

The role of airway infection in chronic obstructive pulmonary disease

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THE ROLE OF AIRWAY INFECTION IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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ABSTRACT

This thesis examines the role of respiratory bacterial and viral infection in the natural history of Chronic Obstructive Pulmonary Disease. The rationale for this study is based upon previous data demonstrating that airway bacterial colonisation is common in stable COPD and that bacterial and viral pathogens are commonly detected at exacerbations.

The methods used have involved the careful characterisation and clinical follow up of a cohort of patients with moderate to severe COPD in the stable state and at exacerbation. Sampling of airway and systemic compartments enabled the detection of respiratory pathogens and quantification of inflammation. Comparisons between clinical indices and evidence of infection were performed to determine the relationships between bacterial and viral infections and disease outcomes including lung function decline and exacerbation severity.

The findings confirmed that lower airway bacterial colonisation is common in stable COPD and is associated with airway inflammation. They demonstrated for the first time a relationship between the degree of bacterial carriage and the rate of disease progression. This study has also described novel evidence for persistence of respiratory syncytial virus in the lower airway and associations with inflammation and lung function decline and impaired anti-viral immune responses. The combined role of human rhinoviral and bacterial infection at exacerbation has been studied and factors influencing responses to exacerbation therapy determined with the importance of early initiation of treatment identified.

The findings in this thesis indicate that both viral and bacterial pathogens may play an important role in the natural history of COPD and are therefore targets for potentially novel interventions. This work suggests that viral and bacterial infections and their interactions play an important role in modulating airway inflammation in stable disease and at exacerbation thus impacting on both disease progression and exacerbation severity. This work has provided a rationale for future investigation into the mechanisms underlying susceptibility to infection in this important disease.

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This work is based on the clinical study of a group of patients without whom it would not be possible. They were at all times committed, enthusiastic, stoical and in many cases an inspiration, I sincerely hope that this and the on going work of the group helps to better the understanding of their disease and perhaps lightens their burden a little.

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I confirm that the work contained within this thesis is my own original endeavour, with contributions from the following individuals, in the specific areas described:

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LIST OF ABBREVIATIONS

A	Adenosine
ATS	American Thoracic Society
bp	base pairs
BAL	broncho-Alveolar Lavage
BTS	British Thoracic Society
C	Cytosine
°C	degrees Celsius
CD	Cluster of Differentiation
cDNA	copy Deoxyribonucleic Acid
CFU	colony forming units
CO₂	Carbon Dioxide
COPD	Chronic Obstructive Pulmonary Disease
CRP	C Reactive Protein
CT	Computed Tomography
dl	decilitre
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
ERS	European Respiratory Society
FEV₁	Forced Expiratory Volume in One Second
FVC	Forced Vital Capacity

g	grams
G	Guanine
GNEB	Gram Negative Enteric Bacteria
GOLD	Global initiative for Obstructive Lung Disease
HBSS	Hanks' Balanced Salts Solution
HRQOL	Health Related Quality of Life
HRV	Human Rhinovirus
ICAM-1	Inter-Cellular Adhesion Molecule-1
ICS	Inhaled Cortico-Steroids
IFN	Interferon
IL	Interleukin
IQR	Inter Quartile Range
kPA	kiloPascals
l	litre
LTB₄	Leukotriene B ₄
LTOT	Long Term Oxygen Therapy
mcg	micrograms
mg	milligrams
ml	millilitres
mM	milliMolar
MPO	Myeloperoxidase
mRNA	messenger Ribonucleic Acid
MRC	Medical Research Council
n	number
ng	nanograms

NHLBI	National Heart, Lung and Blood Institute
NIH	National Institute of Health
p	probability
PBMC	Peripheral blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEF	Peak Expiratory Flow
pg	picograms
PHA	Phyto-Haemagglutinin
PPM	Potentially Pathogenic Micro-organisms
r	Pearson's correlation co-efficient
rho	Spearman's correlation co-efficient
RNA	Ribonucleic Acid
rpm	revolutions per minute
RSV	Respiratory Syncytial Virus
SD	Standard Deviation
SGRQ	St Georges' Respiratory Questionnaire
t	time
T	Thymine
Taq	Thermophilus aquaticus
Th 1	Type 1 'helper' T-Lymphocyte
U	Unit
UV	Ultraviolet Radiation
WHO	World Health Organisation
yr	year

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) occupies a unique position in the epidemiology of major diseases at the beginning of this century. It is the only common cause of death that is currently increasing in prevalence (Murray 1996, WHO 1998).

The World Health Organisation has ranked COPD as the twelfth most prevalent disease worldwide and the sixth most common cause of mortality. The WHO predicts that by 2020 COPD will rise to be the fifth most prevalent disease and the third most common cause of death. COPD is not only a major cause of mortality but causes extensive disability and is expected to become the fifth most important cause of disability adjusted life years (DALYS) by 2020 (WHO 1998). This trend has obvious major, current and future implications for both patients and healthcare providers alike.

COPD is characterised by the development of airway inflammation in response to long term inhalation of noxious gases, primarily tobacco smoke in susceptible individuals. This results in the development and progression of airways obstruction which can eventually lead to persistent symptoms, reduced functional status, susceptibility to exacerbations and to early mortality (WHO 1998, ATS 1995).

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Whilst cigarette smoking has been identified as the most important factor in the aetiology of COPD it remains uncertain why only a proportion of smokers go on to develop the disease.

The pathology of COPD is a combination of a number of processes, namely the development of chronic bronchitis, emphysema and small airways obstruction or bronchiolitis. Chronic bronchitis is due to inflammation of the proximal airways (>4mm in diameter) leading to mucus gland hypertrophy and proliferation, mucus hyper-secretion and epithelial cell disruption, all of which contribute to an environment where bacterial sterility of the airway, which is the normal finding in healthy lungs cannot be maintained (Hogg 2005).

Another key pathological entity in the syndrome which is termed COPD is emphysema. This term describes the destruction of lung tissue distal to the smallest bronchiole and was first described by Laennec in the early 19th century following study of postmortem lungs. Emphysema itself is not a uniform condition and pathologically can be divided into centri-lobular, the commonest type in COPD characterised by dilatation of the bronchiole, and pan-lobular seen more often in alpha-1-antitrypsin deficiency. Emphysema can itself lead to airways obstruction due to the loss of lung elasticity contributing to narrowing and closure of small airways leading to gas trapping and hyperinflation (Strachan 1995).

Recent pathological studies have highlighted that a key site of inflammation in the lungs of subjects with COPD is the small airway (Hogg 2004). Here concentrations of inflammatory cells have been described and it has been postulated that this phenomenon is linked to the role of chronic airway infection.

The mechanisms which determine whether an individual is susceptible to the effects of tobacco smoke and the extent to which they develop bronchitis or

emphysema are not known. The underlying genetic susceptibility to the inflammatory effects of inhaled noxious substances is under study and is likely to prove complex and multi-factorial. To date, the role of genotype in COPD, outside the context of alpha-1-antitrypsin deficiency, remains poorly understood and furthermore knowledge of how prolonged exposure to environmental factors affects the epigenetics of individuals at the organ, cellular, DNA or RNA level remains similarly incomplete. Determining what drives the heterogeneity in phenotype between individuals with COPD, which may either be host specific and pre-determined or modulated by the presence of other environmental factors such as respiratory pathogens, is a vital process in understanding this disease, and a fundamental step in identifying new therapeutic targets in this highly complex and prevalent disease.

1.2 HISTORICAL PERSPECTIVE

COPD is a modern, umbrella term for a disease state characterised by the presence of airflow obstruction due to either the conditions chronic bronchitis or emphysema or both (GOLD 1998, ATS 1995, NICE 2004).

