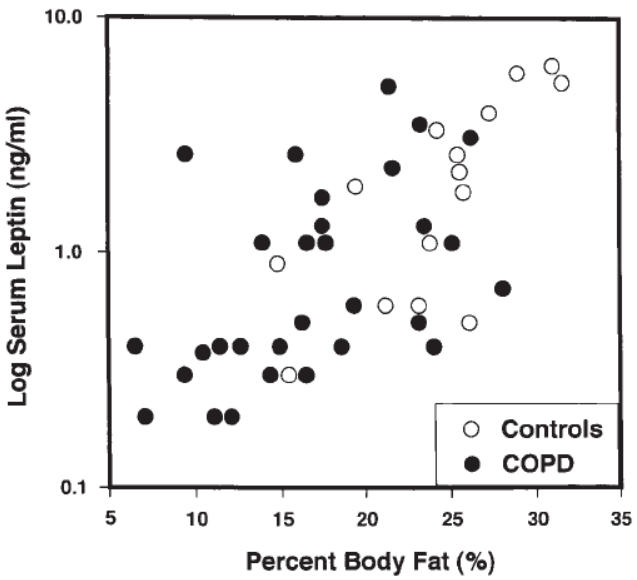


Figure 2. Serum leptin levels (log scale) and percent body fat in patients with COPD and in healthy subjects. Percent body fat was determined as described in METHODS. *Closed circles* represent COPD patients. *Open circles* represent control subjects.

Figure 1.7 Serum leptin levels and percentage body fat. (87)

Takabatake et al investigated serum leptin levels, along with circulating TNF α and its receptor (sTNF-R) in 31 patients with COPD and 15 age-matched healthy controls. The body mass index (BMI) and percent body fat (%fat) were significantly lower in the COPD patients than in the healthy controls. Serum leptin levels were also significantly lower in the COPD patients than in the healthy controls. Importantly, circulating leptin levels did correlate well with BMI and percentage fat (figure 1.7), but not with TNFα or with sTNF-R levels in the COPD patients. This data suggests that circulating leptin is independent of the TNFα system and is regulated physiologically even in the presence of cachexia in patients with COPD. (87)

A study into the differences in leptin levels between patients with chronic bronchitis and emphysema has been performed by Schols et al. (88). They studied patients with COPD and characterised their disease according to spirometry and features on high-resolution CT (HRCT). They found that as expected based on the lower fat mass, that mean detectable plasma leptin was significantly lower in the patients with emphysema compared with patients with chronic bronchitis.

Postmortem studies of patients who died in the Warsaw Ghetto during World War II suggested that death from starvation was associated with pulmonary emphysema. (89, 90) To determine whether anorexia nervosa is associated with an increased risk of pulmonary emphysema, Coxson and co-workers examined BMI, pulmonary function, and CT of the chest in 21 subjects with anorexia nervosa and 16 control subjects (91). They found greater CT measurements of emphysema in subjects with anorexia, a correlation between the BMI and the CT measures of emphysema in all subjects examined, and a correlation between diffusing capacity and CT measurements in subjects with anorexia. These results demonstrate emphysema-like changes in the lungs of chronically malnourished subjects.

7. B-type natriuretic peptide

The structure of atrial natriuretic peptide (ANP) was identified in 1984, and in 1988 a compound was isolated from pig brain that caused natriuretic and diuretic responses similar to ANP. This was called brain natriuretic peptide (BNP) although the main sight of synthesis is the ventricular myocardium. The

stimulus for both ANP and BNP is myocardial stretch, rather than transmural pressure loads. (92)

BNP is a 32 amino acid peptide hormone, the gene of which lies on chromosome 1. In plasma, the intact 108 amino acid prohormone (pro BNP), the biologically active BNP, and the remaining part of the prohormone, N-terminal (NT)-pro BNP can be measured by immunoassay. (93)

Prohormones in normal left ventricular myocytes are not stored in significant amounts, so it takes several hours after acute myocardial stretch for the plasma natriuretic peptide levels to increase significantly after the onset of acute myocardial stretch.

These hormones increase glomerular filtration rate and inhibit sodium reabsorption, causing natriuresis and diuresis. These hormones also inhibit cardiac sympathetic nervous system activity and the renin-angiotensin-aldosterone axis. BNP has been shown to predict cardiac disease state, and prognosis better than ANP. BNP has been shown to be the best discriminatory marker to differentiate cardiac dyspnoea from all other cause of dyspnoea in a whole series of trials. (94) There is also a close association between BNP and functional New York Heart Association (NYHA) class. (95) BNP is also a predictor of all cause mortality in those patients with chronic heart failure. (96)

With regards to ventricular function in respiratory medicine we are more interested in right heart dysfunction, and BNP has been shown to be raised in patients with pulmonary hypertension, cor pulmonale and pulmonary embolism. (93) BNP has been shown to increase in proportion to the degree of right ventricular dysfunction, but to a lesser degree compared to left ventricular dysfunction. In right ventricular dysfunction raised concentrations of BNP is a predictor for increased risk of death, even in cor pulmonale and primary pulmonary hypertension. In pulmonary embolism low BNP levels within 4 hours of admission are associated with an uneventful hospital admission, and may be useful in identifying patients for treatment in an outpatient setting. (97)

In patients with decompensated heart failure treatment with diuretics and vasodilators results in a rapid fall in BNP concentrations. (98) Whether this is true in acute respiratory disease has yet to be studied in a significantly sized

study. It has been shown that plasma BNP levels are significantly raised in patients with chronic respiratory failure complicated by cor pulmonale. In the same study comparisons of patients with lung cancer, and those with chronic respiratory failure not complicated by cor pulmonale, were not significantly different. Plasma BNP levels showed a weak correlation with systolic pulmonary arterial blood pressure, but no link with the degree of hypoxia. (99)

In patient with pulmonary hypertension secondary to COPD, BNP and ANP levels correlate with levels of endothelin 1 in the pulmonary vasculature. ANP and BNP may modulate the pulmonary vascular tone by interacting with endothelin 1 in these patients. (100)

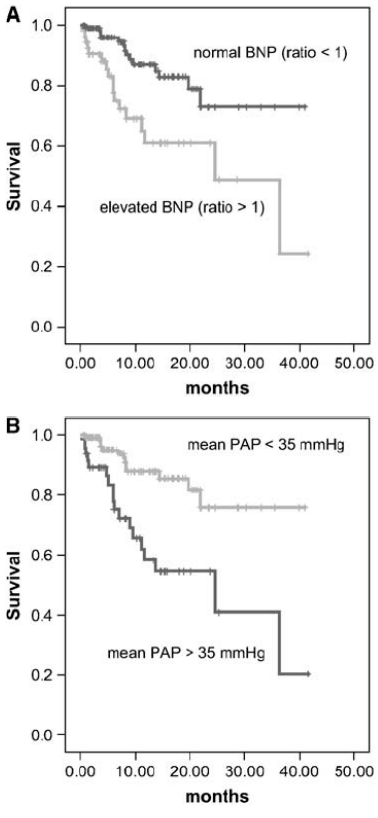


Figure 2. (*A*) Parameters predictive of survival after univariate analysis. Calculation of BNP ratio: measured BNP value/individual normal BNP value. (*B*) Survival estimates based on the presence of significant pulmonary hypertension. Mean PAP = mean pulmonary artery pressure.

Figure 1.8. from leuchte (101) BNP measurements and PAH as predictors of mortality.

Recombinant human BNP is now available for treatment of decompensated heart failure, (102) its benefit over traditional treatments such as dobutamine and milrinone has yet to be proven. Another new treatment, ANP/BNP inhibitors have also been developed, and subject to phase 2 studies comparing traditional treatment with ACE inhibitors (ACEi). The results from this trial were disappointing however and did not show an advantage in primary end points over ACEi. (92) Angio-oedema was also a worrying common side effect (2.2% on BNP inhibitors compared to 0.7% taking enalapril).

A recently published study (101) has studied the possibility of BNP being used as a biomarker; BNP levels were measured in 176 consecutive patients with various pulmonary diseases (50% COPD, 50% restrictive lung disease). Right heart catheterization, lung functional testing, and a 6-min walk test were performed. The mean follow-up time was nearly 1 yr. Significant pulmonary hypertension (mean pulmonary artery pressure > 35 mm Hg) was diagnosed in more than one-fourth of patients and led to decreased exercise tolerance and life expectancy. Elevated BNP concentrations identified significant pulmonary hypertension with a sensitivity of 0.85 and specificity of 0.88 and predicted mortality. Moreover, BNP served as a risk factor of death independent of lung functional impairment or hypoxemia in uni- and multivariate analysis. (figure 1.8)

1.4 Smoking and COPD

Airway Inflammation and smoking cessation in patients with COPD

Smoking cessation is the only currently available treatment that is effective in slowing down the accelerated decline in FEV_1 and thus progression of COPD. However, the exact role of smoking cessation on airway inflammation in COPD is still unknown. A few cross-sectional studies have investigated effects of smoking cessation by comparing smokers and ex-smokers with COPD (38), but there have been few good longitudinal studies comparing levels of inflammation before and after smoking cessation. Airway inflammation in bronchial biopsies and sputum does not differ between smokers and ex-smokers, except for some cytokines (IL- 8, IL- 6, soluble TNF-receptor (sTNFR) 75 and sTNF-R55). (57) Furthermore, Rutgers et al. showed that airway inflammation was more extensive in ex-smokers with COPD than in healthy ex-smokers. (103) Together, these cross-sectional studies suggest that there is ongoing

inflammation in COPD after smoking cessation. A recent study has compared patients with COPD who quit smoking vs. asymptomatic quitters. This study showed that in the 12 patients with COPD who successfully ceased smoking, airway inflammation persisted in bronchial biopsies, while the number of sputum neutrophils, lymphocytes, IL-8 and eosinophilic-cationic-protein levels significantly increased at 12 months. In the 16 asymptomatic smokers who successfully quit, inflammation significantly reduced (i.e. number of sputum macrophages, percentage of eosinophils and IL-8 levels) or did not change. (61)

Histone acetylation

Histones are the core in which chromatin is wound around. In the closed state gene induction is prevented, and excludes the binding of the enzyme RNA polymerase II (RNAPII) which activates the formation of mRNA. Gene transcription only occurs when the chromatin structure is opened up with unwinding of the DNA so that the RNAPII and basal transcription complexes can now bind to the naked DNA to initiate transcription. Each histone core has a long terminal, which is rich in lysine residues that may be acetylated, thus changing the electrical charge of the core histone. This results in acetylation of core histones, thereby reducing their charge, which allows the chromatin structure to transform from resting closed conformation to an activated open form. This allows the binding of TATA box-binding proteins (TBP), TBP-associated factors and, finally, RNAPII. Removal of the acetyl groups is associated with packing of the chromatin and a lack of gene expression or gene silencing. (104)

Histone Deacetylases (HDACs) play a critical role in the suppression of gene expression by reversing the hyperacetylation of core histones. There are 11 different HDACs in mammalian cells. There is evidence that these different types target different patterns of acetylation and, thereby, target different genes. Trichostatin A (TSA) is a nonselective inhibitor of HDACs and in alveolar macrophages it leads to the increased expression of inflammatory genes such as GM-CSF and IL-8. This suggests that HDACs normally act to suppress the expression of inflammatory genes. Atypical nicotinamide adenosine dinucleotide-dependent sirtuins are proteins that deacetylate nonhistone proteins and are thought to play a role in programmed cell death in mononuclear cells.

In COPD there is a marked reduction in the activity of HDAC2 in the lung parenchyma, and this is correlated to disease severity (in those with GOLD stage IV there is more than a 95% reduction in the expression of HDAC2) Reduced HDAC2 is related to a greater intensity of IL-8, and is also related to resistant anti-inflammatory effects of corticosteroids. (84)

Adenovirus increases the expression of inflammatory genes in epithelial cells via the *Adenoviral* E1A protein which interacts with HAT-containing co-activators such as CBP. In COPD there is evidence for latent adenoviral expression and increased expression of E1A. This may partially explain the inflammation in COPD patients. COPD patients are largely unresponsive to corticosteroids. This lack of response to corticosteroids may be explained, at least in part, by an inhibitory effect of cigarette smoking and oxidative stress on HDACs, thus interfering with a critical anti-inflammatory action of corticosteroids. There is a striking reduction in the activity and expression of HDACs in peripheral lung of patients with COPD. Even in patients with COPD who have stopped smoking, the corticosteroid resistance persists and these patients are known to have continuing oxidative stress. The mechanisms whereby oxidative stress and cigarette smoking lead to selective dysfunction of HDACs are currently under investigation. (105). There is evidence that the administration of low-dose theophylline restores the normal level of HDAC in macrophages of patients with COPD, the relevance of this to clinical practice has yet to be fully explored, and this is a novel therapeutic target. (106)

1.5 Measurements of COPD severity

MRC Dyspnoea score

Current guidelines define the severity of COPD in terms of FEV_1, but the correlation between airways obstruction and exercise performance is modest. (48) Health status measurements such as that provided by the St George's Respiratory Questionnaire (SGRQ) and the Chronic Respiratory Questionnaire (CRQ) provide well validated measurements of disability and handicap due to COPD, but these are complex to administer and score. A simple and standardised method of scoring disability that allowa patients and patient populations to be categorised in the manner analogous to the New York Heart

Association grading for disability due to heart failure has been devised, the Medical Research Council (MRC) dyspnoea scale has been in use for many years for grading the effect of breathlessness on daily activities. It is graded as such:

Grade 1: "I only get breathless with strenuous exercise".

Grade 2: "I get short of breath when hurrying on the level or up a slight hill".

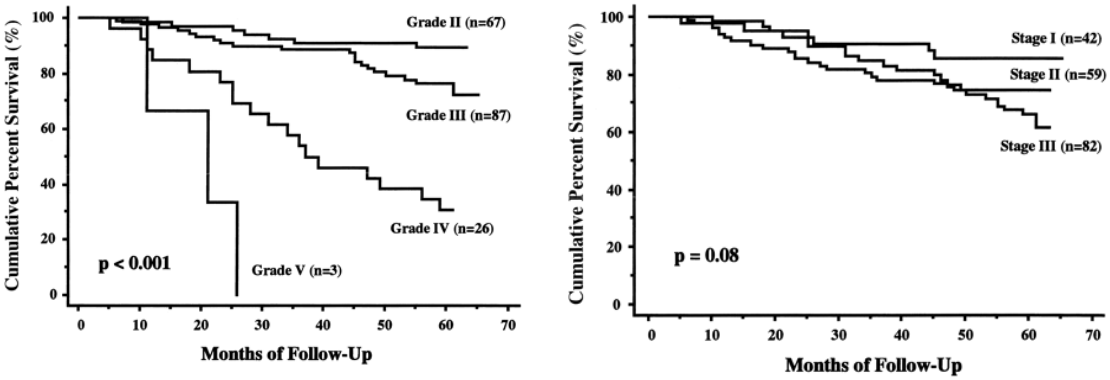
Grade 3: "I walk slower than people of the same age on the level because of breathlessness or have to stop for breath when walking at my own pace on the level"

Grade 4: "I stop for breath after walking 100 yards or after a few minutes on the level".

Grade 5: "I am too breathless to leave the house".

It is particularly noteworthy that the FEV_1 is a poor indicator, and does not relate to disability as measured using the MRC scale. Patients' performance on a shuttle walking test is related to the level of disability, and the mean scores clearly deteriorate as disability increased across the MRC groups. (107)

The MRC scale has also been shown in some circumstances to be a better predictor of mortality than FEV1 (figure 1.9). (108) It is also used as a variable in the BODE index. (109), and has been extensively studied. (110)



MRC Dyspnoea Scale ATS grading based on FEV_1

Fig 1. 9. Patient survival – MRC vs. FEV1 as a predictor for mortality in patients with COPD. (108)

Other measures in COPD

Celli et al. have studied in detail combinations of measures to come up with another scoring system to try and predict life expectancy in patients with COPD. They have devised the BODE index, B – **B**ody Mass Index, O – Degree of airflow **O**bstruction, D – MRC **D**yspnoea score, and E- **E**xercise capacity as measure by the 6-minute walking test. (1) The BODE index is a multi-dimensional 10-point scale in which higher scores indicate a higher risk of death. Celli et al. validated the index in a cohort of 625 patients, with death from any cause and from respiratory causes as the outcome variables. They found that patients with higher BODE scores were at higher risk for death; the hazard ratio for death from any cause per one-point increase in the BODE score was 1.34 (95 percent confidence interval, 1.26 to 1.42; P<0.001), and the hazard ratio for death from respiratory causes was 1.62 (95 percent confidence interval, 1.48 to 1.77; P<0.001). The scoring system is shown below in figure 1.10 below.