Chronic bronchitis has been recognised as a common disease since the early twentieth century. The prevalence of this condition predates the expansion of cigarette smoking in the general population in the UK. Indeed for many years chronic bronchitis was termed the 'English Disease' and the effects of ubiquitous cigarette smoking and environmental pollution seen in UK cities after the Second World War culminated in the excessive morbidity and mortality seen in the London smog of 1952 (Davis 2002). This was a major spur to the epidemiological study of chronic bronchitis was being recognised as an important disease.

Chronic Bronchitis was one of the first conditions to be defined for the purposes of epidemiological research, by the MRC in 1960, leading to the standardised diagnostic criteria of 'chronic productive cough for at least three months of the year for at least two years' (MRC 1965). The presence or absence of the symptom of exertional dyspnoea further characterised the diagnosis to either simple or obstructive bronchitis. It is only therefore in the last forty years that COPD has been defined, studied and also recognised as disease of major importance.

1.3 EPIDEMIOLOGY AND RISK FACTORS

1.3.1 DISEASE DEFINITIONS AND PHENOTYPES

The recorded prevalence of any condition is dependent on the accepted definition of that disease. The prevalence of a condition such as COPD which covers a spectrum of clinical and pathological presentations rather than a discrete and easily classified disease entity (Figure 1.1) has been difficult to determine without standardisation of the diagnosis. COPD has been defined using both clinical and spirometric criteria.

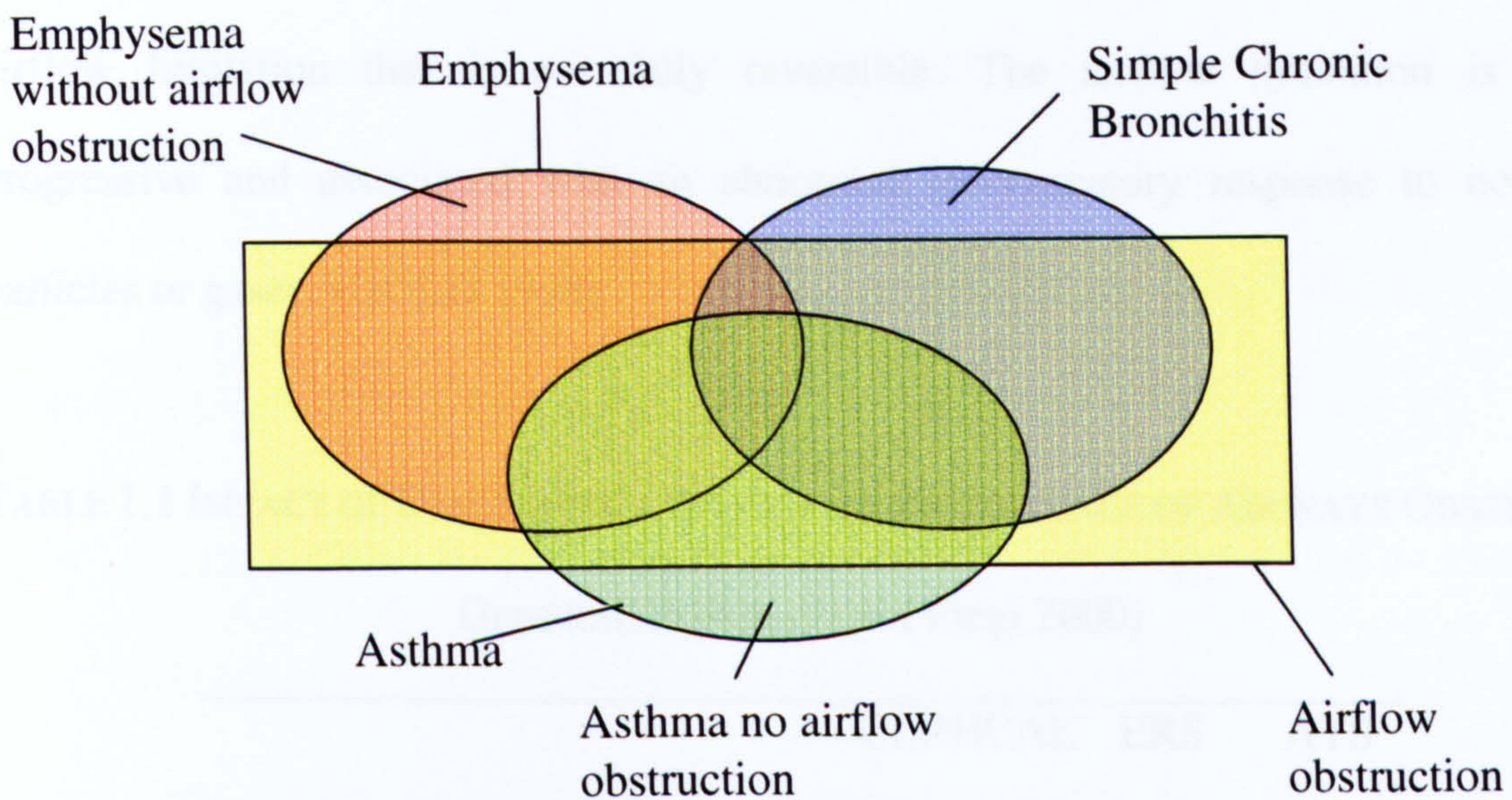
Since the earliest consensus definition was made in the 1960's, there has been an evolution of the definition of COPD. More recent versions have included reference to both the characteristic obstructive lung function and to the progressive deterioration in lung function seen in this condition (GOLD 1998, ATS 1995, NICE 2004).

In 1995 the European Respiratory Society (ERS) consensus statement defined COPD as 'a disorder characterized by reduced maximum expiratory flow and slow emptying of the lungs; features of which do not change markedly over several months. Most of the airflow limitation is due to varying combinations of airways

disease and emphysema; the relative contribution of the two processes is difficult to define in vivo.' (Siafakas 1995).

In comparison also in 1995, the American Thoracic society (ATS) published a comparable disease definition 'COPD is a disease state characterised by the presence of airflow obstruction due to chronic bronchitis or emphysema; the airflow obstruction is generally progressive, may be accompanied by airways hyperreactivity and may be partially reversible.' (ATS 1995). This definition includes the term 'chronic bronchitis' which has lead to some confusion. Reports of longitudinal studies have shown that mucus hypersecretion and progressive airflow deterioration whilst both related to smoking have distinct natural histories. Hypersecretion can occur in the absence of airflow limitation (Fletcher 1976, Vestbo 2002), whilst other work has demonstrated a link between chronic sputum production and subsequent decline in lung function (Vestbo 1996). Whether this association is only an epiphenomenon with sputum production and faster FEV₁ decline both independently associated with increased airway inflammation, or whether excess sputum production could have direct effects on lung function decline remains undetermined and is further discussed later in this introduction.

FIGURE 1.1 DIAGRAMMATIC REPRESENTATION OF COMPONENTS OF COPD AND OBSTRUCTIVE AIRWAYS DISEASE.



1.3.2 IMPACT OF DISEASE DEFINITIONS ON THE PREVALENCE OF COPD

The criteria for the diagnosis of COPD had not been established internationally until recently. Various organisations had established their own spirometric diagnostic criteria for the disease. This point is important to the work discussed in this thesis, as not only will differences in definitions affect the recorded prevalence of the disease but also the interpretation of data from clinical studies that examine the role of infection or inflammation in disease pathogenesis.

An Italian study illustrates the dependence of observed COPD prevalence upon the diagnostic criteria used. 1 727 Patients were assessed using the ERS and ATS criteria for COPD. See Table 1.1 (Viegi 2000). This illustrates that the prevalence of COPD within any population is dependent on the spirometric criteria used for diagnosis and underlines the importance a worldwide standardised diagnosis of COPD.

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In 2000 the global initiative on obstructive lung disease (GOLD) consensus was formed. Its stated goals were to increase worldwide awareness of COPD and to work to decrease COPD related mortality and morbidity. The consensus definition of COPD was issued and is 'a disease state characterised by progressive development of airflow limitation that is not fully reversible. The airflow limitation is both progressive and associated with an abnormal inflammatory response to noxious particles or gases.' (GOLD 1998)

TABLE 1.1 IMPACT OF DIAGNOSTIC CRITERIA ON PREVALENCE OF AIRWAYS OBSTRUCTION IN A GENERAL POPULATION (Viegi 2000)

	CLINICAL	ERS	ATS
AIRWAY OBSTRUCTION	28%	12.2%	57%
MODERATE/SEVERE COPD	4.4%	3.6%	5.2%
Diagnostic criteria;	Clinical	FEV ₁ / FVC < 70%	
	ERS	FEV ₁ / VC < 88% Predicted	
	ATS	FEV ₁ / FVC < 75%	

The spirometric criteria for diagnosis were standardised; postbronchodilator $fev_1 < 80\%$ predicted, $FEV_1 / FVC < 70\%$. The GOLD consensus excludes reversible airflow limitation associated with bronchiectasis, cystic fibrosis, tuberculosis or asthma. The importance of the antecedence of smoking in COPD is not included in the definition above but many clinical investigators exclude never-smokers from their studies.

This international consensus definition has been updated by the ATS/ERS consensus published in 2004 (Celli 2004). Here the disease definition includes an optimistic opening statement and comments on the extra-pulmonary manifestations of

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the disease; *'Chronic obstructive pulmonary disease (COPD) is a preventable and treatable disease state characterised by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and is associated with an abnormal inflammatory response of the lungs to noxious particles or gases, primarily caused by cigarette smoking. Although COPD affects the lungs, it also produces significant systemic consequences.'* In addition, this document rationalised the spirometric classification of disease severity including an at risk pre-disease category, and is comparable to previous GOLD staging criteria. See below:

- **At Risk-** $FEV_1 \geq 80\%$ Predicted, $FEV_1/FVC > 0.7$ who smoke or who have exposure to pollutants, have cough sputum or dyspnoea.
- **Mild COPD-** $FEV_1 \geq 80\%$ Predicted, $FEV_1/FVC < 0.7$.
- **Moderate COPD-** FEV_1 50- 80% Predicted, $FEV_1/FVC \leq 0.7$.
- **Severe COPD-** FEV_1 30-50% Predicted, $FEV_1/FVC \leq 0.7$.
- **Very Severe COPD-** $FEV_1 < 30\%$ Predicted, $FEV_1/FVC \leq 0.7$.

However, use of the FEV_1 as the sole index of disease severity has been criticised and models which include measures of functional status and systemic manifestations of disease have been developed (Celli 2004). These are more useful as prognostic indicators than lung function alone and are likely to become the framework for future consensus statements on estimating disease severity.

1.3.3 STUDIES OF DISEASE PREVALENCE

There are many ways of analysing the prevalence of a condition within a population. These include assessment of the clinical burden either in primary or secondary health care settings, crosssectional sampling studies, screening the "at risk"

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population and correlating the prevalence of a major risk factor such as smoking to the likely disease prevalence. Each of these techniques can only approximate the true prevalence of a disease and has individual weaknesses and inaccuracies. However, it is apparent that in all studies COPD is of major importance and is increasing in prevalence in many countries (Anto 2001, Murray 1996, Feenstra 2001, Stang 2000).

Despite the contribution of COPD to worldwide morbidity and mortality, there have been relatively few large population studies to assess the prevalence of the disease. Those that have been performed almost exclusively describe populations from industrialised countries and diagnostic definitions are often not standardised, making comparisons between studies problematical.

NORTH AMERICA

The USA has collected more epidemiological data on COPD than most countries. The National Centre for Health Statistics published in its 1997 reported that Obstructive Lung Disease was responsible for more than 109 000 deaths in that year alone and estimated that 16 million US citizens have symptomatic COPD making it the fourth most common disease in the US at the time (NCHS 1998).

A comparison between such national data and earlier, regional epidemiological studies gives an interesting insight into the changing prevalence of COPD over the last four decades. One such early prevalence study was performed in Berlin, New Hampshire and published in 1962 (Ferris 1962). This found the overall prevalence of 'Chronic non specific respiratory diseases' to be between 15.4 and 39.1% in men dependent on location and between 15.2 and 20.9% in women. The large spread in the male population was felt to be due to the prevalent occupational risk factors in different locations. The prevalence of irreversible airways obstruction

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(< 60% predicted), representative of COPD ranged from 3.1 to 21.7% in men and 6.2 to 13.9% in women. Other studies were less influenced by pollution or occupational risk factors; in Tecumseh, Michigan in a study of 9000 men and women, 14% of adult men and 8% of adult women had chronic bronchitis, COPD or both (Higgins 1984). A study from a population at altitude, Glenwood Springs, Colorado published in 1971 found that 17% of men had symptoms of dyspnoea, cough, expectoration or wheeze and 13% had COPD with an FEV₁/FVC ratio less than 60%. In women, the prevalence of chronic bronchitis (10%) and COPD (4%) was lower, reflecting the gender differences in smoking behaviour (Mueller 1971).

The third National Health and Nutrition Examination Survey (NHANES III) is a large, national, US study including 20 050 adults which ran from 1988-1994 and included data on lung function, previous respiratory diagnoses and responses to a comprehensive questionnaire on respiratory symptoms. Data on 16 084 patients was analysed and characterised by gender and race. An umbrella diagnosis of Obstructive lung disease (OLD) (chronic bronchitis, emphysema or asthma) was used and defined according to spirometric criteria of an FEV₁/FVC ratio less than 70% and an FEV₁ of less than 80% predicted (low lung function) (NHANES 1998, Petty 2000).

The overall prevalence of low lung function was 6.8% and 10% in the population over 45 years. Overall, 8.5% reported some degree of obstructive lung disease, when analysed for smoking behaviour the rate was 12.5% of current smokers, 9.4% of former smokers, 6.1% of pipe or cigar smokers and 5.8% of never smokers. The prevalence of OLD increased with increasing age and was higher in whites than blacks. There was considerable overlap between asthma and COPD with a number of patients reported both conditions, this group having lower lung function and more respiratory symptoms.

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The study's authors report the most interesting finding as the marked under-diagnosis of OLD in the study population; 63.3% of the subjects with documented low lung function had no prior or current diagnosis of OLD. This was most marked in those with mild to moderate disease.

These studies suggest that COPD is highly prevalent in the US and furthermore diagnosed cases may represent only a small proportion of those who have early to moderate disease (Mannino 2000, Petty 2000). Numerous authors have concluded that spirometric screening of the at risk population i.e. smokers may identify the true disease prevalence and allow early intervention and appropriate resource allocation and may also help to identify which factors may predispose smokers to the development of the disease.

EUROPE

Since 1995 when the European Respiratory Society published its consensus statement on COPD (Siafakas 1995) there has been renewed drive to improve awareness of COPD in Europe and improve the problem of underdiagnosis which is apparent on both sides of the Atlantic (Rennard 2002).

To this end the IBERPOC Project (Estudio Epidemiologico de le EPOC en Espana) was carried out in 1997. 4 035 randomly selected 40 to 69 year olds in Spain. The prevalence of smoking was 26% current and 24% ex smokers, whilst the prevalence of COPD defined by the ERS spirometric criteria was 9.1% overall, 15.8% in males and 5.5% in Females (Sobradillo 1999).

The majority of COPD cases were in the older age group, approximately half being between 60 and 69 years old. Again the proportion of patients with a positive spirometric diagnosis during the study who had previously been identified with

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COPD was very low at 2.3%. The failure to diagnose COPD was not because patients were experiencing few symptoms. There was a high prevalence of reported cough (13.5%), sputum expectoration (10.7%), exertional dyspnoea (10.4%) and wheezing (40.2%) in this population.