Table 2. Variables and Point Values Used for the Computation of the Body-Mass Index, Degree of Airflow Obstruction and Dyspnea, and Exercise Capacity (BODE) Index.*

Variable	Points on BODE Index			
	0	1	2	3
FEV_1 (% of predicted)†	≥65	50–64	36–49	≤35
Distance walked in 6 min (m)	≥350	250–349	150–249	≤149
MMRC dyspnea scale‡	0–1	2	3	4
Body-mass index§	>21	≤21		

Figure 1.10. From Celli, Cote et al. 2004 BODE index (1)

The group concluded that the scoring system was better than FEV_1 at predicting risk of death. They have shown the results graphically comparing the ATS grading system using FEV1 and the BODE index. (figure 1.11)

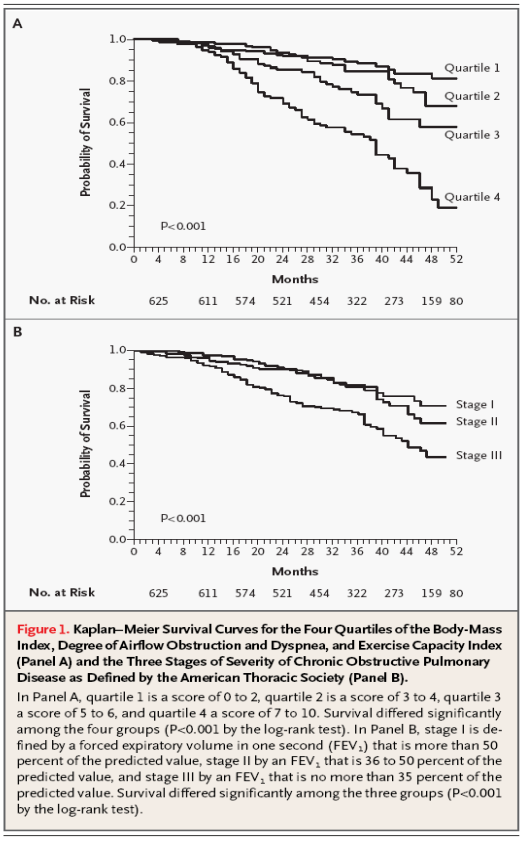


Figure 1. Kaplan–Meier Survival Curves for the Four Quartiles of the Body-Mass Index, Degree of Airflow Obstruction and Dyspnea, and Exercise Capacity Index (Panel A) and the Three Stages of Severity of Chronic Obstructive Pulmonary Disease as Defined by the American Thoracic Society (Panel B).

In Panel A, quartile 1 is a score of 0 to 2, quartile 2 is a score of 3 to 4, quartile 3 a score of 5 to 6, and quartile 4 a score of 7 to 10. Survival differed significantly among the four groups ($P<0.001$ by the log-rank test). In Panel B, stage I is defined by a forced expiratory volume in one second (FEV_1) that is more than 50 percent of the predicted value, stage II by an FEV_1 that is 36 to 50 percent of the predicted value, and stage III by an FEV_1 that is no more than 35 percent of the predicted value. Survival differed significantly among the three groups ($P<0.001$ by the log-rank test).

Figure 1.11 Comparison of survival curves BODE index vs. American Thoracic Classification (1)

1.6 Sputum microbiology

Induced Sputum – Microbiology and Cell Counts

The method of inducing sputum in patients with COPD, even during acute exacerbations has been found to be safe if conventional methods and safety procedures are utilised. (111, 112). Hypertonic saline is a potent bronchoconstrictor; this knowledge is invaluable if to be used in patients already suffering from an acute exacerbation. Induced sputum also contains a higher proportion of viable cells than spontaneous sputum. It has also been shown that there are no significant differences between the sputum samples obtained at seven minutes, and at 14 minutes of hypertonic saline nebulisation in patients with COPD, as opposed to asthma where there are significant differences. (113) With regards to measurement of inflammatory markers there is no obvious difference between those obtained from spontaneous as opposed to induced sputum.

Inducing sputum in high risk patients involves a slightly modified technique to reduce the risk of significant bronchoconstriction during the procedure.

Bacteria in sputum

The bacterial flora in COPD patients in the stable and exacerbation states has been extensively studied. The sputum in healthy control subjects is an almost sterile environment whilst patients with COPD harbour a variety of pathogenic and non-pathogenic organisms.

The three most common bacterial isolates are *Haemophilus influenzae*, *Streptococcus pneumoniae*, *and Moraxella catarrhalis*. The role of bacteria in causing exacerbations is controversial because the same organisms may be isolated in up to 30% of patients when clinically stable. This phenomenon, termed ‘‘bacterial colonisation’’, is associated with continued cigarette smoking and increasing severity of underlying disease. (114) Much of the evidence that bacteria are an important cause of exacerbations is, therefore, extrapolated from the benefit seen in trials of antibiotics. The presence of colonisation is not benign and may itself modulate exacerbation frequency. A recent study by Sethi has suggested that exacerbations may be the result of acquisition of new strains of bacteria. (115) In a study by Patel in patients with stable COPD 50% were colonised by a possible pathogen. (116)

Induced sputum seems to be the method of choice to acquire samples; however, there are no convincing data that it achieves a different result from spontaneously produced samples. There are now many studies that show that induced and spontaneous sputum provide results that are comparable. (116-118) A recent study by Marin et al. on 32 patients with COPD showed that bacterial isolation using both methods achieves the same result; however, induced sputum techniques allow 1/3 of patients unable to expectorate spontaneously to produce sputum. (119) Stable COPD patients often have lower airway bacterial colonisation which may be an important stimulus to airway inflammation and thereby modulate exacerbation frequency. There are several arguments regarding the findings of bacteria in the lower airway and exacerbations of COPD. The main one centres on the fact that bacteria are isolated at the same frequency in stable COPD patients and during an acute exacerbation. (120) So is the finding of bacteria in the lower airways an epiphenomenon? (121) If bacterial pathogens were playing a role in AECOPD, one would expect a neutrophilic inflammatory response in the airways to the acute infection. Therefore, one would expect that airway inflammation during AECOPD should be related to sputum culture results.

Risk factors for colonisation include the degree of airways obstruction, and current smoking. (122) The presence of bacterial colonisation is also related to the colour of the expectorated sputum (figure 1.12). In a study of patients with COPD presenting to primary care, the presence of green (purulent) sputum was 94.4% sensitive and 77.0% specific for the yield of a high bacterial load and indicated a clear subset of patient episodes identified at presentation that was likely to benefit most from antibiotic therapy**.** (123) In an editorial review on the relationship of bacteria to lung host defences, it was suggested that it should be possible to separate the presence of bacteria as commensals in the airway from those causing an infection. (124) The latter would be expected to be accompanied by activation of secondary host defences, which include increased neutrophil recruitment to the airways. This neutrophil influx should be associated with a change in secretions from mucoid to purulent (because the myeloperoxidase from the neutrophils is green), and the process would reverse after antibiotic therapy that reduced or eliminated the bacterial load, thereby leading to resolution of the secondary host response.

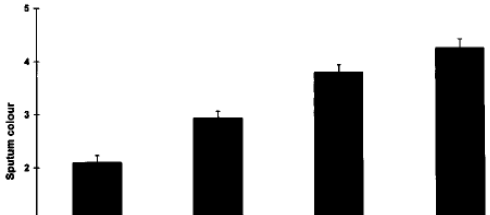


Figure 1.12 The relationship between sputum colour and total neutrophil count from patients with an exacerbation of COPD. From (123)

Serologic evidence of atypical bacterial infection, mostly by *Chlamydia pneumoniae*, is seen in 5 to 10% of exacerbations. (125) However, a recent study in AECOPD using Real-time PCR to detect *C.pneumoniae* or *Mycoplasma* failed to identify either organism in the sputum. (126) Serologic evidence of atypical bacterial infection, mostly by *Chlamydia pneumoniae*, is seen in 5 to 10% of exacerbations. (125) However, a recent study in AECOPD using Real-time PCR to detect *C.pneumoniae* or *Mycoplasma* failed to identify either organism in the sputum. (126)

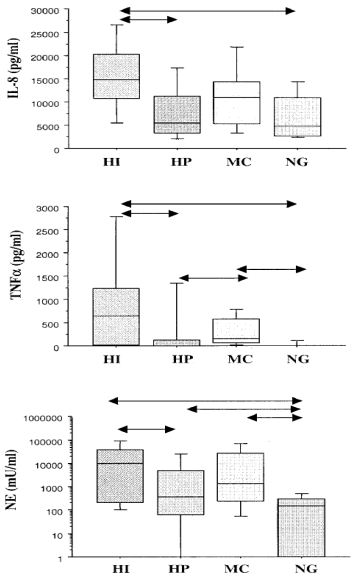


FIGURE 2. Box plots of sputum inflammation in the unpaired groups. The vertical bar represents 10th and 90th percentile values, the box encompasses the 25th to 75th interquartile range, and the horizontal line in the box represent median values. The arrows represent significant ($p < 0.05$) differences by the Mann-Whitney *U* rank test. HI = *H influenzae* isolated as the sole pathogen on culture; HP = *H parainfluenzae* isolated as the sole pathogen on culture; MC = *M catarrhalis* isolated as the sole pathogen on culture; NG = only normal flora isolated on culture.

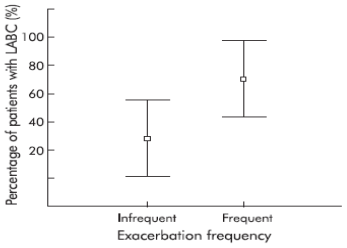


Figure 2 Relationship between lower airway bacterial colonisation (LABC) by a possible pathogen in induced sputum and frequent (>2.58 exacerbations per year; n=14) and infrequent exacerbations (<2.58 exacerbations per year; n=14) with 95% confidence intervals.

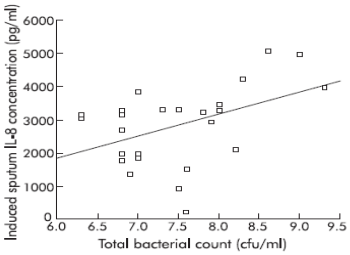


Figure 3 Relationship between total bacterial count (colony forming units/ml) and induced sputum IL-8 levels (Spearman's rho=0.459, p=0.02). The bacterial count data have been logarithmically transformed.

Figure 1.13. *H.Influenze* is associated with high IL-8 levels when a colonizing bacteria in COPD. (127) Bacterial colonisation is associated with exacerbation frequency and a higher IL-8 concentration. (118)

Bacterial pathogens are isolated from sputum in about 50% of exacerbations. The prevalence of each in the study by Patel was as follows, *Haemophilus influenzae* (53.3%), *Streptococcus pneumoniae* (33.3%), *Haemophilus parainfluenzae* (20%), *Branhamella catarrhalis* (20%) and*, Pseudomonas aeruginosa (*20%). The presence of lower airway bacterial colonisation in the stable state was found to be related to exacerbation frequency. Patients colonised by *H influenzae* in the stable state reported more symptoms, increased sputum purulence at exacerbation, and had higher IL-8 levels than those not colonised. (118) (see figure 1.13) They also found that patients colonised by *H. influenzae* in the stable state reported more symptoms and increased sputum purulence at exacerbation than those not colonised.

The elevation in inflammatory markers in patient colonised by bacteria was confirmed by Sethi et al. who examined sputum from 45 patients with acute exacerbations of COPD managed as outpatients. The correlation of inflammatory markers to bacterial type is shown in the figure (1.13 above). The authors of this study concluded that increased airway inflammation associated with isolation of *H influenzae* and *M catarrhalis* supports an aetiological role of these pathogens in AECOPD. Both these organisms are increased in those who continue to smoke. (127)

H influenzae and its products reduce mucociliary clearance, increase mucus secretion, and cause bronchial epithelial damage *in vitro*. The lipo

oligosaccharide of *H influenzae* induces IL-8, IL-6, and TNF-α secretion from bronchial epithelial cells *in vitro*. (128) It is, therefore, likely that a similar inflammatory process is being engendered by these mucosal pathogens during exacerbations of COPD.

Several bronchoscopic studies of distal airway bacterial flora in AECOPD have shown that *H influenzae* and *M catarrhalis* are often present in concentrations of > 10^3/mL in the distal airways during AECOPD. It may be that the particular strain of an organism is different during exacerbation than when isolated as a coloniser in the stable condition. This has been shown with *Moraxella catarrhalis, Streptococcus pneumoniae*, and *Haemophilus influenzae*. (115, 129)

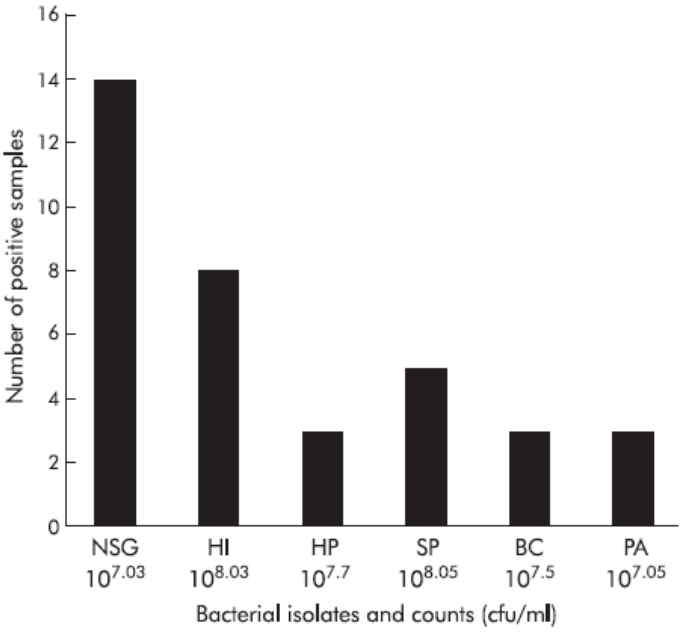


Figure 1 Possible pathogens recovered in induced sputum. Mean bacterial counts (colony forming units per ml, cfu/ml) in each group are expressed below isolates. NSG=non-specific growth; HI=*Haemophilus influenzae*; HP=*Haemophilus parainfluenzae*; SP=*Streptococcus pneumoniae*; BC=*Branhamella catarrhalis*; PA=*Pseudomonas aeruginosa*.

Figure 1.14. Shows the pathogens recovered in induced sputum during the stable state. (39 patients) from (118)

The frequency of exacerbations also seems to contribute to the long term decline in lung function of patients with moderate to severe COPD. In a study by Donaldson et al., 109 patients with moderate to severe COPD were followed over 4 years (FEV_1 0.7-1.3L). These patients experienced 757 exacerbations. Patients with frequent exacerbations had a significantly faster decline in FEV_1 of –40.1 ml/year than infrequent exacerbators in whom FEV_1 changed by –32.1 ml/year. Frequent exacerbators also had a greater decline in FEV_1 if allowance was made for smoking status. Patients with frequent exacerbations were more often admitted to hospital with longer length of stay. Whether these declines in FEV_1 are clinically important is debatable – but they do represent a 25% difference between the 2 groups. (130) (figure 1.15)

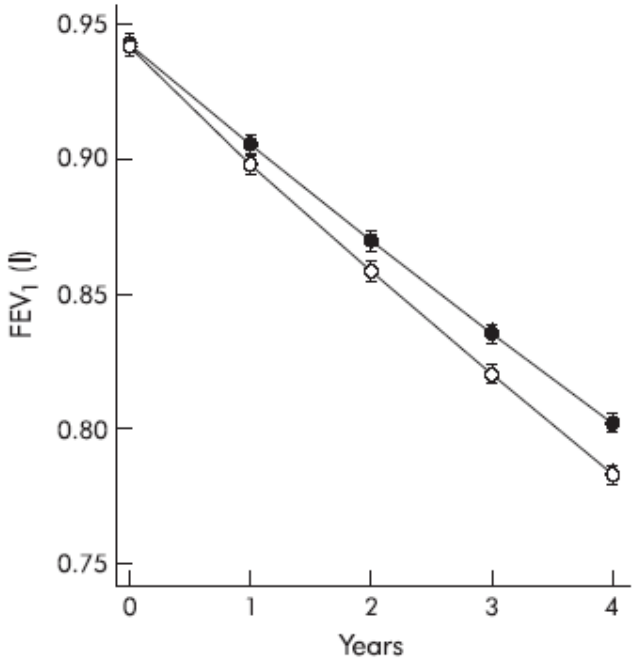


Figure 2 Percentage change in FEV_1 with standard errors over 4 years. Open circles represent infrequent exacerbators; closed circles represent frequent exacerbators. *

Figure 1.15. Frequent exacerbators have a greater decline in FEV_1 if allowance was made for smoking status. * The original legend from the paper is wrong, open circles represent the frequent exacerbators and closed circles infrequent exacerbators (1)

Haemophilus influenzae colonizes the respiratory tract of patients with COPD, often presenting with a pattern in which periods of negative sputum cultures are

preceded and followed by isolation of apparently identical strains. To investigate whether the strains preceding and following episodes of negative cultures were indeed the same, Murphy and colleagues performed molecular typing on isolates of *H. influenzae* collected monthly in a prospective study. (131) During a 7-year study involving 104 patients, they detected 122 episodes of negative cultures lasting 1 month or more, which were preceded and followed by isolation of an apparently identical strain of *H. influenzae*. Seventeen such episodes of negative cultures, lasting 6 months or more, were studied in detail to test the hypothesis that these periods of negative cultures represented continuous colonization by the same strain of *H. influenzae*. Molecular typing by three independent methods established that the strains preceding and following the episodes of negative cultures were indeed identical. Strain-specific *H. influenzae* DNA was detected in some of the sputum samples that had yielded negative cultures. These results show that some patients with COPD are persistently colonized with *H. influenzae*, and that sputum cultures underestimate the frequency of colonization of the respiratory tract by *H. influenzae*. The study also suggests that chronic bacterial colonization may significantly contribute to airway inflammation and to the course and pathogenesis of COPD. To test the hypothesis that the immune response to the homologous (infecting) strain of *H. influenzae* may have a limited ability to kill other (heterologous) *H. influenzae* strains, and thus it may protect against recurrent exacerbations caused by homologous strains, Sethi et al. collected sputum and serum samples in 81 patients with COPD monthly and during exacerbations. They found that, after exacerbation, an immune response to homologous *H. influenzae* occurs in 61% of cases with newly acquired strains as compared with 21% of cases with pre-existing strains. New bactericidal antibodies developed after COPD exacerbations were highly strain-specific, showing bactericidal activity for only 12% of heterologous strains. (129) These results suggest that, after an exacerbation of COPD associated with *H. influenzae* strain, serum antibodies to the infecting strain develop in the majority of cases with newly acquired but homologous strains, and that the immune response to the homologous strain may not protect against infectious exacerbations by heterologous strains of *H. influenzae*. These observations further support the role of *H. influenzae* in a large proportion of COPD exacerbations and, by showing the limited immune response to heterologous strains of *H. influenzae*, may explain the mechanism of recurrent exacerbations with *H. influenzae* in COPD. In fact, although patients produce strain-specific

antibodies to homologous strains of *H. influenzae* after exacerbations, the immune response leaves the host susceptible to reinfections by other heterologous strains of *H. influenzae.*

Phagocytosis of non typeable Haemophilus influenzae (NTHI) by alveolar macrophages of smokers has been accompanied by increased bactericidal activity compared with alveolar macrophages of nonsmokers. In a study of the effectiveness of alveolar macrophages to clear NTHI they found that alveolar macrophages from ex-smokers displayed immunological properties that were more like alveolar macrophages of non-smokers. (132)

Viruses

Evidence accumulated over the past few years has to point to a clear association between viruses and AECOPD. Virus infection, identified by a four-fold rise in antibody titre or by viral isolation, was found almost 30 years ago by Buscho to cause up to a third of exacerbations. (133) Since then, with the development of PCR techniques, viral infection has been found in most studies to be the cause of an exacerbation in over 50% of patients. Viral infections seem to be associated with smoking status, being more common in current smokers. (134) Viruses also seem to be responsible for secondary bacterial infections by facilitating the attachment of bacteria to the epithelium. (135) The most common viruses involved in AECOPD are shown in table 1.3.

Rhinoviruses are one of the main virus species isolated during acute exacerbations. Rhinoviruses are an RNA picornavirus and are responsible for the common cold. They are spread from person to person by infected respiratory secretions. Studies in childhood asthma have shown that viruses, especially *rhinovirus*, can be detected by polymerase chain reaction (PCR) in nasopharyngeal samples from a large proportion of these exacerbations. However, the role of *rhinovirus* in COPD exacerbation has been unclear until recently. In a study by Seemungal, it was found that rhinovirus was associated with 23% of COPD exacerbations; the virus being detected with higher frequency in induced sputum from the lower airway, compared to upper airway specimens. *Rhinoviruses* were associated with a greater rise in lower airway IL-6 levels and total symptom score at COPD exacerbation. (136) *Rhinoviruses*

are also associated with active smoking and the patient's pack year history. (137).

There are a number of ways that *rhinovirus* infection may be involved in exacerbations. *Rhinovirus* attaches to airway epithelium by ICAM-1 which promotes the recruitment of inflammatory cells via NFκB. (138, 139) *Rhinovirus* also interacts with the bacterial flora by increasing the expression on epithelial cells of platelet aggregation receptor, this allows for greater adherence by *Streptococcus pneumoniae* at least in in-vivo studies. (table 1.4)

Viruses	Rhinovirus
	Coronavirus
	Influenza A and B
	Parainfluenza
	Adenovirus
	Respiratory Syncitial Virus
Atypical Organisms	Chlamydia Pneumoniae
	Mycoplasma Pneumonia

Table 1.3. Respiratory viruses and atypical organisms involved in exacerbations of COPD

Other viruses associated with AECOPD are *RSV, influenza A* and *B, parainfluenza, adenovirus*, and *metapneumovirus*. Lower levels of influenza as a cause of exacerbations are associated with the increasing uptake of the influenza vaccine. (140)

Human metapneumovirus is a paramyxovirus that was discovered in 2001 in the Netherlands. Epidemiological (UK English) studies have shown it to be a major cause of acute respiratory tract infection in normal infants and children worldwide, with a seasonal occurrence and spectrum of clinical illness most similar to the closely related *respiratory syncytial virus.* The greatest prevalence of severe disease requiring hospitalization in otherwise healthy children

appears to be in those aged between 6 and 12 months, older than the peak age of hospitalizations for *respiratory syncytial virus*. *Human metapneumovirus* is also a significant cause of acute respiratory disease in adults, particularly the elderly and those with co morbid conditions such as chronic obstructive pulmonary disease, asthma, and cancer. Because there is no rapid diagnostic assay, RT-PCR is most widely used.

In a recent study 194 samples from patients with acute exacerbations of COPD were analysed using RT-PCR assays for *picornaviruses, coronaviruses* 229E and OC43, i*nfluenza A and B viruses, respiratory syncytial virus, parainfluenza types 1–3* viruses, and *human metapneumovirus* and a PCR assay for *adenoviruses*. RT-PCR assays identified an additional 35 respiratory virus-associated illnesses not identified previously by cell culture or serology (*n*=46). *Picornaviruses* and *coronaviruses* were the most common viral infections identified only by RT-PCR. Overall, 41.8% of the acute respiratory illnesses evaluated were associated with a respiratory virus infection, with *picornaviruses, coronaviruses* and *influenza viruses* being the most common infections recognized. No *Human metapneumovirus* infections were identified by RT-PCR assay. Another study, however, found the presence of *metapneumovirus* in 7% of patients during an acute exacerbation. (59). A recent paper also reported detection of *hMTP*, this virus was only present in combination with another *rhinovirus*. (141)

Potential mechanisms of viral infection to cause exacerbations of COPD

Upregulation of ICAM1
Increase expression of platelet aggregation receptor
Increase recruitment of inflammatory cells
Increased oxidant stress
Increased systemic and airway endothelin-1

Table 1.4 Potential mechanisms of viral infection to cause exacerbations of COPD

1.7 Cell counts

Cell Counts In Induced Sputum.

Airway inflammation is also thought to play a key role in the pathogenesis of COPD. The cellular inflammatory response is characterised by an increase in neutrophils, macrophages and CD8 positive T-lymphocytes in small and large airways, as well as observed in the lung parenchyma itself. The major type of cell in induced sputum is the neutrophil, the quantity of which is associated with the severity of the airflow limitation. Sputum represents a non invasive marker that is potentially useful for disease monitoring; however there has been observed independence in factor analysis between airways inflammation (as measured by cell count in induced sputum, and not cytokines) and lung function. (28)

Neutrophils and eosinophils predominate in sputum, macrophages and lymphocytes predominate in bronchoalveolar lavage, and lymphocytes predominate in bronchial biopsies. In cigarette smokers with COPD, the degree of neutrophilia relates loosely to the degree of chronic airflow limitation. (69) Some patients with chronic bronchitis or COPD have an increase in the proportion of eosinophils in sputum, as is characteristically seen in uncontrolled asthma and in non-smokers with eosinophilic bronchitis without asthma. (142). Eosinophils have also been reported to increase in some exacerbations of COPD. (122) The prevalence of sputum eosinophilia in COPD has not been examined in a population study. Sputum eosinophilia is important because it appears to be associated with clinical improvement. When eosinophils have been observed in the sputum of patients with COPD they are also associated with eosinophilic cationic protein and eosinophilic peroxidase suggesting that the eosinophils have degranulated. This maybe the result of neutrophil elastase levels in COPD. Although some exacerbations of COPD are associated with sputum eosinophilia, they are associated more usually with neutrophilia.

A recent study published by Papi, who examined the aetiology of 64 patients hospitalised with COPD, showed exacerbations with a viral origin, alone or in combination with a bacteria exhibit a sputum eosinophilia compared to sputum from patients with only bacterial detected. Neutrophil counts were similar in all groups. (Figure 1.16) (141)

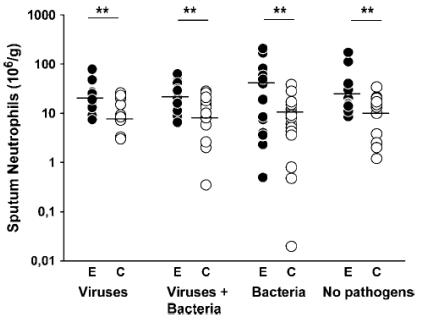


Figure 3. Airway neutrophil counts are increased during COPD exacerbations independent of exacerbation etiology. Sputum neutrophil counts in patients with COPD during severe exacerbation requiring hospitalization (E) and during stable convalescence (C). Subjects were grouped according to the presence of respiratory viruses alone, bacteria alone, both viruses and bacteria, or no pathogen in the sputum during exacerbation. *Bars* represent median values. Data from all 64 patients. **p < 0.01.

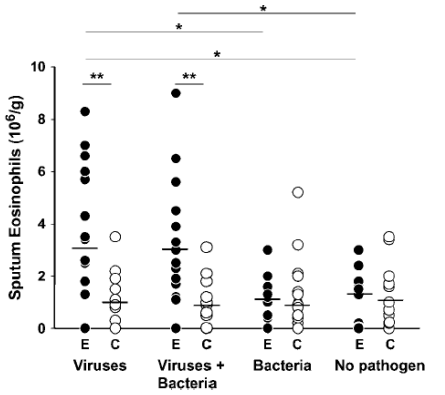


Figure 4. Airway eosinophil counts are only increased during COPD exacerbations associated with virus detection. Sputum eosinophil counts in patients with COPD during severe exacerbation requiring hospitalization (E) and during stable convalescence (C). Subjects were grouped according to the presence of respiratory viruses alone, bacteria alone, both viruses and bacteria, or no pathogen in the sputum during exacerbation. Data from all 64 patients. *Bars* represent median values. *p < 0.05, **p < 0.01.

Figure 1.16. A recent study published by Papi observed exacerbations with a viral origin, alone or in combination with a bacteria exhibit a sputum eosinophilia compared to sputum from patients with only bacterial detected. (141)

Induced sputum in COPD contains mainly neutrophils at a percentage that is almost reciprocal to that of macrophages in healthy volunteers. (112) These observations are in line with previous findings of high percentages of intraluminal neutrophils and confirm previous studies demonstrating an increased number of neutrophils in bronchoalveolar lavage fluid. (143) The finding that the highest neutrophil percentages are found in patients with the worst airflow obstruction is in agreement with the hypothesis that neutrophilic inflammation of the small bronchi are an important factor in the pathogenesis of airflow limitation in COPD. It has been shown that small bronchi and bronchioli are the major site of increased airflow resistance in COPD and that inflammatory changes at these sites correlate with airflow limitation. (144) Assuming that induced sputum contains material from the lower airways, this technique may be a useful tool to study airway inflammation in COPD.

Peripheral blood and sputum lymphocytes

The functional activities of both CD4+ and CD8+ T-cells are initiated by the binding of specific antigen presented in association with the major histocompatibility complex (MHC) on the target cell to T-cell antigen receptors.

Once activated, T-cells migrate into the peripheral tissue, the lung in this case, which is the site of antigen entry and persistence.

In contrast to the numerous studies which show that lymphocytes play an important role in the pathogenesis of asthma, few papers have looked into their role in COPD. One of the earliest papers published looking into CD4 and CD8 cell ratios was published by De Jong, he showed that the percentage of CD8+ lymphocytes was significantly higher in non-smoking COPD subjects compared to non-smoking healthy control subjects. These authors failed to find a difference between ex- and current smokers with COPD. (145)

Other groups have found differences between current and ex-smokers with COPD. Thus, Lapperre and colleagues studied 114 patients with COPD GOLD stage II and III. They performed bronchoscopies on these patients and measured the number of inflammatory cells in bronchial biopsy specimens; they found that ex-smokers had higher number of CD4+ lymphocytes and plasma cells than current smokers. They also found that those who stopped smoking more than 3.5 years ago also had lower numbers of CD8+ lymphocytes. Their observations led them to comment that T-lymphocyte numbers respond to current smoking status. (146) These patients had mild disease, were steroid naïve and were not exacerbating at the time of the study.

The number of CD4+ T-cells in the lungs of smokers increases significantly after 30 yrs of smoking as do the CD8+ T-cells, suggesting that the CD4+ T-cell might be playing a role in the inflammatory process. (147) CD4+ T-cell help is required for the priming of CD8+ cytotoxic T-cell responses, for maintaining their memory and for ensuring CD8+ T cell survival, (148) suggesting that even low numbers of CD4+ T-cells could be essential for the development of the CD8+ T-cell inflammatory infiltrate found in smokers.

To look at the effect of cigarette smoking on T-cell subsets Tollerud et al recruited 282 healthy subjects and performed FACS analysis on their peripheral blood. They found that cigarette smoking was associated with a selective increase in CD4+ cells compared to non-smokers. These findings were reproduced in other study by Bijl and colleagues. (149, 150)

In another study samples from lung were obtained at surgery from 15 patients who smoked and 6 who had never smoked (147). Neutrophils were found to be were the predominant cell in the lung parenchyma of non smokers and smokers without emphysema. In smokers with emphysema, the CD3+ and CD8+ were the predominant cells ($p<0.05$) in the alveolar wall. It was concluded that the pathogenesis of emphysema might be mediated by T-lymphocytes, mainly CD8+ T-cells.

In some ways these studies are contradictory, or the profile of T cell subtypes in the blood and lungs are different.

CHAPTER 2: METHODS

Chapter 2: Methods 1

2.1 The pilot project

This project evolved from a pilot study located at Poole Hospital NHS Trust, Dorset, UK (note this thesis is for an international audience). As part of a systems review for the inception of a COPD at home project (where patients are discharged earlier than would be expected with supportive care at home from an experienced nursing team), it was decided to perform full spirometry on all patients during their exacerbation to try and determine which patients were recovering sufficiently well to enable a safe discharge. This pilot study was performed in 2002 and published as an abstract at the ERS conference in Stockholm. (2)

A total of 40 patients were followed through their exacerbation. I performed the following daily measurements of lung function: FEV_1, FVC, and PEFR. The results from this study revealed that ex-smokers recovered from their exacerbation much more quickly than current smokers in the first few days of treatment.

From this pilot we formulated a study hypothesis.

2.2 The Hypothesis

In a group of patients with COPD, requiring hospital admission for the treatment of an exacerbation I hypothesise that…….

1. patients with COPD who are ex-smokers (for more than 1 year) recover more rapidly from exacerbations than current smokers (as shown by lung function tests), due to either a different response to conventional treatment (as smokers are known to be partially resistant to steroid treatment) or a different disease mechanism;

2. this could be due to a difference in the bacterial flora and/or viral infection at the time of exacerbation;

3. it is possible to identify biomarkers that are differentially expressed in current and ex-smokers.

2.3 The aims of the study

Based on the hypotheses the aims of my study were to:

1. follow ex- and current smokers through their acute exacerbation, and research differences between the 2 groups that could account for the differences observed in the pilot study;

2. see if the findings of the pilot study can be repeated;

3. identify other measures of lung function that may be useful in measuring response to treatment during acute exacerbations;

4. identify potential markers that may be of value in monitoring response to treatment;

2.4 Ethics, and research and development

Ethics approval was obtained from the Southampton and South West Hampshire Research Ethics Committee on 6th July 2006 (ref: 05/Q1702/68). The project was accepted on the 4th July 2006 by the hospital research and development committee (ref: RHM MED0636).

2.5 Power calculation

My pilot study allowed me to undertake power calculation to determine the numbers necessary to recruit to find a statistical difference between the 2 groups at the 5% level.

The primary outcome for the pilot study was average percentage change in FEV_1 from baseline averaged over the first week. Using the average percentage changes from 20 subjects collected in Poole in 2002, I determined the mean (SD) % changes in FEV_1 of 8.55(12.70) in ex smokers and 2.17(5.85) in current smokers. Based on the pooled standard deviation of 9.9, 28 subjects per group should be sufficient to give 80% power in the two sample t–test performed at the 5% level, if the mean difference is 6%. This calculation is backed up by a previous study by Calverley et al. (151)

2.6 The Study Population

The population studied were those subject to severe exacerbations of COPD admitted to Southampton General Hospital. The project had not been designed to look at the whole population of COPD patients because many with mild to moderate disease will not experience an exacerbation severe enough to warrant admission to hospital. The population represents the more severe end of the disease spectrum and it is these patients that are most at risk from exacerbations in terms of morbidity and mortality. The patients with severe disease also have the most impact on secondary health care in terms of bed days and overall expenditure.

Smoking and treatment history were taken and blood cotinine values obtained to confirm smoking status. Patients were recruited from either the Respiratory Centre of the Southampton General Hospital (which assesses patients suffering acute exacerbations in a day hospital setting) or the Acute Medical Unit where patients are hospitalised from the Accident & Emergencies Department.

2.7 Exclusion Criteria

To achieve robust data it was necessary to have strict inclusion and exclusion criteria. Patients with a history of asthma, bronchiectasis, carcinoma of the bronchus, or other significant respiratory disease were excluded from the study. A comprehensive history, chest x-ray and reversibility studies were performed and used to exclude those patients with other lung pathology.

Other patients excluded were those unable to perform spirometry, those with severe type 2 respiratory failure requiring non-invasive ventilation, and patients with consolidation on plain chest x-ray.

Patients with other causes of dyspnoea such as cardiac disease were excluded following a comprehensive history, examination, ECG, measurement of BNP levels and chest x-ray. The BNP level and ECG were used as an initial screen for congestive cardiac failure, if BNP levels reach those expected to be seen in left ventricular dysfunction, then echocardiography was performed to confirm/refute cardiac failure.