A high prevalence of respiratory symptoms in mild or 'pre-clinical' COPD was demonstrated by the findings of the European Respiratory Society study on COPD (EUROSCOP) (Yernault 1992). The study was a three year placebo controlled double blind trial to investigate the effect of inhaled corticosteroids on lung function decline. The 1 277 patients, all smokers, (mean age 52, 74% men) were mainly recruited by media advertising rather than from a clinic population. The mean FEV₁ was 77% predicted with an obstructive ratio of 62% and very little reversibility to inhaled beta-agonists. More than 75% of patients with pre-clinical COPD reported respiratory symptoms. These data and current studies suggest that whilst there are some differences in COPD prevalence between European countries, the overall prevalence is high, and chronic symptoms and significant airflow obstruction are common findings at diagnosis.

The history of COPD in the UK is different from many other industrialised nations. The prevalence of COPD has been very high in the UK throughout the last century and has actually declined over recent years. Historically the prevalence of smoking in the UK was much higher much earlier than elsewhere in Europe or the US (Pride 2002). The UK cohort of patients who had the highest smoking prevalence this century were those men born in 1900-1910 and those women born 1920-1930. The survivors of this female group contribute to the current increase in COPD mortality seen in the female population whilst the majority of the male cohort would have

contributed to the peak prevalence of COPD seen some decades ago (Pride 2002, Doll 2004).

The only major UK study assessing lung function across a broad age range is the Health and Lifestyle Survey (Cox 1987). This study commenced in 1987 and assessed the lung function of 2484 men and 3063 women using turbine spirometry performed at the subject's home. The subjects were a representative population sample and were aged 18-65 years. 10% of men and 11% of women overall demonstrated an FEV₁ two or more standard deviations below the predicted value for their age and height. The proportion with poor lung function increased with age particularly in smokers. The prevalence of COPD in these studies was high, and was related to tobacco usage and age demographics. However, a key feature was that social class, even when differences in tobacco usage are adjusted for, is an independent predictor of disease prevalence. This finding may suggest that environmental causes other than smoking are important in the development of COPD.

THE REST OF THE WORLD

The Global Burden of Disease Study conducted by the WHO and the World Bank estimated the worldwide prevalence of COPD to be 9.34/1,000 in men and 7.33/1,000 in women in 1990 and this data is discussed in the GOLD consensus document (Murray 1996, WHO 2000). This document reminds us that such overall prevalence data considers all age groups and under estimates the true prevalence in older adults. Outside the developed world there is little data on COPD prevalence, however, the higher the per capita tobacco consumption the higher the prevalence of COPD.

Whilst there is evidence to suggest that the tobacco markets in the USA and Europe may have matured with consumption levels no longer rising, this is not the case in Third world economies where low levels of tobacco use is rapidly increasing. China, in particular, as an emerging economy already has a very high prevalence of smoking amongst males. Physician diagnosed COPD rates are high with 7.2% in men and 4.7% in women (Liu 1998, Xu 2005). These levels underestimate the true disease prevalence and even with a major smoking cessation initiative the future burden of COPD in this country alone will be enormous.

There are differences in COPD prevalence and in related death rates between countries that may not be explained simply by the differences in tobacco consumption. There have been many theories to further explain these differences including genetics, pollution, climate, tobacco type and the frequency and nature of respiratory infections. However, mortality figures are dependent on local certification practices and awareness of particular conditions. The GOLD consensus aims to combat such difficulties in international comparisons, thus enabling improved epidemiological study which may uncover important genetic or geographic predisposing factors more prevalent in certain populations.

1.3.4 COPD ASSOCIATED MORTALITY, MORBIDITY AND HEALTH CARE COSTS

Mortality data is almost universally collected in developed countries however the reliability of the diagnosis on death certificates is at best variable and often incomplete. Furthermore, the interpretation of this data and its relationship to COPD is difficult. The analysis of the certified cause of death alone does not allow for other clinically important conditions which have contributed to but are not the primary

cause of mortality. This is especially true when considering a chronic and disabling condition such as COPD.

The ICD9 classification system which is internationally recognised does not have a specific code for COPD. ICD9 codes 490-496 (unspecified bronchitis 490, chronic bronchitis 491, emphysema 492, asthma 493 etc.) cover airways obstruction and have been analysed as a means of assessing mortality related to COPD.

A review comparing ICD 9 coded mortality data for different countries found death rates from codes 490 – 496 to be highest in the UK, Australia, and Eastern Europe and lowest in Southern Europe, Scandinavia and Japan. Published figures for the UK 1992 reveal 6.4% of male deaths and 3.9% of female deaths to be certified as due to these coded diseases (NHSE 1996).

When all contributing causes of death are considered the number of cases with chronic respiratory disease involvement is high. Analysis of multiple cause mortality from US national figures indicates that 8.2% of deaths had a coding for obstructive lung disease however less than half (43%) of these cases had COPD as the registered cause of death (NHLBI 1998). Mortality from COPD is underestimated, but even when considering current figures it is ranked as the sixth most important worldwide cause of death as is predicted by the WHO to rank third within twenty years (Feenstra 2001, Murray 1996).

Due to the chronic and progressive nature of COPD, it is an important cause of long term symptoms. In more severe disease these can limit functional capacity, impair health related quality of life and incur considerable health care costs. The costs of COPD care have been estimated in a number of countries and have been estimated to total \$1.9 billion per year (Pauwels 2004). However the additional costs due to

disability, lost working days (up to 24 million dollars per year) and reduced productivity were estimated to be an additional \$3 billion.

The majority of COPD related costs are incurred by patients with more severe disease with a significant proportion of outpatient costs incurred by the provision of long-term oxygen therapy in the community (Guest 1999) and a similarly significant proportion of inpatient expenditure incurred in treating patients with severe exacerbations (Oostenbrink 2004). Therefore identification of factors which may modify progression to severe disease and the incidence and severity of exacerbations may have a major impact on the health care resources required by patients with COPD.

All studies of COPD related costs are likely to underestimate the true economic impact of the disease, as patient care by family members of patients with chronic and severe disease is not usually included but is often significant, particularly in countries with less developed health care services.

1.3.5 RISK FACTORS FOR THE DEVELOPMENT OF COPD

SMOKING PREVALENCE; THE RELATIONSHIP TO COPD PREVALENCE

Consideration of the epidemiology of COPD illustrates the difficulties of direct and accurate estimation of the disease prevalence due to problems inherent in detecting the disease in its early stages. This is particularly true in non-industrialised countries where access to spirometry is limited, symptoms due to other respiratory disease are common and health resource utilisation data underestimates true prevalence.

As cigarette smoking has been identified as the most important determinant of the pathogenesis of COPD, with age of starting smoking, and pack year history

predictive of COPD mortality. Therefore a model using smoking prevalence to estimate that of COPD would be a useful epidemiological tool. It is commonly quoted that 10 to 15% of smokers go on to develop COPD. This figure is taken from cross sectional US national data and Fletcher and Peto's seminal longitudinal work which is discussed later in this chapter (Fletcher 1976). However, a smoking model using up to date diagnostic criteria and validated against US prevalence data (NHANES III) was published by Stang et al. This study postulates that in current smokers 40 years old the true prevalence of COPD is 17% rising to 43% at age 75 upwards, with a higher prevalence in females for any given age (Stang 2000). If these figures are correct, these data confirm that the number of people diagnosed with COPD is only a small proportion of the actual number of people who have the disease. Furthermore any prevalence data must be qualified by the age of the population it describes.

If the links to cigarette smoking and the development of COPD are incontrovertible the effects of smoking cessation on the course of the disease are less well described. However a longitudinal key study with a 5 year follow up of over 5000 patients 'The Lung Health Study', has described the benefits of smoking cessation in slowing disease progression, with sustained smoking cessation achieving the largest benefit (Anthonisen 1994). The effects of a reduction rather than a cessation in smoking are less marked with some benefits on lung function but little effects on symptoms seen in patients who reduce but do not stop daily tobacco use (Simmons 2005). This may suggest that once airway obstruction develops smoking cessation may not entirely limit disease progression, therefore factors which contribute to worsening lung function after cessation may be important.