2.8 Acute Exacerbations – the definition

For this project we used the definition of an acute exacerbation of COPD from the East London group.

The following symptom pattern had to be experienced for at least two consecutive days for the diagnosis to be made: any two of three major symptoms (increase in dyspnoea, sputum purulence, and increased sputum volume) or at least one major symptom together with at least one of the following minor symptoms - a cold (increase in nasal discharge or congestion), wheeze, sore throat, cough, or fever. These criteria have become standard for defining acute exacerbations in the research. (38)

With our own definition:

`An acute exacerbation of COPD is a sustained worsening of the patient's condition which could be attributable to their COPD, 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. It often presents with the combination of increasing dyspnoea, and an alteration in sputum production.`

Questionnaires

The MRC dyspnoea score and St George`s Respiratory Questionnaire (SGRQ) were recorded at the time of admission. Both these scores have been validated and used extensively in studies.

2.9 Spirometry

Equipment

1. Micromedical Spirometer: MicroLoop Cat. No. ML3535
2. Disposable mouth pieces Micromedical Ltd 66.5mm X 27.8mm X 30mm
3. Spida 5 Software Cat. No. SD5000

All from Micromedical Ltd, Rochester, Kent

Methods

Spirometry was performed daily during the admission using the micromedical spirometer ML3535 (Micromedical Ltd, Rochester, Kent). This spirometer has been validated in previous studies and meets the American Thoracic Society (ATS) criteria for performance. (152)

The following measurements were performed daily at 10am after their morning nebuliser (if prescribed): FEV_1, FVC, FEV_6, IC and PEF. The best of 3 attempts was used as standard. The results were recorded in the patients file, and uploaded for analysis using the Spida 5 software (Micromedical Ltd, Rochester, Kent). The Spida 5 software is an electronic database that allowed us to store and analyse the pulmonary function tests from the ML3535 handheld spirometer. Some of the patients admitted to hospital with their COPD were discharged early (within 7 days) using the COPD early discharge scheme implemented in the hospital. In such cases, spirometry was recorded in the community by myself or one of the community outreach nurses from the Respiratory Centre using the same spirometer.

To standardise the spirometric measurements body weight, height and ethnic origin was also recorded. Percentage predicted for each variable was formulated using the spida 5 software and recorded.

To obtain the measurements necessary subjects were asked to perform the following manoeuvres.

1. Insert mouthpiece and perform tight lip seal. Nose clip was then placed to prevent air leakage.
2. The patient was then instructed to perform tidal breathing for 3 tidal breaths.
3. At the end of the last tidal breath the patient was instructed to perform a full inspiratory manoeuvre followed by a maximum expiratory manoeuvre.

This is shown graphically below

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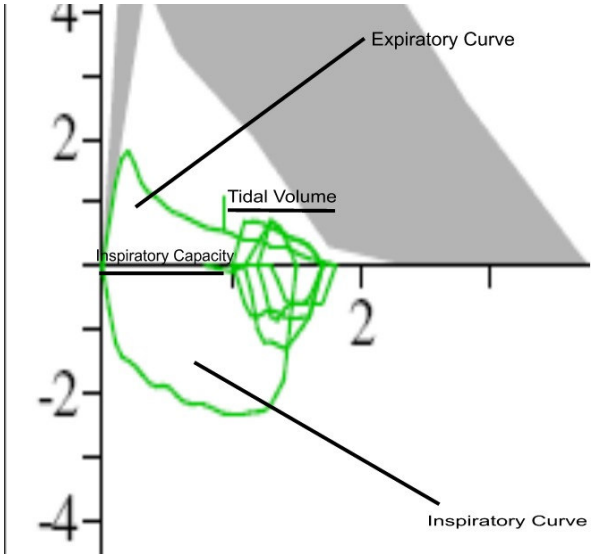


Fig. 2.1 Flow volume loop from subject – from Spida 5 software package.

2.10 ECG

A baseline 12 lead ECG was performed on admission day 1 and recorded in the patient's notes.

2.11 Blood Tests

Venesections were performed on day 1 of the admission and at the 3 monthly follow up visit.

Equipment

Vacutainer Systems safety-Lok™ Blood Collection Set REF 367282 Becton Dickinson and the following vacutainers were used: 3ml EDTA tube (for BNP, Full Blood Count), 8ml LH tube (for flow cytometry) 4.5ml Sodium Citrate Tube (for clotting, fibrinogen, and to process plasma, 10ml (Z) CAT Tube (to process serum).

Methods

Some of the blood collected was sent to the laboratories of Southampton Hospital and analysed for full blood count, CRP, indirect fibrinogen and urea and electrolytes. An EDTA sample was also processed and stored at -28°C in the biochemistry lab and analysed for BNP. A lithium heparin sample was obtained and Cotinine levels were measured in the biochemistry lab. Serum and Plasma was processed according to the following method and stored at -80°C for later analysis.

Plasma

1) Blood was collected into one 10ml vacutainer for serum (Z) and two 4.5ml vacutainers for plasma (sodium citrate). Once collected the samples in the citrate vacutainers were inverted gently 8 times each, but the Z vacutainer was allowed to rest undisturbed at room temperature for 30 minutes without agitation until fully clotted.

2) The citrate vacutainers were then centrifuged immediately at 3,000 rpm (1,750g) for 10 minutes at 20°C. The plasma was collected without disturbing the red pellet and pooled into a 15ml tube, and then aliquoted into screw-capped 2ml tubes. 3 x 500µl samples were stored and frozen immediately at -80°C.

3) The z serum tubes were processed and centrifuged in the same way once the 30 minutes had passed and the blood had fully clotted.

2.12 Arterial blood gas

Equipment: heparinised syringe for arterial blood puncture and an Arterial Blood Gas Machine.

Methods

Arterial blood collected in a heparinised syringe using the radial artery puncture method. The samples were analysed immediately and results obtained for pH, PaO_2 , $PaCO_2$, bicarbonate, and base excess.

2.13 Sputum Induction

Sputum induction was performed on day 1 and, whenever possible, 3 months after AECOPD.

Equipment: Nebuliser machine – standard ward based, elephant hosing, face mask – standard, salbutamol 5mg, 5 mls 0.9% Normal saline, petri dish, Peak flow metre and pulse oximeter.

Methods

Sputum was induced at the patients' bedside because of the severity of their illness. This patient population is especially susceptible to the bronchoconstriction that can be induced by nebulisation with hypertonic saline. An alternative but validated method was used for this population. (111, 113, 117) Due to the fragility of this patient group, prior to sputum induction, all patients were pre medicated with 5mg of salbutamol.

Nebulisation with 0.9% saline was commenced using a procedure modified from that of Pin *et al* but using a conventional ward based nebuliser. After 3 minutes of nebulisation measurement of oxygen saturation and spirometric tests were performed and nebulisation continued if the FEV_1 had not fallen by more than 20%. Patients were instructed to blow their noses and rinse their

mouths out with water before expectorating sputum. Sputum samples were collected throughout the procedure and then placed in a Petri dish.

This method has been shown to be a perfectly safe for sputum collection in patients with acute COPD. (111)

2.14 Sputum Processing

Equipment: Forceps, falcon tubes, bench rocker, 70µm filter, pipettes various eppendorf tubes with screw caps, cytospin, slides, cover slips, haemocytometer, trypan Blue, 0.63M dithioerythritol (DTE) in HEPES buffered saline (48mg DTE in 50ml HBS) (Sigma, Poole, UK), HEPES buffered saline (50mM HEPES pH 7.4, 140mM NaCl) (Sigma), and Protease inhibitor cocktail (Sigma cat no: P8340) (sigma)

Methods

Sputum processing was performed within 2 hours of induction. A sample of sputum was sent to microbiology for cultures using standard guidelines. (153) The maximum time allowed from production of sputum to plating for culture was 2 hours.

The remaining sputum was placed in a Petri dish and forceps were used to select the mucoid portions and transfer these into another Petri dish. The samples were then mixed thoroughly using forceps and divided into two. Sample A was solubilised with 4X sputum weight of DTE while sample B was solubilised with 4X sputum weight of PBS. A protease inhibitor cocktail was then added to the samples in the ratio of 22.5µg per g of sputum. After mixing for 30 minutes on a bench rocker and filtering both samples through a 70 µm filter the samples are then centrifuged at 1,500 rpm (400g) for 10 minutes to form cell pellets. The supernatant was carefully removed and put into labelled 1.5ml eppendorf tubes, these were then stored for future analysis at –80°C.

The cell pellet from the DTE treated sample was resuspended in 1 ml PBS. A total cell count is obtained by mixing10 µl of cell suspension and mixing with 90 µl of trypan blue in an eppendorf. Total cell count and viability per ml was then calculated. The HBS was then diluted to obtain approximately 1 x 10^6 cells / ml. Cytospins are assembled and 70 µl of diluted cell suspension is added to slides.

This was then spun at 450 rpm (for Shandon Cytospin 2 or as appropriate for cytospin model) for 6 minutes. One slide was stained with Diff-Quick or May Grunwald Giemsa and air dried. One slide was frozen at -80°C.

CHAPTER 3: METHODS – PART 2

Chapter 3 – Methods 2

3.1 RNA extraction from sputum

Step A

Equipment: Microprep kit (Absolutely RNA® Microprep Kit Catalog number 400805 Stratagene, La Jolla, USA) this contains: Lysis Buffer, β-Mercaptoethanol, (β-ME) (14.2 M)**,** RNase-free DNase I (lyophilized)**,** DNase Reconstitution Buffer, DNase Digestion Buffer **,** High-Salt Wash Buffer (1.67×), Low-Salt Wash Buffer (5×), Elution buffer (10 mM Tris-HCl, pH 7.5), RNA-Binding Spin Cups and 2-ml receptacle tubes, 1.5-ml collection tubes

Method

The RNA from the sputum was extracted according to the following method from cells obtained during the sputum processing at a concentration of $1x10^6$cells/ml, and also from 200 µl of the DTE prepared supernatant. There were 3 conditions used under which the RNA was extracted. Cells, supernatant, and then cell and supernatant.

To minimise cross infection an independent RNA area was used.
Gloves were sterilized and the pipette disinfected prior to the procedure. The gloves and bench were sprayed with RNA'se away spray and filtered tips were used at all times.

In an eppendorff (1.5ml) 400 µl of lysis buffer from the kit was mixed with 2.8 µl of β-Mercapthoethanol to formulate the lysis mix. Two 1.5 ml eppendorfs were used and 1ml of cells ($1x10^6$cells) was centrifuged in the micro-centrifuge for 3 minutes at 3000 rpm. The supernatant was then discarded. For one of the 2 tubes the pellet was resuspended with the 200µl of sputum supernatant and with 200 µl of PBS for the other tube. In each tube 200 µl of the Lysis mix was added, mixed and then vortexed for 5 seconds. The 2 tubes are then stored at -80°C.

The RNA was then extracted using a column based method as per the kit instructions. In summary an equal volume (usually 100 µl) of 70% ethanol is added to the cell lysate and mixed thoroughly by vortexing for 5 seconds. This mixture was then transferred to the RNA-Binding Spin Cup that has been seated within a 2-ml collection tube. This was then placed in the microcentrifuge for 30–60 seconds at maximum speed. The spin cup was removed and the filtrate discarded. 500 µl of High-Salt Wash Buffer was added to the spin cup and placed in a microcentrifuge at maximum speed for 30–60 seconds. The filtrate was then discarded. The spin cup was replaced in the collection tube. 600 µl of Low-Salt Wash Buffer was then added. The sample was then placed in a microcentrifuge at maximum speed for 30–60 seconds. We then remove and retain the spin cup**,** discard the filtrate, and replace the spin cup in the collection tube. 300 µl of Low-Salt Wash Buffer was added. The sample was placed in a microcentrifuge at maximum speed for 2 minutes to dry the fiber matrix. 30 µl of Elution Buffer was then added directly onto the fiber matrix inside the spin cup and incubated at room temperature for 2 minutes. The sample was then placed in a microcentrifuge for 1 minute. This elution step was repeated to increase the yield of total RNA. The RNA was then stored at–80°C for long-term storage.

3.2 Viral PCR

Step B

Equipment

Primers *meta-pneumovirus, adenovirus B & C, influenza virus AH1-AH3-B, parainfluenza virus 1-2-3, respiratory syncytial virus A & B*, and *rhinovirus* (all sub-types) Were all from PrimerDesign Ltd (Southampton, UK). Buffer components were also from PrimerDesign Ltd), while iQ5 Real-Time PCR Detection System was from Bio-Rad Laboratories) (Hemel Hempstead, UK). Absolutely RNA® Microprep Kit (Stratagene, Amsterdam Zuidoost, The Netherlands)

Mehods

RNA was extracted from 10^6 sputum cells and/or 200 µl of sputum supernatant using the Absolutely RNA® Microprep Kit (Stratagene, Amsterdam Zuidoost, The Netherlands) in RNase-free conditions and directly followed by a liquid-phase DNase treatment (Ambion/Applied Biosystems, Warrington, UK). Reverse transcription reactions, and polymerase chain reactions, were carried out using virus-specific primers (PrimerDesign Ltd). For each sample, 5 µl of cDNA in 20 µl volume was used for independent TaqMan® based quantitative PCR. In each case, the reaction was optimized with respect to enzymes, buffer components (PrimerDesign Ltd), and cycling parameters in iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories). For all PCR amplifications positive and negative controls were included.

Sputum factors inhibits PCR Virus DNA detection

All samples were tested by PCR using primers/probes detailed above. After the initial run, no signals were positively detectable. We then tested the detection kits by performing spiking experiments with short DNA sequences coding for a part of the virus genome. We introduced a sample with 12500 or 800 copies of

the RHV positive control DNA sequence. After comparison with the PCR signal obtained with non RNA samples, we observed the presence of intrinsic factors, which strongly inhibited the PCR reaction in spiked sputum samples (figure 3.1). We observed this inhibition for all viruses in both the cellular and fluid conditions (data not shown).

This inhibitory effect was reduced by diluting the post reverse transcriptase reaction product (cDNA) samples. Spiking experiments were then carried out on serial dilutions of both, cellular and fluid samples. Dilution was found to increase the recovery of the signal with a maximal recovering after 64-fold dilution of the samples for the RHV detection. At this point the recovery is near to 100% to compare with no RNA spiked positive controls. The recovery rate was dependent on the virus, and was also shown to be sample dependant.

We formulated a hypothesis that if the dilution of the sample increased the recovery rate of detection of virus DNA sequence by PCR, then this process also dilutes the cDNA. To prove this we perfomed two different spiking experiments run in parallel. Initially we diluted the sample and then spiked all conditions with a known amount of virus DNA sequence; this was compared then to spiking the samples and then diluting it to compare the recovery rate of detection with the right shift of the signal due to dilution. It was discovered that for some types of virus the dilution factor necessary to obtain a maximal recovery rate induced a right shift of the PCR signal below the primers/probe PCR sensitivity kits.

The demarcation point between the signal recovery and its disappearance had to be determined for all virus detection kits. An example of this is shown in figure 3.3, which shows the effect of dilution on 2 samples for RHV and FluAH3. For these two samples, a dilution factor of 8 has been applied which allowed us to detect true positive PCR signals.

An experimental strategy was devised to analyse all samples at the same time. We initiated the work by evaluating the sensitivity of all the virus detection kits. For this we performed a standard PCR curve for every kit using different dilutions of the virus positive sequences. Figure 3.1 shows PCR results obtained using serial dilution, from 12500 to 24 copies, of the RSVA positive cDNA sequences.). We then performed PCR on all undiluted samples, every

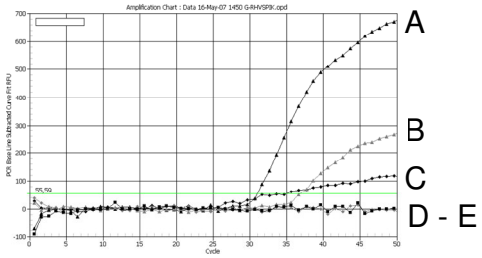
sample contained a cDNA equivalent of 10,000 sputum cells or 2 µl of sputum fluid. Figure 3.3 shows the PCR curves obtained for Rhinovirus and Flu AH3. In all the samples and with each detection kit most of results were negative, partially due to the presence of inhibitatory factors.

To analyse the effect of inhibition we calculated for each virus detection kit the minimal quantity of virus that could be detected in the samples. To determine the "threshold" of detection, according to the standard curve, three different quantities of control virus sequences were arbitrary chosen and used to spike 6 samples chosen at random.

This experimental strategy was then applied for every virus kit, Table 3.1 shows the threshold values for each virus detection kit.

RNA was extracted from 30 fluid- and 28 cellular-fraction. The results of which are shown in the main paper.