GENETICS AND EPIGENETICS

The most conclusive proof that genetic factors play a role in the aetiology of smoking related airways obstruction comes from the model of alpha-1-antitrypsin (A1AT) deficiency. This autosomal recessive condition, first described in 1963 (Eriksson 1963), is characterised by the development of early onset and rapidly progressive panacinar emphysema. A1AT is a serpine protease inhibitor and its key role is to inhibit neutrophil elastase. The Z variant, most frequently associated with lung disease, results in normal mRNA and synthesis of antitrypsin, but only 15% is released into the circulation. The deficiency occurs because about 85% of synthesized AAT is blocked in the terminal secretory pathway of the hepatocyte (Lomas 2005, ATS 2003).

The observed differences in the development of airway obstruction between individuals who develop COPD and have normal AAT levels suggests that differences in the extent of cigarette smoking account for only 15% of the observed variability in the development of airways disease in smokers (Bascom 1991). The role of genetic factors in the pathogenesis of COPD have also been suggested by association studies, with first degree relatives of patients with early onset COPD displaying a higher risk of developing COPD than controls matched for smoking behaviour (Silverman 1998). However, identification of polymorphisms associated with susceptibility for COPD has proved to be an elusive goal with inconsistent findings from one study to the next. Polymorphisms in matrix metalloproteinase genes may play a role in the pathogenesis of COPD. MMP1 and MMP12 genes, but not MMP9, have been suggested to be related to smoking-related lung injury (Silverman 1998, McCloskey 2001, Sandford 2002). Whilst an association between an MMP9 (Minematsu 2001), IL-11(Klein 2004), TGF-beta1 (Wu 2004),

polymorphisms and the development of COPD have been identified. However findings are rarely reproduced in different populations and further study is required. It is likely that multiple genes may be involved in determining susceptibility to the disease and that different gene expression patterns may explain the observed heterogeneity of clinical and pathological phenotypes.

Epigenetics is the study of reversible changes in gene function or other controls of cell phenotype that occur without a change in DNA sequence. These non-coding changes occur in response to environmental factors, and may play a vital role in the development of diseases such as COPD. Whilst the long term effects of cigarette smoking have been described in terms of pathological and inflammatory changes, the way in which such exposure could lead to non-sequence modification of DNA and RNA expression and translation requires investigation. There is to date, some evidence that tobacco smoke exposure can lead to telomere shortening in a dose related fashion, suggesting important effects on nucleic acid conformation which may confer downstream effects on gene expression and hence phenotype or inflammatory profile (Morla 2006). A possible molecular mechanism by which cigarette smoke drives pro-inflammatory gene transcription and an inflammatory response in the lungs has been postulated following work in rats. Here, histone acetylation and de-acetylation, key steps in the regulation of the specificity and duration of gene transcription, were shown to be disrupted by smoke, resulting in excessive transcription of specific pro-inflammatory genes in the lungs (Marwick 2004). Further studies into the mechanisms underlying modification of gene expression and post-translational modification of protein products are required, especially with the advent of technologies which may provide new therapeutic opportunities to alter post translational genomic expression (Fire 1998).

ENVIRONMENTAL POLLUTION AND OCCUPATIONAL EXPOSURE

Inhalation of particulate matter from environmental or occupational sources has been implicated as a risk factor for the development of COPD. Exposure to biomass smoke particularly in women has been identified with this disease in a number of population studies performed in different populations largely in non-developed countries (Amoli 1998, Pandey 1984, Perez-Padilla 1996, Smith 2000). Similarly exposure to occupational pollution is a risk factor for the development of airways obstruction and the effects are compounded by tobacco smoking (Kauffman 1979).

However, the contribution of outdoor pollution as an aetiological factor remains uncertain. Some studies have demonstrated a link (Tashkin 1994) however the effects are likely to be relatively inconsequential in comparison to inhalation of tobacco smoke.

AIRWAY INFECTION

The role of airway infection in the natural history of COPD is the key area of investigation for the studies described in this thesis and a discussion of the many studies into this is presented later in this introduction. The role that lung infections in early life may play in the later development of COPD is less well described. There is however, considerable epidemiological evidence that socioeconomic status is a risk factor for COPD. This link is likely to be multi-factorial and includes prenatal exposures, housing conditions, air pollution, environmental tobacco smoke, diet, in addition to possible infective factors such as frequent lower respiratory tract illnesses in childhood which associate with lower social class (Prescott 1999). There have been a number of studies which have found a relationship with lower respiratory tract infections in childhood and the development of subsequent COPD (Shaheen 1994,

Shaheen 1995). Whether these relationships reflect causality or an epiphenomenon due to the effects of low birth lung function, maternal smoking, respiratory tract infections and the development of subsequent wheeze or retardation of lung growth is uncertain. It is interesting to speculate on the possible mechanisms of these associations however evidence does exist that inflammatory episodes due to infections in early life predict the development of subsequent disease (Finch 2004). Whether this is due to long-term modulation of immune responses or even to persistence of infective pathogens remains uncertain.

1.4 THE NATURAL HISTORY OF COPD

1.4.1 LUNG FUNCTION DECLINE

Any clinician who has cared for patients with COPD will be aware of the progressive nature of the disease, and the majority of consensus definitions of COPD now allude to the progressive nature of lung function decline seen in the condition. The majority of patients are diagnosed in the fourth or fifth decade of life and are symptomatic at the time of diagnosis. In normal subjects lung function is maximal between 25 and 30 years of age with a gradual decline from then on with age.

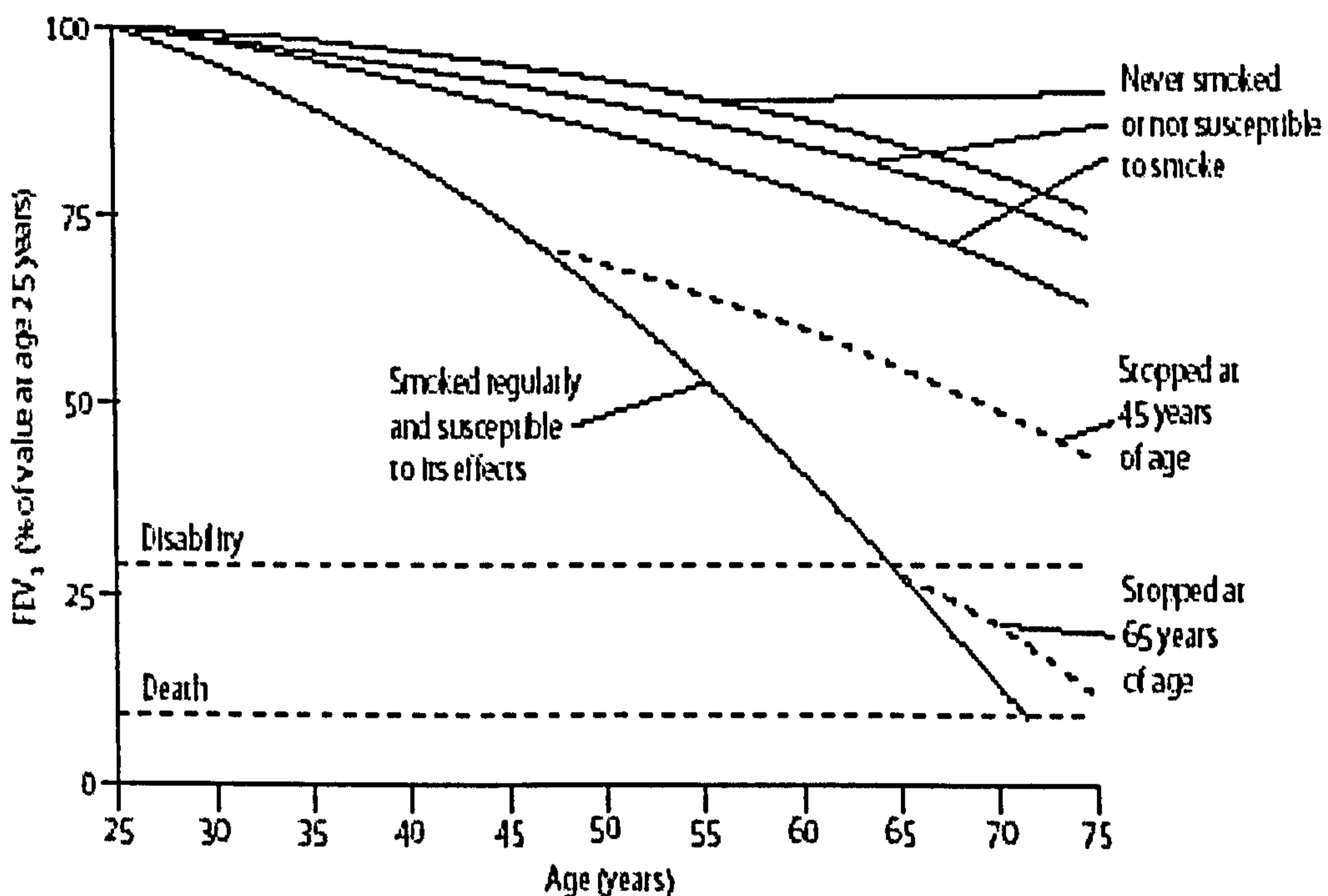
A landmark study published in the BMJ in 1976 by Fletcher and Peto was the first to describe the natural history of this decline in a suitably large population (792 'healthy' males aged 30 to 59) based on 8 years of follow up. The well recognised curve is illustrated below (Fletcher 1976).