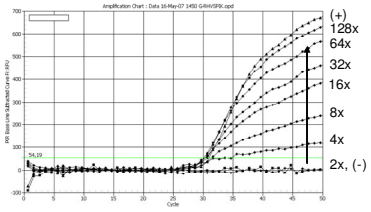
Proof of inhibition – Spiking experiment



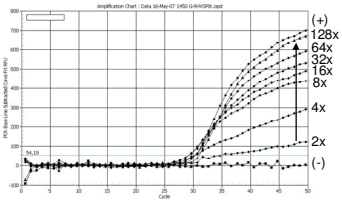
A: Sample without RNA + 12500 copies of the positive sequence
B: Sample without RNA + 800 copies
C: Sample with RNA + 12500 copies
D: Sample with RNA + 800 copies
E: Sample without RNA + 0 copies

Dilution leads to better recovery of the signal

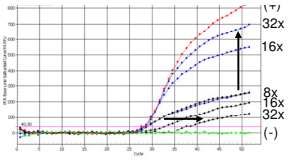
Cellular



Supernatant



But you can lose the signal



Control+ (12500 copies)

Dilution →spike constant (on observe l'effet d'inhibition)

Spike → dilution (on observe l'éloignement du signal)

Figure 3.1. Experiments to show proof of inhibition. Detection kits were tested by performing spiking experiments with short DNA sequences coding for a part of the virus genome. Dilution can lead to better recovery of the PCR signal in both the cellular and supernatant fractions. If the samples are diluted too much then the signal can be lost

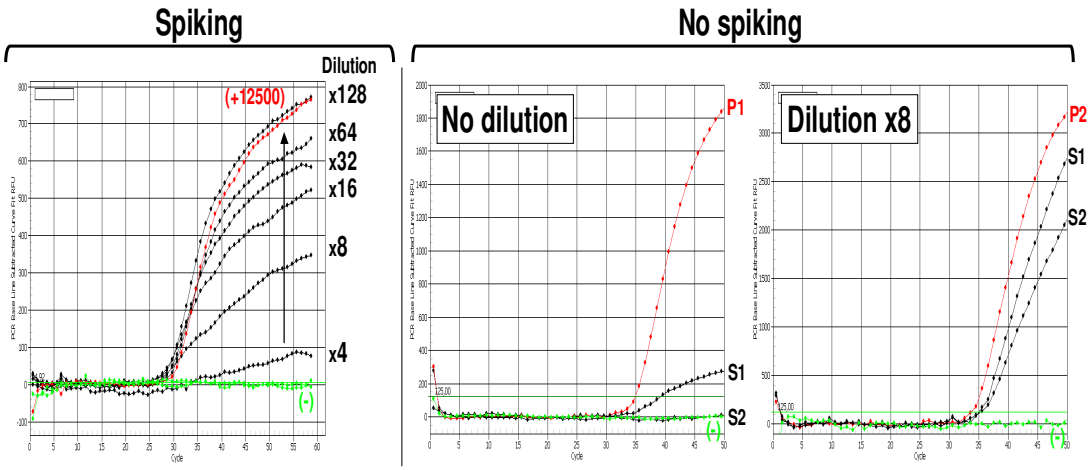


Figure 3.2: Intrinsic sputum inhibition factor effects on PCR reaction (example shown is for experiments using the rhinovirus detection kit).
Left panel: a series of dilutions of a single RNA sputum sample was spiked with 12,500 copies of a rhinovirus specific DNA sequence. PCR curves analyses showed that the inhibition effect is reduced with sample dilution. (+): positive control: PCR carried out on 12,500 copies of the synthetic DNA control sequence in water; (-): PCR carried out on water.
Right panel: Effect of dilution on real (non-spiked) samples. Rhinovirus PCR was carried out on 2 different samples (S1, S2) undiluted and diluted 8 times. The analysis of PCR results showed no (or very weak) signals on undiluted samples, but after dilution (and reduction of inhibition factors) the PCR results became positive.

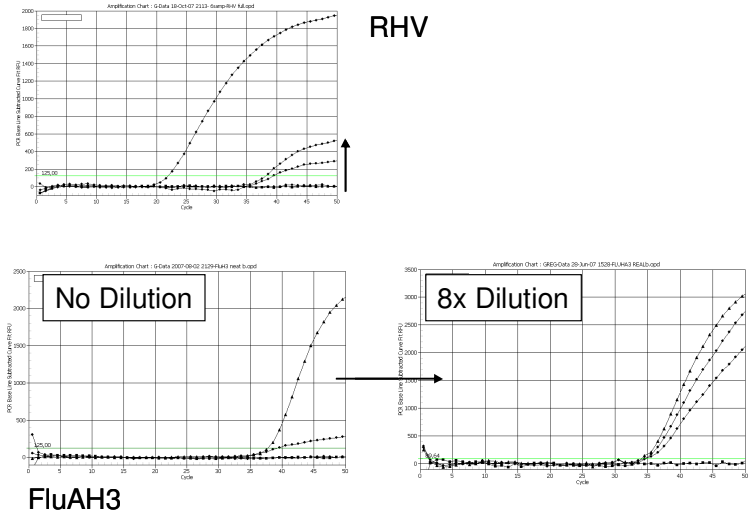


Figure 3.3: The effect of dilution on 2 samples for RHV and FluAH3. For these two samples, a dilution factor of 8 has been applied which allows detection of true positive PCR signals.

Table 1. List of virus and the PCR threshold of detection

Name	Threshold (number of copies)
Adenovirus B	100
Adenovirus C	12
Influenza AH1	12
Influenza AH3	100
Influenza B	50
Metapneumovirus	50
Para-influenza virus 1	12
Para-influenza virus 2	12
Para-influenza virus 3	12
Respiratory syncitial virus A	50
Respiratory syncitial virus B	400
Rhinovirus (all subtypes)	800

Table 3.1: The PCR threshold was optimised for each individual virus

3.3 Flow Cytometry

Flow cytometry using the Four-colour FACS Calibur Cell Sorter was used to detect the types of cells present in the sputum whether the cells were activated or not.

Equipment cluster

Four-colour FACS Calibur Cell Sorter (BD biosciences)

Antibodies used (Table 3.2):

Surface marker	Antibody	Isotype control	Fluorochrome labelled
Granulocytes activation markers	CD66b	moIgM	Fluorescein isothiocyanate–conjugated (FITC)
	CD11b	ratIgG2b	Peridinin chlorophyll protein (PerCP)-cyanin (Cy)5.5
	CD62L	moIgG1	Allophycocyanin (APC)
T-cells phenotyping markers	CD3	moIgG1	APC
	CD4	moIgG1	PerCP-Cy5.5
T-cells activation markers	CD25	moIgG1	R-Phycoerythrin (PE)
	CD69	moIgG1	FITC
	HLA-DR	moIgG2a	PerCP

All antibodies purchased through BD biosciences Oxford UK.

Methods

Freshly processed sputum cells were suspended in 1 ml of ice-cold Phosphate Buffered Saline (PBS) with Ca2+ and Mg2+ (Sigma). Cells were then washed with flow cytometry staining buffer (FBS) (PBS, 2% heat-inactivated foetal calf serum, 0.09% sodium azide) and incubated for 15 min on ice with 2 mg per millilitres of polyclonal human Immunoglobulin (Ig)G (Sigma) to block any non specific binding of antibodies to Fcγ receptors. For blood analysis, 100µl of whole blood was diluted twice with FBS. For both samples, specific staining was then performed with specific fluorochrome-coupled antibodies (anti-CD62L, -CD11b, -CD66b for granulocytes and anti-CD3, -CD4, -CD25, -CD69, -HLA-DR for T-cells and incubated for 45 min on ice. Unbound antibody was removed

by washing once with FBS. For whole blood samples, a haemolysis step was performed to eliminate red blood cells with a lysis buffer (BD biosciences) for 6 minutes on ice and then washed with FBS. Cells were analysed on four-colour FACS Calibur using Cell Quest software (both from BD Biosciences)

FACS gating strategy for analyses of granulocytes surface marker expression in blood and sputum samples.

For both the granulocyte and lymphocyte population only samples with >50 cells was used for anaylsis.

Initially, cell debris which bind antibodies non-specifically, are excluded by their low size and granularity. In respect to their short life, in sputum samples, dying granulocytes are gated out by virtue of their staining with the DNA-binding agent, propidium iodide. The living granulocyte population is then gated positively in the forward and side-scatter plot (high size and granularity). Remaining contaminant monocyte/macrophage cells are excluded using their autofluorescent property and by staining with CD66b which is not expressed by these cells. Different granulocytes activation markers were tested.

FACS gating strategy for analyses of T-cells surface marker expression in blood and sputum samples.

As for granulocytes, cell debris were excluded by their very low size and granularity and lymphocytes cell T cells are gated positively in the forward and side-scatter plot (low size and granularity). CD3+ cells are positively selected by staining with anti-CD3 antibody. From CD3+ cells, CD4+ T-cells are detected by using anti-CD4 antibody, CD8 cells were assumed as CD3+ CD4- cells. Activation markers expression were analysed on the CD3+ population, low size and low granularity.

3.4 Luminex – Multiple cytokine analysis

The Luminex system allows for the analysis of multiple cytokines within a very short period of time using antibody binding within a flow cytometry gating method. It has excellent reproducibility and correlates well with single ELISA methods for cytokine detection. (154)

Equipment

Luminex assay (Bio-Rad, Hemel Hempstead, UK), recombinant cytokines IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNFα, IFNγ (R & D systems, Abingdon, UK), capture and detection antibodies for TNFα (R&D) IL-2, IL-4, IL-13 capture antibody; IL-5, IL-10, IL-12p70, IL-13 detection antibody (Endogen, UK), IFN-γ, IL-6, IL-8, IL-10, IL-12p70 capture antibody; IFN-γ, IL-4, IL-6, IL-8, IL-12p70 detection antibody. (GlaxoSmithKline, Stevenage, UK), 96-well filter plate (Millipore, Watford, UK), Antibody-conjugated xMAP Carboxylated Microspheres (Applied Cytometry Systems, Sheffield, UK), 50 µl of streptavidin-PE (1 µg/ml in assay buffer - BD Biosciences), STarStation 2.0software (Applied Cytometry Systems).

Methods

Luminex assay (Bio-Rad) was used to determine the concentrations of multiple cytokines (IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNFα, IFNγ) in patient serum samples diluted 1:5 according to the manufacturer's instructions. Recombinant cytokines for standard curve generation were purchased from R&D systems (Abingdon, UK). Capture and detection antibodies were from R&D (TNFα), Pharmingen, UK (IL-2, IL-4, IL-13 capture antibody; IL-5, IL-10, IL-12p70, IL-13 detection antibody), Endogen, UK (IFN-γ, IL-6, IL-8, IL-10, IL-12p70 capture antibody; IFN-γ, IL-4, IL-6, IL-8, IL-12p70 detection antibody), and GlaxoSmithKline (IL-5 capture antibody). HEPES buffered saline (50mM HEPES pH 7.4, 140mM NaCl) (Sigma)

50 µl of standards or diluted samples were placed on a 96-well filter plate (Millipore, Watford, UK) pre-wet with 50 µl of assay buffer (PBS, 1% BSA,

0.025% Triton-X100). 50µl of appropriate antibody-conjugated xMAP Carboxylated Microspheres (Applied Cytometry Systems) were added to each well ($1x10^5$ microspheres/ml for each conjugated microsphere set) and the plate covered and incubated at 4ºC overnight. After two washes with wash buffer (PBS, 0.05% Tween-20), 50 µl of a cocktail of appropriate detection antibodies (500 ng/ml for each antibody) was added to each well and incubated on a shaker at 700 rpm for 1 h at room temperature. After two further washes, 50 µl of streptavidin-PE (1 µg/ml in assay buffer - BD Biosciences) and incubated on a shaker for 30 min at room temperature at 700 rpm. The plate was then washed twice and 120 µl of sheath fluid was added to each well before reading on a Luminex xMAP100 machine (Bio-Rad).

Results were analysed using STarStation 2.0software (Applied Cytometry Systems).

CHAPTER 4: RESULTS

Results

I screened a total of 138 patients presenting with an acute exacerbation of COPD to the acute medical unit. From these patients I recruited to the study 31 current smokers and 27 ex-smokers. Exceeding the number required from the power calculation by 2. Details why certain patients were excluded are shown below in figure 4.1

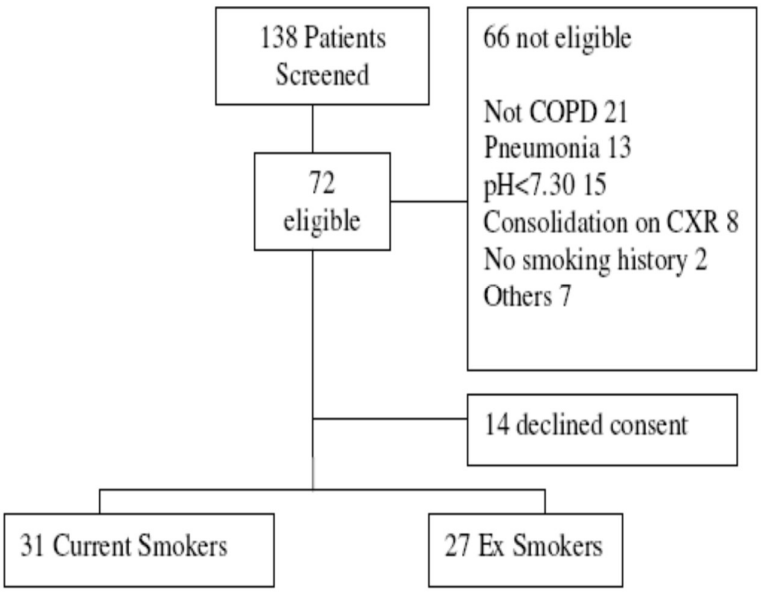


Figure 4.1 : Study design

The ex-smokers and current smokers were matched for age, sex, FEV_1, exacerbation rate, and use of inhaled treatment. The only statistically significant difference between the 2 groups was in the total number of pack years. Not

surprisingly the current smokers had smoked more cigarettes than the ex smokers. ($p<0.05$) (table 4.1)

	Current Smokers (n=31)	Ex-smokers (n=27)	P value
Age (years)*	69.1 +/- 2.2	66.1 +/- 1.84	0.83
Male:Female	17:14	15:12	0.88
FEV_1 on admission#	0.7 (0.23-1.63)	0.71 (0.25-1.0)	0.93
FVC on admission#			
Smoking pack years*	55.7	38.22	0.02
Patients on inhaled Steroids	84%	85%	0.84
Patients on long-acting β_2- agonists	68%	60%	0.54
Patients on long term oxygen therapy	4%	5%	0.83
Past Exacerbation Rates (yr^{-1}) *	2.35 +/- 0.3	2.11 +/- 0.18	0.85
MRC Score*	3.62 +/- 0.27	3.00 +/- 0.37	0.36
SQRQ Score*	57.21 +/- 6.54	52.87 +/- 9.05	0.71
pH on admission*	7.43 +/- 0.01	7.38 +/- 0.01	0.66
CO_2 on admission (kPa)*	5.53 +/- 0.51	6.84 +/- 0.43	0.36
0_2 on admission (kPa)*	8.09 +/- 1.25	8.20 +/- 1.28	0.73
Bicarbonate (mmol)*	26.9 +/- 1.46	29.4 +/- 1.50	0.73
Years ex smoking*	NA	3.59 +/- 5.8	NA

Table 4.1 Demographics. #= Median, *= Mean

Ninety five percent of the recruited patients completed 6 days of follow-up after admission, allowing analysis of lung function tests during the period immediately following the acute exacerbation. The remaining patients (3) died

during admission. If the patients were discharged sooner than 6 days, measurements were obtained using the same equipment within their own homes. Only 36 of these patients could be reassessed after 42 days: 5 patients had died and 17 patients refused follow-up within the study. This was felt to be insufficient for analysis of clinical and disease mechanism outcomes.

All the patients received standard treatment for an acute exacerbation, consisting of a course of prednisolone (30mg OD), nebulised bronchodilators (salbutamol 2.5mg QDS and PRN and ipratropium 500µg QDS) and antibiotics (doxycycline 200mg BD for 2 days then 200mg OD for 5 days). None was admitted into the high dependency unit for non-invasive ventilation, and none was intubated. There were no differences in treatment received by the two groups of patients.

Length of stay in hospital and recovery of lung function

The mean (SEM) length of stay of ex-smokers was 3·1+/- 0·12 days which was significantly (P<0.001) different from current smokers (5·6 +/- 0·27 days) (figure 4.2). In current smokers FEV_1 recovered significantly (P<0·05) more slowly than in ex-smokers. This pattern was also seen when analysing the recovery of FVC and IC (Figures 4.3 a, b and c).

To work out the statistical analysis for the changes in lung function we used 2 valid statistical methods. The first was to use a simple t-test (the values were normal distributed) which gave a p-value of P<0.01. The problem with this however is that because we are measuring serial data there is an increased chance of finding a positive result so we also used the slopes of the individual patients in a linear regression analysis and then compared the 2 groups. This also gave p-value of P<0.01.

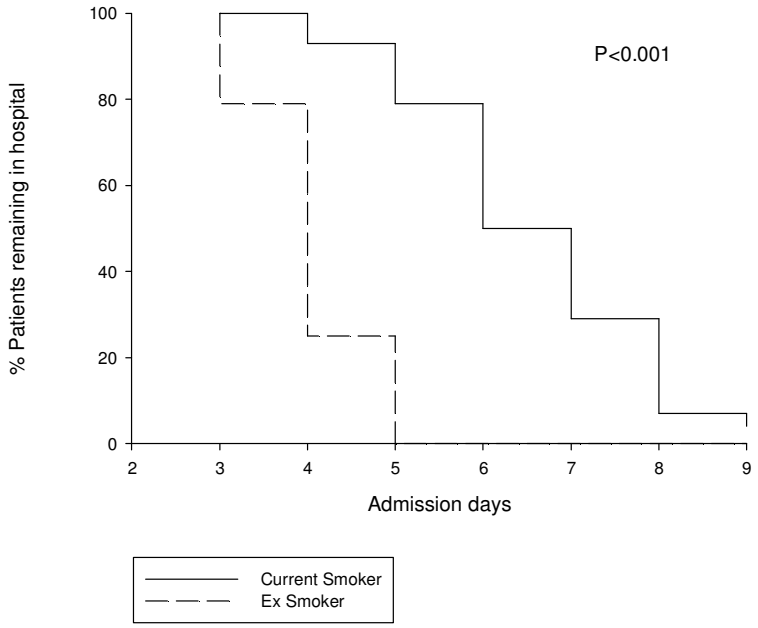


Figure 4.2: Kaplan**-**Meier Graph showing numbers of days patients occupied a hospital bed according to smoking status. Ex-smokers were discharged much earlier than current smokers. ($p<0.001$)

ID	Smoker	FEV 0	FEV 1	FEV 2	FEV 3	FEV 4	FEV 5	FEV 6
1	1	2.1	1.5	1.48	1.5	1.6	1.69	1.71
2	1	0.9	0.7	0.7	0.68	0.79	0.81	0.84
3	1		1.63	1.57	1.59	1.68	1.71	1.74
4	2	1.68	0.98	1.1	1.15	1.15	1.28	1.3
5	1	0.71	0.6	0.6	0.58	0.61	0.64	0.66
6	1	1.78	1.5	1.48	1.5	1.6	1.69	1.71
7	2	0.93	0.88	0.81	1.1	1.12	1.17	1.17
8	1	0.54	0.41	0.38	0.38	0.38	0.34	0.32
9	1	0.33	0.45	0.5	0.49	0.33	0.33	0.33
10	1	0.8	0.45	0.41	0.41	0.45	0.51	0.52
11	1	0.99	0.83	0.83	0.83	0.9	0.83	0.91
12	2	0.76	0.51	0.55	0.61	0.68	0.68	0.69
13	2	1.22	0.9	0.97	1.03	1.05	1.02	1.1
14	2	1	0.71	0.66	0.73	0.8	0.81	0.82
15	1	0.97	0.79	0.68	0.72	0.71	0.73	0.81
16	1	1.1	1.06	0.97	0.9	0.9	0.83	0.9
17	2	1.29	0.9	0.93	1	1.02	1.08	1.25
18	2	0.89	0.47	0.47	0.67	0.68	0.66	0.76
19	1	1.28	1.13	0.88	1.1	1.1	1.15	1.15
20	1		0.58	0.57	0.5	0.61	0.63	0.64
21	1	0.31	0.23	0.2	0.2	0.31	0.37	0.38
22	1	0.71	0.56	0.48	0.58	0.56	0.58	0.6
23	2	0.7	0.26	0.38	0.38	0.41	0.47	0.49
24	2		0.74	1	1.03	1.1	1.11	
25	1	1.13	0.7	0.72	0.61	0.72	0.61	0.81
26	1	1.16	1.36	1.4	1.4	1.47	1.4	1.5
27	1	0.89	0.51			0.49	0.61	
28	1		0.78	0.8	0.83	0.9	0.9	0.92
29	2		0.34	0.41	0.47	0.48	0.5	0.5
30	1		0.38	0.37	0.37	0.4		
31	1		0.7	0.69	0.72		0.73	0.81
32	1	0.89	0.53	0.55	0.55	0.6	0.61	
33	2	0.99	0.81	0.88	0.88	0.9	0.92	0.94
34	1		0.33	0.3	0.35	0.37	0.37	
35	1	0.82	0.55	0.58	0.6	0.6	0.62	
36	2		0.7	0.77	0.79	0.83	0.83	
37	2	1.01	0.8	0.9	0.92	0.94	0.96	0.99
38	2		0.25	0.29	0.37	0.44	0.44	0.46
39	2	0.8	0.59	0.56	0.6	0.61	0.68	
40	1	0.79	0.49	0.52	0.5	0.52	0.49	0.53
41	2		0.49	0.53	0.54	0.56	0.58	
42	2	1.2	0.96	0.98	1.01	1.12	1.14	1.14
43	1		0.83	0.53	0.79	0.8	0.84	0.86
44	2	1.1	0.68	0.72	0.7	0.79	0.81	
45	2		0.51	0.54	0.6	0.6	0.69	0.74
46	1	0.91	0.6	0.59	0.61	0.65	0.65	0.81
47	1	1.08	0.8	0.7	0.79	0.81	0.85	0.85
48	2	1.37	0.8	0.82	0.9	1.01	1.1	1.23
49	1		0.57	0.59	0.58	0.59	0.61	0.62

50	2	0.72	0.44	0.5	0.52	0.5	0.6	0.61
51	1	0.76	0.58	0.6	0.6	0.62	0.65	
52	2		1	1.15	1.18	1.19	1.19	
53	2		0.6	0.64	0.72	0.73	0.77	0.81
54	2	1.1	0.91	0.93	0.97	1.02	1.03	1.04
55	1	1.2	0.91	0.9	0.83	0.9	0.94	0.97
56	2		0.58	0.6	0.83	0.88	0.88	0.93
57	2	1.43	0.7	0.8	0.97	1.17	1.28	1.29
58	2		0.71	0.93	1.15	1.2	1.26	1.27

Table 4.2 Absolute changes in lung function for patients 1-58. Smoking classification 1 – Current smoker 2 – Ex smoker. All values expressed in litres.

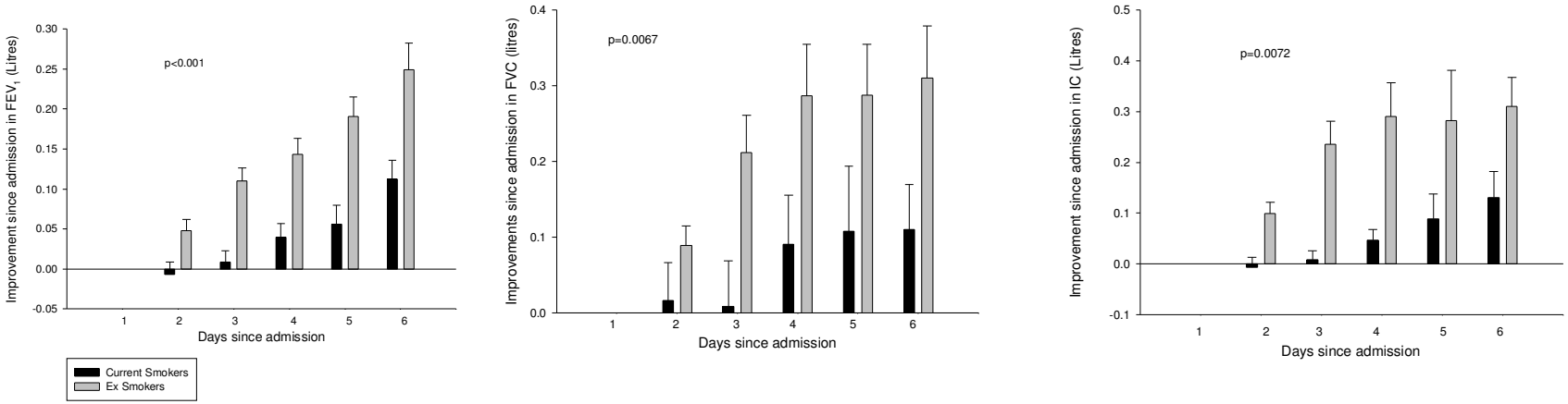


Figure 4.3a: Improvement in lung function a) FEV_1 b) FVC and c) IC during an acute exacerbation since admission to hospital. Ex-smokers recovered much more quickly than current smokers in all parameters.

Detection of bacteria and viruses

Of the 58 samples submitted for culture, 52% grew significant pathological bacteria, with current smokers having significantly ($p<0.05$) more pathogenic bacteria than ex-smokers (Figures 4.4 a and b). This was especially true for *Haemophilus influenzae,* which was also associated with a significantly higher CRP level at day 1 than any other organism detected (Figure 4.5b) ($p<0·05$) Sputum from 18 current and 13 ex-smokers were analysed for viruses. When pooling the data from the cell and fluid sputum phases, a total of 30 viruses were detected in 31 patients (figure 4.4 c and d). Some patients were infected with more than one species. The rate of detection tended to be higher in ex-smokers (76·9%) than in current smokers (50%), but this was not statistically significant ($p=0·21$). There was a particularly low prevalence of *S. pneumoniae* in this study, only 2 current smokers cultured this organism. A greater prevalence of these bacteria may have been detected if we used PCR techniques.

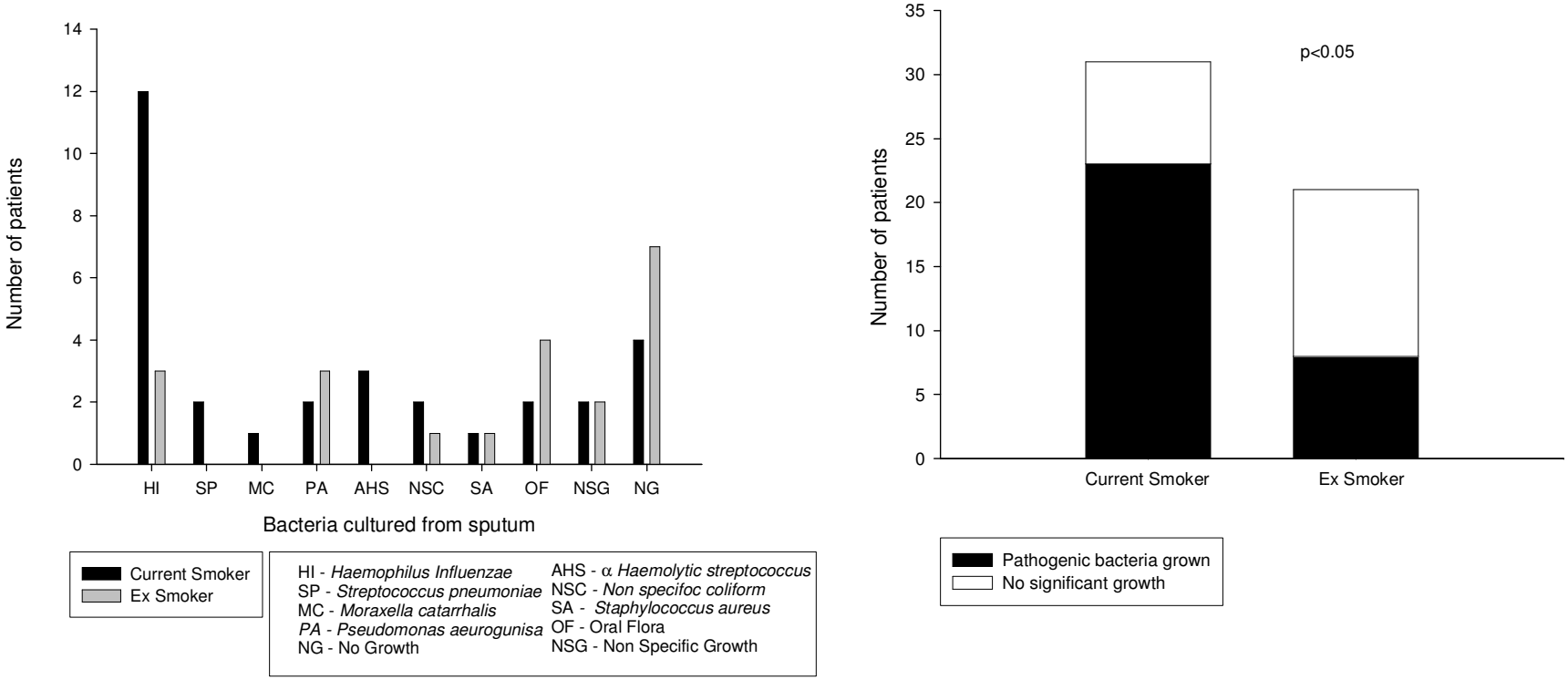


Figure 4.4: Microbiological analysis of induced sputum in current and ex-smoking COPD patients undertaken on day 1 of admission with an acute exacerbation: a) bacteria species grown in culture b) differences in quantity of pathogenic bacteria grown in the sputum culture. Current smokers had a greater burden of bacteria.

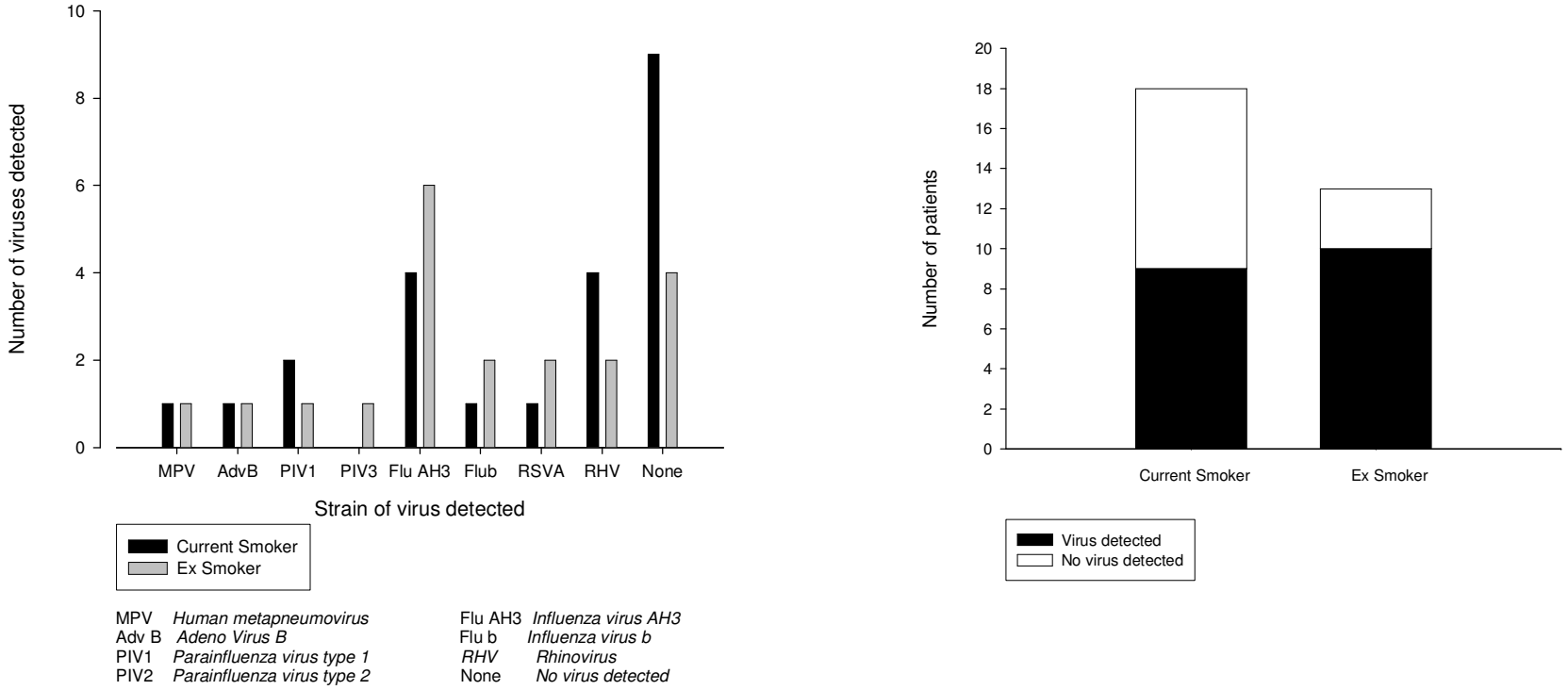


Figure 4.4: cont.. c) virus species detected by PCR in sputum d) Quantity of virus detected. Whilst ex-smokers tested more frequently for virus infection this was not statistically significant

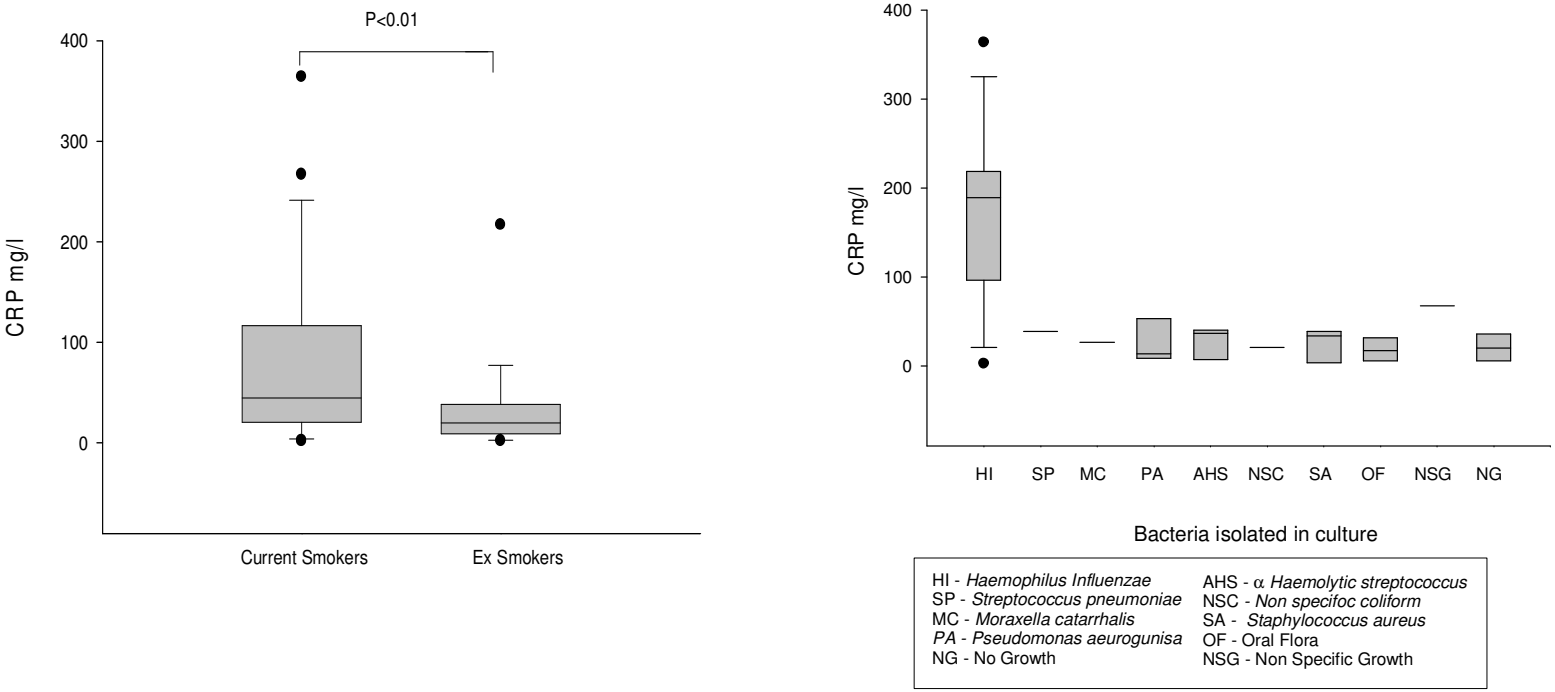


Figure 4.5: a) Serum concentrations of CRP at day 1 of acute COPD exacerbation. Current smokers had significantly higher CRP levels than ex-smokers. ($p<0.01$) and b) CRP levels were highest in patients in whom *H .influenzae* was detected ($p<0.05$).