The annual decline of FEV₁ in non-smokers was 25 ml and in smokers 50 ml per year. A subset population which exhibited an accelerated decline of 100ml per year developed significant symptomatic airways obstruction during the study. The

curve also demonstrates the possible effect of smoking cessation on the observed accelerated decline in lung function, suggesting a return to the rate of decline back to normal. This represents important evidence as to the benefits of smoking cessation at any stage of COPD. However, despite being recognised as an important study, the published curve relies largely on extrapolation at its extremes.

FIGURE 1.2 THE FLETCHER-PETO CURVE OF HYPOTHETICAL DECLINE IN FEV₁ THE NATURAL HISTORY OF CHRONIC BRONCHITIS AND EMPHYSEMA.

Adapted from Oxford University Press 1976.



This is particularly important as the subjects' age and the deterioration in lung function becomes more marked. This section of the curve relies upon mathematical extension of the acquired data; the majority of patients were less than 60 on completion of the study and did not yet have severe airways obstruction. An accurate model of the changes in lung function at this clinically significant later phase of COPD is not yet available.

The annual rate of decline varies greatly between studies from as low as 7ml per year in the Six Cities Study (Xu 1992) to 91 ml/yr in smokers in the Tucson population study (Lebowitz 1989). Some of these differences will be due to statistical noise created by simple variation in spirometry readings. The shorter the follow up period, the more likely this may mask any true effects as the 95% confidence interval for FEV₁ decline in an individual is 190ml, which is several years decline even in a population of smokers (Tweedale 1984). Hence, with annual spirometric readings, which has been the norm in larger studies, a follow-up period of at least 4 years is recommended (Pride 1995).

It is believed that only certain individuals are susceptible to cigarette smoking and several years of accelerated lung function decline result in the spirometric impairment seen when COPD is diagnosed. If it is assumed that individual subjects tend to maintain their percentile ranking over time then an individual with an initially low FEV₁ will remain in the lower percentiles as his lung function declines a phenomenon known as tracking.

An alternative theory is that lung function does not deteriorate significantly until damage to the airways has become very widespread. Thus due to the large functional reserve of the lungs, susceptible smokers might only develop an accelerated decline in FEV₁ after years of airway inflammation. Therefore until middle age, one would expect even susceptible subjects to exhibit normal annual changes in lung function. Techniques to assess changes in small airways more sensitively than spirometry are necessary to assess such early damage.

Single breath nitrogen testing is such a technique. Indeed, ten year follow up data using this technique revealed that almost all patients who develop airways obstruction had abnormal nitrogen breath testing when there FEV₁ was still normal.

However the negative predictive value of this test was poor and as a technique it is less suitable for large population studies than spirometry (Buist 1988). Newer techniques such as high resolution CT scanning have yet to be evaluated in terms of longitudinal studies.

1.4.2 'THE BRITISH HYPOTHESIS'

In the 1950's and 1960's British respiratory researchers hypothesised that recurrent respiratory infections were the differentiating factor between those smokers who developed airways obstruction and those who did not, with the development of chronic bronchitis and hence innate immune defences a key stage in the process. The study by Fletcher and Peto (Fletcher 1976), found that chronic bronchitis (cough and sputum production, and episodes of acute bronchitis) as defined by the MRC (MRC 1965), was not associated with the development of airways obstruction in a population of British workmen. They concluded that bronchitic symptoms were not related to disease progression in this patient group and these conclusions led to a change in emphasis for the role airway infection in the pathogenesis of COPD.

The finding that uncomplicated chronic bronchitis does not predict development of subsequent COPD has been confirmed in a further study (Vestbo 2002), however, an analysis involving patients with established airways disease has demonstrated that smokers with chronic bronchitis do develop airways obstruction more rapidly than those without these symptoms (Vestbo 1996). It is likely therefore that the role of mucus hypersecretion and its clinical correlate of chronic bronchitis in the development of airflow obstruction may vary depending on disease stage and potentially therefore on the prevalence of other factors which may upregulate the inflammatory response such as bacterial colonisation.

1.4.3 EXACERBATIONS AND DISEASE PROGRESSION

Exacerbations of COPD can be defined as ‘an event in the natural course of the disease characterised by a change in the patients baseline dyspnoea, cough and/or sputum beyond day-to-day variability sufficient to warrant a change in management.’ (Celli 2004). These events have important consequences for patients and health care professionals alike, the key features of the aetiology and pathophysiology of these events are discussed later in this introductory chapter, however the evidence that exacerbations contribute to disease progression in COPD will be considered in this section.

Analysis of data from the Lung Health Study (Kanner 2001) revealed that exacerbations were associated with an increased rate of decline in FEV₁ in current but not in ex-smokers. Data from our own group has confirmed that patients with more frequent exacerbations did have a decline their FEV₁ at a faster rate, irrespective of smoking status (Donaldson 2002). The key difference between these two studies was the severity of disease, with a mean FEV₁ percent predicted of 78% in the LHS and 38% in the East London Study. Airway inflammation, which is a key process in the development of progressive airflow limitation is greater in patients with more severe disease (O’Donnell 2004) and in smokers (Tanino 2002). Exacerbations are associated with increased levels of markers associated with (Bhowmik 2000) airway inflammation and this is the likely mechanism by which they accelerate FEV₁ decline. It is interesting to postulate that higher numbers of airway inflammatory cells in stable smoking and severe COPD patients potentiate a greater rise in inflammation at exacerbation with consequences for disease progression in these groups.

1.5 LOWER AIRWAY INFLAMMATION

There is great heterogeneity in the pathology of COPD from one individual to the next with differing contributions from the key processes of chronic bronchitis, small airways disease and emphysema to the pattern of disease in a particular individual. What is common to all subjects with COPD however is the finding of pulmonary inflammation which has been demonstrated using biopsy, sputum, lavage and post mortem tissue studies (Maestrelli 1995, Thompson 1989, Hunninghake 1983, Hogg 2004, O'Donnell 2004). A number of pathological studies have described inflammatory profile associated with the development of airway obstruction in COPD. However, the factors which determine the degree of the inflammatory response to inhaled noxious gases are not understood.

Tobacco smoke inhalation affects ciliary function (Verra 1995) and may therefore impair mucous clearance from the airway, reducing the ability of the mucociliary escalator to remove particulates from the airway. Smoke inhalation also reduces the efficacy of the protective respiratory epithelial cell layer which may augment the overall acute inflammatory response due to facilitating the persistence of pathogens in the lower airway (Qvarfordt 2000).