Measures of airway and systemic inflammation

Analysis of sputum cells showed predominant neutrophilic infiltration with no differences between the current and ex-smokers both in cell counts and degree of activation (figure 4.6). There was no difference between ex- and current smokers in granulocyte populations or cell surface markers of activation in blood (figure 4.7). However, current smoking was associated with significantly ($p<0{\cdot}01$) higher CRP concentrations (figure 4.5a). When assessing the proportions of CD4+ and CD8+ T cells in sputum the differences between the two patient groups were marked, with a predominance of CD8+ T cells in ex-smokers and CD4+ T cells in current smokers (figure 4.8), resulting in a significant ($p<0{\cdot}01$) difference in CD4+:CD8+ T cell ratios. In contrast, there was no difference in CD4:CD8 ratios in blood. Although there was a tendency for current smokers to have higher concentrations of most cytokines on admission, only the raised levels of IL-12 in smokers reached statistical significance ($p<0{\cdot}05$) (figure 4.9a)

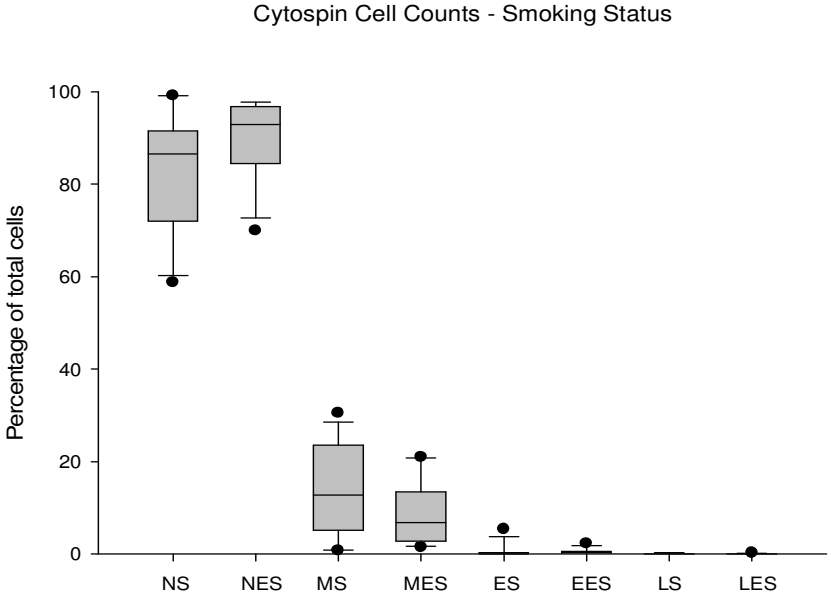


Figure 4.6: The dominant cell type was the neutrophil. There were no differences between ex- and current smokers in the cell populations obtained by cytospins.
NS – Neutrophil smokers NES – Neutrophil ex-smokers MS – Macrophage smokers MES Macrophage ex-smokers ES – Eosinophils smokers EES – Eosinophils ex-smokers LS – Lymphocyte smokers LES - Lymphocyte ex-smokers

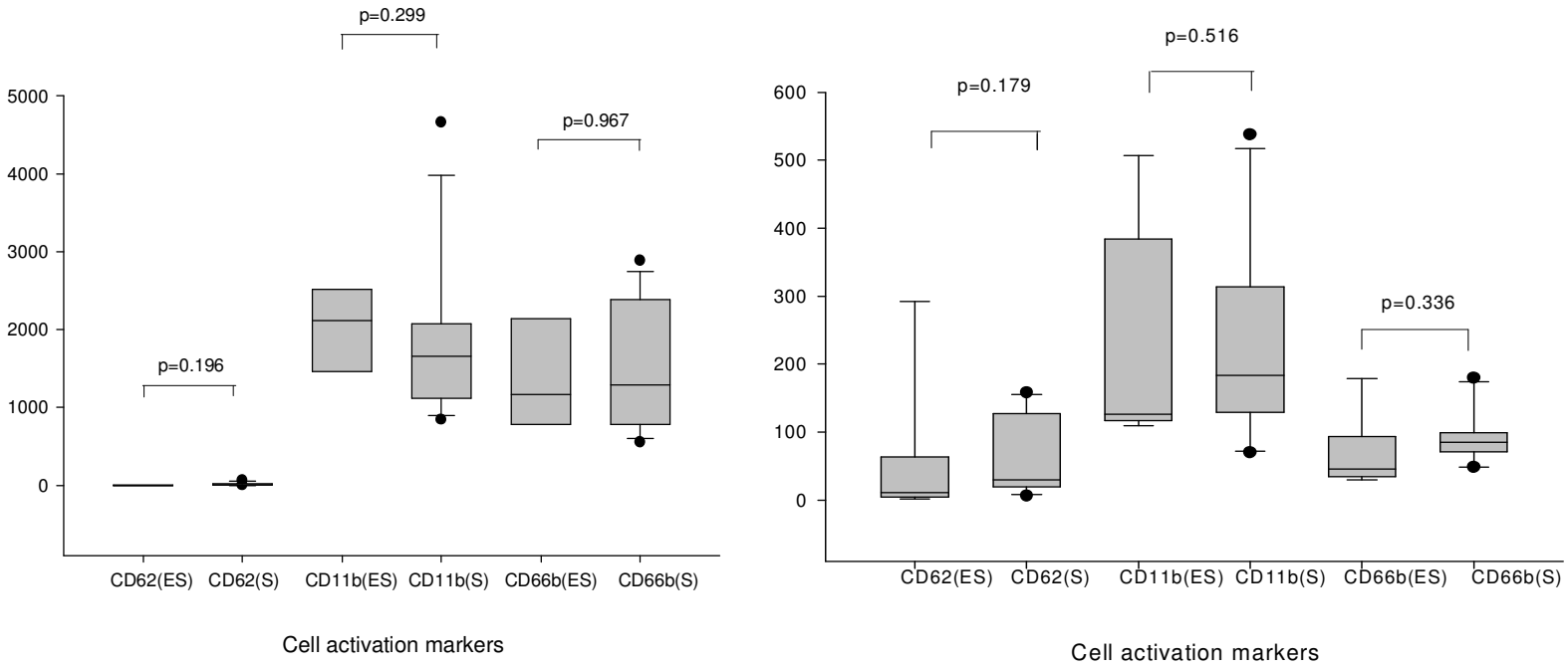


Figure 4.7: Granulocyte cell surface markers observed in a) sputum and b) Blood via flow cytometry.
(ES) = Ex-smokers (S) = Current smokers. There were no statistical differences between the 2 groups in any of the activation markers.

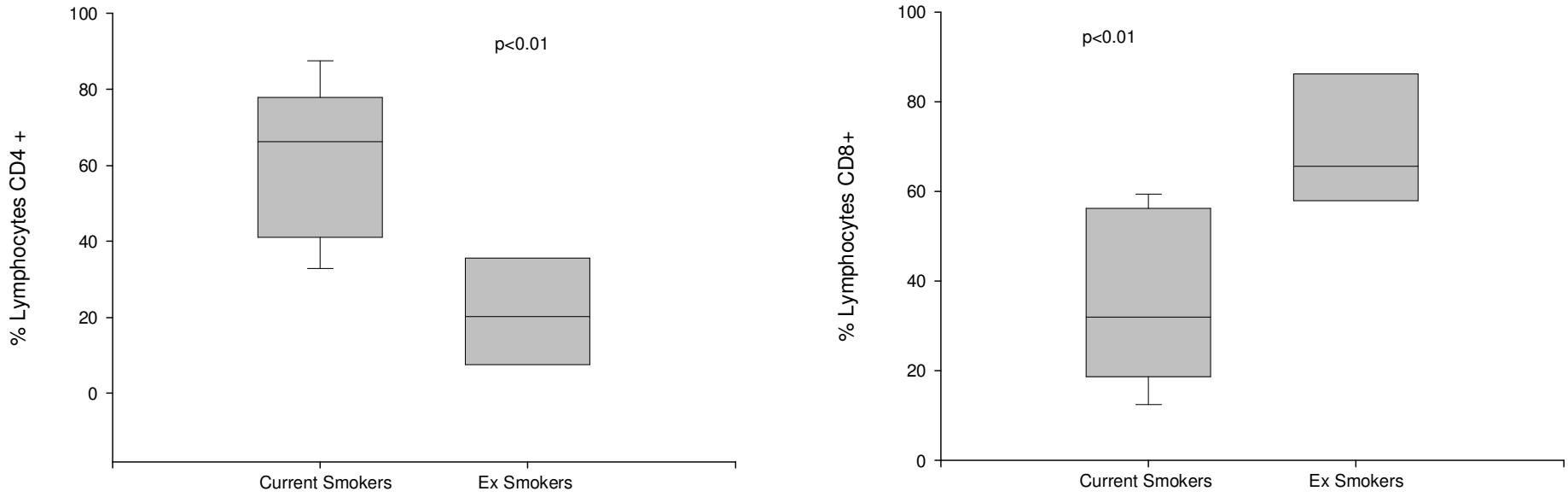


Figure 4.8: Graphs showing the differences between ex-smokers and current smokers in the distribution of CD4+ and CD8+ lymphocytes in sputum.

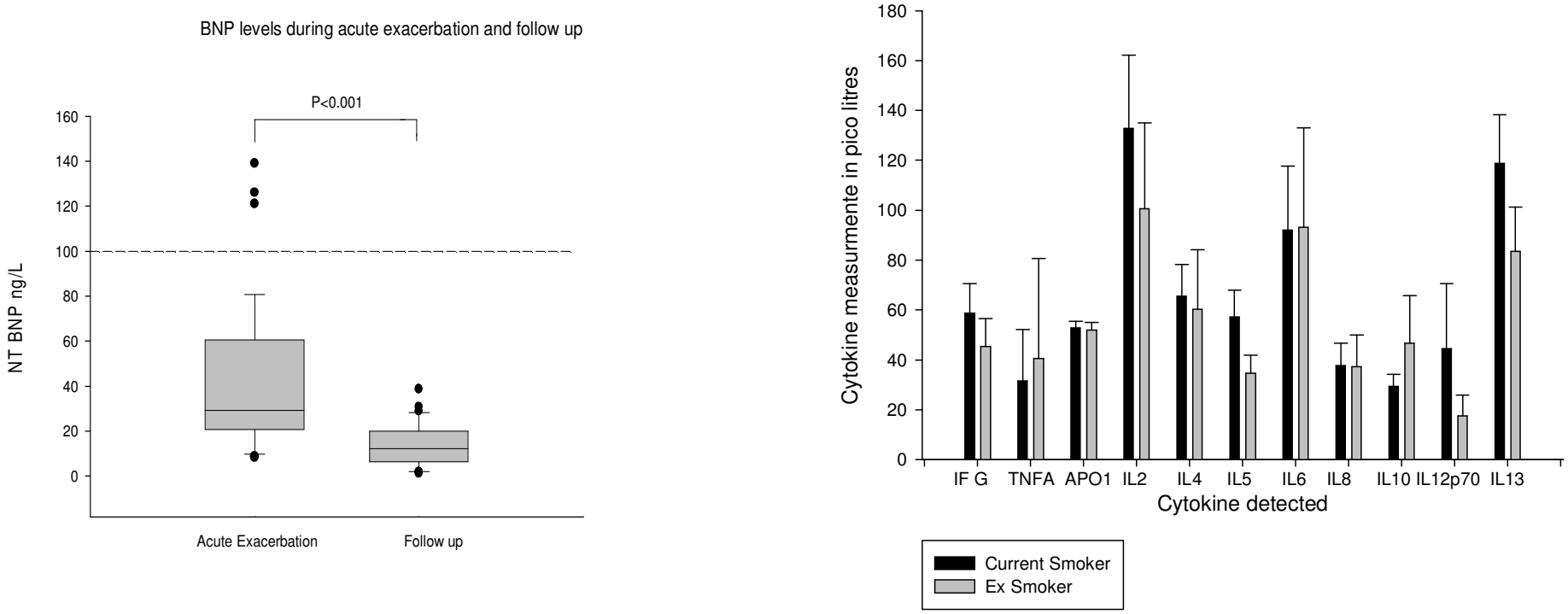


Figure 4.9 a. BNP levels are shown from the acute exacerbation and then at follow up six weeks later. BNP levels are higher during the acute exacerbation than at follow up (P<0.05). Only 3 patients reached values associated with congestive cardiac failure (dotted line), their chest x rays showed clear lung fields and ECG normal sinus rhythm with no acute changes. b) Luminex cytokine panel results. The only statistically significant cytokine difference was observed for IL12 p70 which is associated with bacterial infections.

Post-hoc analysis of mortality

13 current smokers (smoking at the time of admission) and 6 ex-smokers died within one year of admission (figure 4.10) (p=0·053). The cause of death was felt not be sufficiently reliable to be included in the analysis.

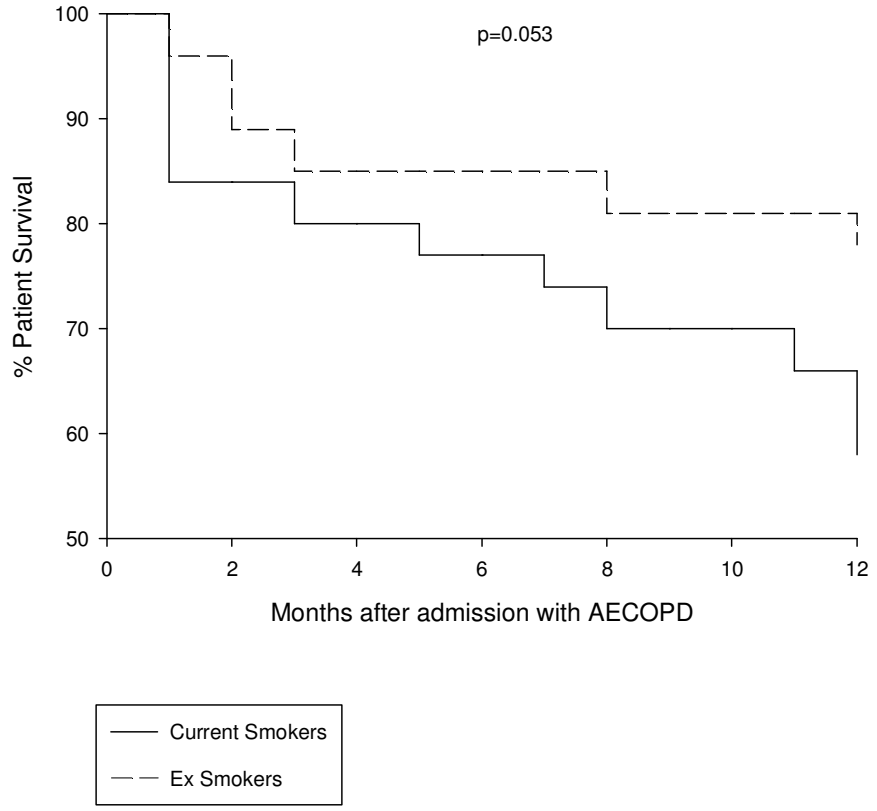


Figure 4.10: Kaplan**-**Meier graph showing the mortality over a one year period after being admitted with an acute exacerbation of COPD. The mortality in the current smokers was higher than ex-smokers, but this was not significant. (p<0.053)

CHAPTER 5: DISCUSSION AND CONCLUSION

Chapter 5 Discussion and Conclusion

5.1 Demographics and recruitment

The majority of the patients were recruited from the Acute Medical Unit in Southampton General Hospitals NHS Trust (SGH), only 5 patients were recruited through our Respiratory Centre which receives patients from either self referral or via their GP. The exclusion criteria had to be rigorous in order to ensure that all the patients recruited were admitted with an exacerbation of COPD and that no other co-morbidity or other infectious process was at the centre of their admission. Criticisms have often been made of papers that fail to make the diagnosis concrete. To this end, I have not only confirmed the diagnosis of COPD on history and spirometry, but also excluded pneumonia with a chest x-ray and other significant co-morbidities (figure 4.1) with a detailed history and examination supplemented by routine blood investigations and ECG (to exclude arrhythmias). These were combined with BNP and chest x-ray to confirm that congestive cardiac failure was not the cause of the dyspnoea. Three patients reached the levels of BNP that might be associated with heart failure, but were only slightly over the threshold. In these patients echocardiography was performed on admission and normal ventricular function was confirmed. The rise in BNP levels may in some part be due to hyperinflation. Smoking status was also confirmed with plasma cotinine levels thus not relying totally on history. Finally, arterial blood gas measurements were made to assess the severity of the exacerbation.