Neutrophils, airway macrophages and mast cells are the key innate immune response cells which are involved in the initial phase of the immuno-inflammatory cascade. A number of studies have demonstrated increased numbers of neutrophils in the airways of patients with COPD compared to smokers who do not develop airway obstruction (O'Shaughnessy 1997, Baraldo 2004). Furthermore, experimental work has shown that a key product of activated neutrophils, neutrophil elastase can induce key pathological features representative of those seen in the COPD lung, including mucus hypersecretion, goblet cell metaplasia and the development of emphysema

(Sommerhoff 1990, Amitani 1991, Smallman 1984, Snider 1985, Hill 2000). Indeed work with animal models of smoke induced emphysema have demonstrated that alpha-1-anti-trypsin ameliorates the severity of changes suggesting not only a key role for neutrophil elastase in this process but also the importance of protease- antiprotease imbalance in the pathogenesis of COPD (Churg 2003).

Another key cell in the innate defences of the lung is the macrophage, these are the most numerous cells present in the airway when sampled using bronchial or broncho-alveolar lavage. Macrophages are the key phagocytic cell and it is this process which is fundamental in orchestrating the innate immune response. Furthermore macrophage derived matrix-metalloproteases (MMPs) are important in the protease mediated epithelial and alveolar cell damage which contributes to the development of airways obstruction. Evidence for the importance of MMPs in the development of emphysema is available from in vitro studies. Mice without MMP12 are not susceptible to the development of emphysema following cigarette smoke exposure (Hautamaki 1997). However it is likely that contributions from both macrophage and neutrophil are fundamental in the pathology of the disease; both MMPs and neutrophils being required for matrix breakdown in models of emphysema via a TNF alpha mediated pathway (Hogg 2004, Dhami 2000, Finkelstein 1995).

The role of eosinophils and their precursor; mast cells in the pathobiology of COPD is less certain. The evidence that eosinophils or ECP are elevated in COPD (Fujimoto 1999, Linden 1993, Lams 1998) or not (Di Stefano 1998, O'Shaughnessy 1997, Lacoste 1993, Saetta 1999) depends on which part of the conflicting evidence base one reads. A study with more sophisticated approach than merely counting cell numbers has suggested that whilst eosinophil numbers may be higher in COPD the degree of eosinophilic activation was no greater than that of normal controls (Rutgers

2000). An important clinical correlant of eosinophilic involvement in the pathology of COPD would be a link to steroid responsiveness. Generally, COPD is considered a 'steroid resistant disease' when compared to asthma, a largely eosinophil mediated and steroid responsive disease. Indeed study subjects with greater eosinophil numbers have been shown to be more responsive to steroids (Fujimoto 1999, Chanez 1997, Pizzichini 1998) but whether this patient group represents a population with co-existent asthma or truly a subset of COPD remains uncertain. Similar controversy as to the role of mast cells exists and clearly further clinical and mechanistic studies are required in this area.

Presentation of antigen allows activation of the adaptive immune response which itself is both apparent and abnormal in the COPD lung. Adaptive immune cells in regional pulmonary lymph nodes and in bronchial associated lung tissue (BALT) are activated by circulating antigen presenting cells and/or directly from the epithelium. An increase in T cells particularly CD8+ cells has been a finding in many (O'Shaugnessy 1997, Saetta 1999, Majo 2001, Hogg 2004) but not all studies of COPD airways, with higher numbers of CD4 + cells a less frequent finding. The exact role of CD8+ lymphocytes in the pathology of COPD is uncertain. They are an important component of the anti-viral immune response and can kill virally-infected cells directly by cytolysis or by the induction of apoptosis via the release of granzyme into the target cell (Abbas 2000). The presence of large numbers of CD8+ T cells in the airway of COPD patients has been linked to the possibility of a role for airway viral infection in the aetiology of airway inflammation and obstruction. This is discussed further below.

B lymphocytes may play an established role in maintaining airway immunity both through the production of mucosal IgA and through circulating Ig M and Ig G,

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all of which can neutralise and opsonise microbial epitopes aiding phagocytosis by the innate immune system. Hogg's publication on small airway inflammation which showed greater numbers of not only T cells but also B cells in airways of patients with more severe disease suggests a role for this arm of the adaptive immune system in disease progression (Hogg 2004).

The key site for development of airflow limitation are the small airways (Hogg 2004) and it is here that airway inflammatory cells concentrate. At this site there has been shown to be a relationship between the number of cells in the lumen and the severity of COPD, in terms of airway obstruction (Hogg 2004, Yanai 1992). Indeed it has been postulated that mucus secretion and plugging of these small airways results is an important factor in the development of airways obstruction.

A different and probably complementary mechanism in the development of airway obstruction is airway wall remodelling. Pathological studies have shown connective tissue deposition in the adventitia in severe disease (Matsuba 1989). This process of the small airways is seen in combination with local accumulation of BALT in more severe disease suggesting a role for infection in the development of this component of COPD (Hogg 2004).

KEY INFLAMMATORY MARKERS ASSAYED IN THIS STUDY

The cellular inflammatory response seen in the lungs of COPD patients is orchestrated by the production on an array of signalling peptides and proteins termed cytokines and chemokines. The array of possible profiles is immense and this project which aims to determine the effects of airway infection in COPD has chosen a limited range of soluble mediators which when quantified reflect rather the overall degree of airway inflammation which may then be related to clinical and infective parameters.

IL-6

IL-6 is a 26-kiloDalton cytokine with pleiotropic activity in a range of systems. It is produced by a wide array of cells including lymphocytes, epithelial cells and macrophages. It is a key mediator in the acute-phase response (Geiger 1988) and is elevated in both acute (Chollet Martin 1996) and chronic inflammation (Ridderstad 1991). IL-6 has been shown to be elevated in airway (Bucchioni 2003) and systemic (Debigare 2003) compartments in COPD and to rise further at exacerbation, particularly those associated with viral infection (Bhowmik 2000, Wedzicha 2000). IL-6 has been shown to augment antibody production and also to induce neutrophil deformability and sequestration to the lung (Suwa 2001) and therefore may play an important role in controlling the immune response to airway infection.

IL-8

Interleukin 8 is a chemokine produced by an array of cells including the airway epithelium, neutrophils, macrophages and lymphocytes (Pease 2002). The stimuli to IL-8 release include lipopolysaccharide, IL-1beta and tumor necrosis factor-alpha. Regulation of the IL-8 production is under the control of nuclear factor kappaB. IL-8 is a potent chemotactic agent for neutrophils and exerts its effects by binding with high affinity to two receptors on its cell surface, the chemokine receptors CXCR1 and CXCR2.

IL-8 is elevated in the COPD airway and the levels are higher in patients with more severe disease (Yamamoto 1997). Levels are also related to the degree of airway bacterial colonisation (Hill 2000) and rise during exacerbations (Aaron 2001, Fujimoto 2005, Wilkinson 2006). IL-8 is likely to play a key role in mediating the neutrophilic inflammatory responses which are key in the pathology of COPD.

Myeloperoxidase (MPO).

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Myeloperoxidase is a hemoprotein secreted during activation of neutrophils, which plays an important role in the immune defences by catalyzing the production of hypochloric acid (HOCl) which is toxic to pathogenic organisms which have been phagocytosed by the neutrophil. Levels are elevated in the airway of COPD patients and relate to disease severity and is a specific marker of neutrophil activation (Di Stefano 1998). It has been postulated that unregulated release of MPO may play a key role in driving oxidative stress which may be important in the development of airways obstruction (Drost 2005).