The 2 groups were evenly matched in all important parameters (displayed in Table 4.1), apart from pack years. Not surprisingly the current smokers had smoked significantly more cigarettes than the ex-smokers. The target of 56 patients in the power calculation derived from the pilot study was exceeded by 2.

Overall the patients had very severe disease with a median FEV_1 of 0.7 on the day of admission.

5.2 Spirometry – Improvement in Lung Function

The micro medical hand held spirometer was ideal for use in this study, and the majority (95%) of the patients completed spirometry up until day 6 of their admission. Even though the patients were suffering with a severe exacerbation they were able to understand and perform most of the manoeuvres without difficulty.

The improvement in spirometry during the AECOPD is shown in figure 4.3 a,b, and c. Ex-smokers recovered much more quickly from their exacerbation than current smokers in all spirometric parameters. In the ex-smoking population recovery of lung function is shown to occur from day 1 onwards. In the current smoking population, recovery to even the baseline of the admission test can take 2 or 3 days. Even at day 6 there is a large difference between the populations. Thus, the results of this study confirmed what I had previously found in the pilot study which led to initiating this research.

FEV_1 was the easiest measurement to perform for the patient. When performing an FVC during an acute exacerbation there was great difficulty completing the manoeuvre in the first attempt due to coughing; however many patients found this therapeutic and were able to expectorate sputum following this. Once the patient was trained sufficiently, IC (some patients did find the manoeuvre difficult to understand and perform) was also well reproduced during the 3 attempts, however improvements were seen in all 3 parameters and the most reproducible of the 3 was FEV_1.

The graphs are all quite similar apart from the larger SEM observed in the FVC graph which is not surprising considering the larger values obtained, and the greater difficulty in performing the test.

The theory about why this discrepancy is seen between the 2 groups is discussed in the conclusion. These results support the use of FEV_1 in COPD studies, as neither FVC nor IC contributed additional information, and are not as easily reproduced. The improvement in IC however does support the body of research postulating that a reduction in hyperinflation during an acute exacerbation allows the patient to breath easier from a lower resting lung volume. Indeed it is often the high respiratory rate from the exacerbation that

causes the significant lung trapping leading to hyperinflation. Reassurance, rest, nursing care and pharmacotherapy (as well as NIV in the serious cases with type II respiratory failure and respiratory acidosis) help reduces the overall respiratory rate, improve bronchodilation, increase expiratory time, and thus allowing deflation to occur.

5.3 Length of hospital stay and mortality

The ex-smokers stayed far fewer days in hospital (mean 3.08) compared to current smokers (mean 5.59 days). This reduction was very significant ($P<0.001$). This correlates is in keeping with the improvements in lung function observed between the 2 groups. This has an obvious socioeconomic impact, however does not lead to a significant cost saving to primary care due to the changes in the tariff structure since payment by results was introduced.

At one year I followed up the mortality data to see if there was a significant difference between the 2 groups. One year all cause mortality after the admission for an acute exacerbation of COPD was higher in current smokers than ex smokers but did not reach quite reach significance ($P=0.053$). I realise that this is a post-hoc analysis and that the study was not powered to look at this end point, but the result is interesting none the less because apart from smoking status the 2 groups were evenly matched before the exacerbation. Several studies of smoking status and mortality have been published, none with more subjects than the recent study published by Doll in the BMJ (155) which showed that ex-smokers had a significantly reduced all cause mortality rate compared to their current smoking colleagues. It may be there are significant improvements in the immune defence in the ex-smokers to account for the discrepancies seen. This area needs to be further studied in a much larger prospective study.

5.4 Bacteriology

Overall the results from the bacterial cultures support current literature that *H.influenzae* is the most common bacteria pathogen isolated during acute exacerbations. This study, however, has shown for the first time that there is a large difference in the bacterial pathogen and the frequency isolated between current and ex-smokers. Bacterial pathogens are far more likely to be isolated in the current smokers than the ex-smokers ($P<0.05$ – graph 4.4) and especially *H.influenzae* which was isolated in 12 out of the 31 current smoking

patients as opposed to only 2/27 of the ex-smokers. *H.influenzae* was also associated with a much higher CRP level at day one than any other organism (figure 4.4b) and contributed to a much higher CRP level overall in the current smoking population (figure 4.4a). As mentioned in the introduction to this thesis phagocytosis by alveolar macrophages of smokers has been shown to be accompanied by increased bactericidal activity compared with alveolar macrophages of non smokers. In a study of the effectiveness of alveolar macrophages to clear *H.influenzae* (132), the authors found that ex-smoker alveolar macrophages displayed immunologic properties that were more like alveolar macrophages of non smokers. This supports the theory that current smokers are less able to clear this very influential pathogen. The elevated cytokine levels associated with *H.influenzae* above any other organism have been published (116), however there is little in the way of publications looking specifically at the organism isolated at the time of exacerbation and corresponding CRP levels; especially in patients admitted with a severe exacerbation. In fact in this study if a patient is admitted with an acute exacerbation, has a CRP level above 100, and is a current smoker then the organism isolated was always *H.influenzae*

Higher levels of cytokines IL2, IL6 and IL8 were observed in patients in whom a bacteria was detected ($P<0.05$).

5.5 Virology

The detection of viruses from sputum is notoriously difficult and this study was no exception. Initially the detection of viruses from our experiments was poor until it was discovered that there was significant interference at play – see methods section for more details. The most commonly virus isolated in our population was *Influenza AH3* closely followed by *Rhinovius* (figure 4.4 c,d), these findings are broadly in line with previous studies outlined in the introduction. The lack of detection of *Rhinovirus* may have been due to technical problems. The exacerbations in ex-smokers were caused by a virus in 77% of cases, as opposed to 50% in current smokers, but this difference was not significant ($P=0.21$). The study was almost certainly underpowered to detect such a difference. Nevertheless, the results of my study suggest that the infectious cause of AECOPD may differ between smokers and ex-smokers, with bacterial agents being more frequently associated in smokers whilst viruses appear to play a more important role in ex-smokers. Whether and to what

extent this could be linked with the observed differences in CD8+ T cells in smokers and ex-smokers is unclear as the specificity of CD8+ T cells has not been determined, but it is tempting to speculate that the CD8+ T cell population could contain virus-specific T cells.

The difference in the microbiological insult may go some way in explaining the difference between the 2 groups; with current smoker exacerbations mainly due to bacterial pathogens (with *H.influenzae* the main bacterium) and ex-smokers mainly due to viral infections. There is certainly a large difference between these 2 groups in overall systemic inflammatory response denoted by CRP caused by *H.influenzae* which is a good reason why recovery may take much longer in the current smoker.

5.6 Flow cytometry and inflammatory cell counts

There are few papers exploring the use of flow cytometry methods in sputum but this is an expanding field. Flow cytometry allows us to distinguish cell types present in the sputum, their subtypes and also allows us to detect markers of cell activation. As discussed the only difference between the 2 groups in this study was in the T-cell CD4:CD8 ratios in sputum and in blood (figure 4.8). Neutrophils, eosinophils and macrophages were observed in similar quantities in both groups; their markers of activation were also non significant (figure 4.7). However, my numbers of samples assessed by flow cytometry are small as I only introduced this to the study half-way through recruitment as validation of the technique took much longer than expected. The detection of low numbers of eosinophils does deserve a mention as a few studies have shown an increase in eosinophils during acute exacerbations.(141)

The number of eosinophils detected by flow cytometry was similar in both groups and did not depend on whether the pathogen was viral or bacterial. This was backed up by the analysis of our slides obtained from the cytospins; these showed a low eosinophil level (<1%) but a large neutrophil predominance. There is no obvious reason for this discrepancy however the populations studied previously tended to be at the more mild/moderate end of the disease spectrum compared to the severe patients studied here.

The major difference observed between the 2 groups was seen in the

T-lymphocyte population with current smokers having a much higher CD4:CD8 ratio than ex smokers. This can partly be explained by the difference in pathogen isolated from the patients (viral infections tend to move t-lymphocytes towards CD8 predominance) and also possibly by a difference in the immune mechanisms at play between the 2 groups. (Figures 4.8).

The white cell count and neutrophil count were higher in the current smokers than ex-smokers; however the difference was not significant. The higher values in the current smokers were to be expected with bacterial infections the predominant infective aetiology.

5.7 Cytokines

The development of the luminex system has allowed us to investigate a whole spectrum of cytokines quickly and with great accuracy. I found that apart from TNFα and IL10 the cytokines were seen in greater quantities in the current smoking population. The only significant result however was seen with IL12p70 which is significantly higher ($p<0.05$) in current smokers than ex smokers (Figure 4.11). It is possible that a properly powered study involving larger numbers of patients might detect significant differences.

IL-12 is a heterodimeric 70 kDa glycoprotein (IL-12-p70) consisting of a 40 kDa subunit. It is secreted by peripheral lymphocytes after induction and is produced mainly by B-cells and to a lesser extent by T-cells. The most powerful inducers of IL-12 are bacteria and bacterial products which explains why the levels are higher in the current smoking population.

5.8 BNP

I found that during an acute exacerbation BNP levels were much higher ($P<0.001$) than during the stable state (figure 4.18). BNP levels in only 3/58 patients reached levels associated with congestive cardiac failure. Importantly, BNP levels did not differ between the 2 patient groups, which means that I can exclude with confidence heart failure as an explanation for the worse clinical outcome in current smokers.

BNP has been shown to be the best discriminatory marker to differentiate cardiac dyspnoea from all other cause of dyspnoea in a whole series of trials. With regards to ventricular function in respiratory medicine we are more

interested in right heart dysfunction, and BNP has been shown to be raised in patients with pulmonary hypertension, cor pulmonale and pulmonary embolism. (93) BNP has been shown to increase in proportion to the degree of right ventricular dysfunction, but to a lesser degree compared to left ventricular dysfunction. In right ventricular dysfunction raised concentrations of BNP is a predictor for increased risk of death, even in cor pulmonale and primary pulmonary hypertension. In pulmonary embolism low BNP levels within 4 hours of admission are associated with an uneventful hospital admission, and may be useful in identifying patients for treatment in an outpatient setting. (97)

What my findings add to the field is that BNP levels do not rise significantly above the threshold normally associated with congestive cardiac failure; even during an acute exacerbation of COPD, and therefore BNP is a useful discriminatory test in those cases when the diagnosis is in doubt.

5.9 Limitations of the Study

There at least a couple of ways in which this study could have been improved. The lack of baseline measurements on the subjects would have added greatly to the understanding in improvements in spirometric parameters during the study, allowing us to express these as percentage of baseline.

Serial measurements of symptom improvements in these patients would have been helpful, and with hindsight I should have encouraged the patients to record their symptoms on diary cards with an analogue measurement of dyspnoea scores.

Conclusion

In the first study of its kind, we have shown that smoking status has a major impact on the nature of COPD exacerbations, resulting in slower recovery of lung function and delayed discharge, factors which are of importance to both patients and health resources. Current smoking was associated with a more significant bacterial burden, in particular *H. influenzae*, and higher serum CRP levels, suggesting that cigarette smoke alters both the local environment in the lungs and the systemic consequences of acute infectious exacerbations.

In the UK, COPD accounts for up to 10% of all acute annual admissions,(156) and patients experience on average 1 to 3 exacerbations per year, the highest exacerbation rate being related to low FEV_1 (157). Exacerbations are responsible for 30,000 premature deaths per year in the UK, and mortality is still rising, especially in the female and elderly populations. (3) Exacerbations also account for at least 50% of the treatment cost of COPD; these figures make COPD a priority disease for all acute medical services. (11, 156)

There have been several studies following COPD patients during their exacerbation and monitoring recovery by measuring FEV_1. (158, 159) Our results support FEV_1 as an appropriate measurement for use in COPD exacerbation studies, because in our experience more sophisticated measures, such as IC, are not necessarily reproducible in the critically ill patients as this requires more effort on the patient's part. The exacerbating patient often found it difficult to understand such manoeuvres and to complete. The same was true for FVC where patients found it difficult to reach a plateau in the time-volume curve due to dyspnoea.

In the current study, ex-smokers demonstrated a greater improvement in FEV_1 already one day after being admitted, and this increased linearly during the admission. In contrast, in current smokers first improvement was noticeable only after 2 days. This finding is compounded by the hospital length of stay, with ex-smokers recovering much more quickly during their exacerbation than current smokers, resulting in a hospital stay 2·5 days shorter than current smokers, which equates to a 55% saving in use of hospital beds. This has important socioeconomic implications.

The finding of major differences in the microbiological insult suffered during exacerbation in this study sheds light on possible reasons for why smokers have a different course of exacerbation. Routine culture of sputum showed *H. influenzae* to be the predominant microbe isolated, accounting for 37% of all isolates. Whilst this study does not have the power to sub-analyse the clinical and pathological features of patients positive for *H. influenzae*, the fact that 12 of a total of 30 were positive in current smokers and only 3 of 21 were positive in ex-smokers suggests that smoking might alter the susceptibility to this common bacterial strain. The role of bacteria in stable COPD and during exacerbations has been extensively studied and *H. influenzae* has been consistently shown to be the most frequently isolated bacterium. It has also been shown to produce the greatest inflammatory responses, even when isolated as a colonising bacterium. (121)-(127) Patients whose airways are colonised by *H. influenzae* in the stable state report more symptoms and increased sputum purulence at exacerbation than those not colonized (118). Bacterial colonisation, a feature of COPD, especially in the more severe forms, is known to be more prevalent in current smokers. (160)

This study has shown that current smokers have a considerably greater degree of systemic inflammation, as denoted by CRP serum levels, and thus adds to the considerable body of evidence pointing to the role of systemic inflammation in COPD.(161) Indeed, recent studies have shown CRP to be the most sensitive biomarker of an exacerbation. (58) Whether higher CRP levels in the current study are due to smoking or the presence of *H. influenzae* is unclear. Smokers in general have higher serum concentrations of CRP, (162) but bacterial colonisation with such bacteria as *H. influenzae* and *Psedudomonas Aeruginosa* has also been associated with higher CRP levels. (127) CRP is also linked to worse pulmonary function even in individuals without pulmonary disease. (64)

The finding of differing CD4+ to CD8+ ratios in the sputum on admission seen when comparing ex- and current smokers is also the first observation of its kind and suggests significant differences in the involvement of these central cells of acquired immunity. Ex-smokers were found to have a predominant CD8+ T cell population in sputum, a finding which is in keeping with observations in bronchial biopsies of patients with stable COPD. (146) How this relates to smoking status is unclear. Cigarette smoking has been shown to be associated

with a selective increase in CD4+ cells in peripheral blood when compared to non-smokers. (149, 150) The finding that viruses were more readily detected in ex-smokers, albeit not significantly, while bacteria were more prevalent in current smokers also suggests possible differences in immune responses. It is tempting to speculate that these differences could explain why ex-smokers recover much more quickly than current smokers.

Ever since the publication of the graphs of Fletcher and Peto, depicting the relentless decline in lung function associated with smoking (163), a major effort has been directed at smoking cessation. The findings of our study should provide further incentive and a strong public message to current smokers with COPD to quit. In addition to showing a worse immediate clinical outcome, the study shows a clear trend towards increased mortality and markedly increased costs of treatment of acute exacerbations in smokers with COPD. The current study should be viewed as a pilot study in respect of the respective role of viruses and bacteria in causing exacerbations and should provide incentive to elucidate the role of bacterial colonization as compared to acquisition of new strains of bacteria in smokers. (129) This could have implications for strategies to prevent exacerbations, possibly including selection of patients for prophylactic antibiotic use which has recently been demonstrated to reduce exacerbation rates. (164)

Future Studies

I have several studies lined up on completion of my Doctorate in Medicine. I have a clinical and academic position within Southampton University, The Biomedical Research Unit and Southampton University Hospital as clinical lead for COPD. I have developed an advanced community service structure with the help of the local Primary Care Trust and this provides an ideal opportunity to create a large well phenotyped population of COPD patients to help with basic and translational research. We initially aim to recruit a cohort of 150 patients, 50 mild, 50 moderate and 50 severe. I then aim to see if the results from this study are reproduced in the moderate population and further expand the study with regards to cell activation and ratios using flow cytometry. I am also interested to see if the introduction of the intermediate services and change in service provision has made an impact on the COPD population in Southampton City.

The underlying basic mechanisms for the differences observed in this paper also deserve further research and I plan to further explore the difference in the T-Cell populations in current and ex-smokers, and then extend this to research into macrophage biology.

I am also very interested in looking at decline in lung function in COPD patients in a well phenotyped population and reproduce some of the work by Fletcher and Peto to confirm that the attenuation of lung function decline with cessation of smoking is seen within a well phenotyped population with COPD.

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