Fibrinogen

Fibrinogen is produced by hepatocytes in response to stimulation by systemic inflammatory cytokines chiefly interleukin 6 (IL-6) (Castell 1989, Gabay 1999). Elevated plasma fibrinogen is an established risk factor for coronary heart disease (Meade 1993, Ernst 1997, Salomaa 2002). It has been shown to be elevated in response to infection and during exacerbations of COPD (Wedzicha 2000) and therefore factors which modulate fibrinogen levels may also be important in the incidence of increased cardiovascular morbidity associated with COPD.

1.6. COPD EXACERBATIONS

The natural history of COPD is characterised by sometimes frequent episodes of worsening symptoms and lung function termed exacerbations. These episodes are not only an important cause of morbidity and mortality (Guest 1999), but also are a frequent cause of physician consultation in primary and secondary care and a major cause of hospital admission (Garcia-Aymerich 2000, Pearson 1994). They also adversely affect health related quality of life (Seemungal 1998).

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There remains no generally accepted definition of an exacerbation; however in recent years attempts to agree on a consensus definition have been made. Early definitions had largely been made in the context of clinical trials such as that used in the antibiotic trial by Anthonisen and colleagues of “At least two of increase in SOB, sputum purulence, sputum volume or any one above and one of: URTI, wheeze, cough, increase in respiratory or pulse rate” (Anthonisen 1987). Later definitions have been less specific; following a consensus meeting in 2000, a definition of “a sustained worsening of COPD patient’s condition from stable state necessitating a change in regular medication” (Rodriguez-Roisin 2000) was proposed. However, this definition required health care utilisation to occur in order for an exacerbation to be diagnosed. This definition has been reworked against the background of clinical findings that COPD patients will often fail to report episodes of worsening symptoms and that these unreported episodes or exacerbations were similar in nature and severity to those that did receive additional therapy (Seemungal 1998, Seemungal 2000). Consequently, the consensus definition has evolved to “An exacerbation of COPD is an event in the natural course of the disease characterised by a change in the patient's baseline dyspnoea, cough and/or sputum beyond day-to-day variability sufficient to warrant a change in management.” (Celli 2004), which is widely but not universally accepted.

The definition used in the context of a clinical study is required to be much more specific than the approaches of consensus statements above. The East London COPD group has used, since its inception, a symptom based definition based upon the original Anthonisen definition and is “the presence for at least two consecutive days of increase in any two “major” symptoms (dyspnoea, sputum purulence, sputum amount) or increase in one “major” and one “minor” symptom (wheeze, sore throat, cough,



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symptoms of a common cold) (Seemungal 1998, Seemungal 2000, Bhowmik 2000). This definition has been validated against a number of important outcomes including health related quality of life (Seemungal 1998), rate of disease progression (Donaldson 2002), airway (Bhowmik 2000) and systemic inflammatory changes (Wedzicha 2000).

There have been a number of studies which have described the symptomatic, physiological and inflammatory changes which may occur during exacerbations of COPD. The first in depth description of the time course of symptoms during COPD exacerbations was published by our group in 2000. This analysis of 504 exacerbations in 101 patients with moderate to severe COPD, this showed that there was a short prodrome before the onset of exacerbation associated with a deterioration in the symptoms of dyspnea, sore throat, cough, and symptoms of a common cold but not of lung function. Larger falls in PEFr were associated with symptoms of increased dyspnea, colds, or increased wheeze. Median recovery times were 6 days for PEFr and 7 days for daily total symptom score. Recovery of PEFr to baseline values was complete in only 75.2% of exacerbations at 35 days. In the 404 exacerbations where recovery of PEFr to baseline values was complete at 91 days, increased dyspnea and colds at onset of exacerbation were associated with prolonged recovery times (Seemungal 2000).

A recent study in 20 patients demonstrated that acute exacerbations are associated with worsening airflow obstruction and lung hyperinflation. Reductions in dyspnoea following onset are associated with reductions in lung hyperinflation and consequent increase in expiratory flow rates (Parker 2005). However what determines the degree of deterioration in lung function at a given exacerbation or the modulation of recovery back to baseline is not understood.

It is also important to note that a not all symptomatic exacerbations, only 49.6% in the Seemungal study, was actually reported to the investigators indicating that COPD patients often do not seek therapy for exacerbations which may have important consequences which have not been investigated.

1.6.1. AIRWAY AND SYSTEMIC INFLAMMATION AT EXACERBATION

COPD exacerbations have been associated with heightened levels of airway and systemic inflammation (Hurst 2006). The variable nature of the findings of studies investigating the inflammatory changes at exacerbation highlights the heterogeneity of these events. Biopsy studies have confirmed greater numbers of inflammatory cells in subjects at exacerbation compared to stability. The role of the eosinophil remains controversial as there is evidence that airway mucosal eosinophil numbers rise significantly at exacerbation (Saetta 1994) although the nature of the eosinophils seen differs in terms of cytokine expression from those in the asthmatic airway (Saetta 1995). These biopsies also revealed evidence of neutrophilic airway inflammation along with increases in CD3+ T cells. There is also evidence that the degree of airway inflammation is directly related to both the severity of the exacerbation and of the underlying disease (Drost 2005). Studies of inflammation in our patient population with moderate to severe COPD using induced sputum failed to show a rise in sputum neutrophils at exacerbation, however soluble markers such as interleukin-8 did rise suggesting a contributory role for the airway epithelium in driving the inflammatory response (Bhowmik 2000).

A link between the aetiology of an exacerbation and the inflammatory profile can be hypothesised from studies linking symptoms of putative viral infections such as cold symptoms to rises in IL-6 (Bhowmik 2000) or the presence of purulent

sputum, higher isolation rates for bacterial pathogens and greater levels of LTB₄ (Gompertz 2001).

A number of studies have also shown heightened levels of systemic inflammation at exacerbation. Work from our group has demonstrated rises in plasma fibrinogen at exacerbation (Wedzicha 2000) and patterns of systemic inflammation were seen to be related to possible infective aetiology, with a trend towards greater increases in systemic inflammation in virally associated exacerbations (Seemungal 2001).

Systemic inflammation at exacerbation may be an important part of the acute inflammatory response for a number of reasons. It is likely to be mechanistically linked to the muscular weakness associated with exacerbations with an association between systemic levels of CXCL8 and IGF-I and the development of peripheral muscle weakness in severe exacerbations (Spruit 2003). It may also contribute to the excessive cardiovascular morbidity associated with exacerbations (Sin 2003).

1.6.2 BACTERIAL INFECTION AND EXACERBATION

The hypothesis that airway infection may play an important role not only in the aetiology of exacerbations but also in the genesis and progression of airways obstruction has existed for a considerable time, indeed as a component of the original 'British Hypothesis' discussed above. An association between the presence of airway pathogens and the occurrence of exacerbations has been established. However, despite the high incidence of these events, there is very little data available on how airway infection may modulate their severity or how different groups of pathogens may interact. It is also important to consider that a proportion of events diagnosed as exacerbations may be due to concurrent pathologies such as pulmonary emboli or

worsening of heart failure. The true incidence and indeed severity therefore of infective or inflammatory exacerbations may be affected by these additional aetiological factors.

A number of studies have identified lower airway bacteria in sputum at exacerbations of COPD (Sethi 2000, Miravittles 1999, Monso 1995, Soler 1998). The most commonly identified organisms being *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* with isolation rates of potentially pathogenic organisms varying between 25 and 75%, and dependent on the severity of disease and the prevalence of smoking (Rosell 2005, Monso 1995).

The role of bacterial infection at exacerbation is complicated by the presence of bacteria colonising the lower airway in the stable state. Studies have shown however that the prevalence of potentially pathogenic organisms and that the airway bacterial load are higher at exacerbation than in the stable state (Monso 1995) which may strongly suggest but do not prove a role for bacteria in the aetiology of these events. Other work in this area has shown that the isolation of a bacterial pathogen at exacerbation was associated with the presence of sputum purulence which is itself a measure of neutrophilic inflammation, with 84% of subjects with purulent sputum and 38% of those with mucoid sputum during an exacerbation cultured positive for a potentially pathogenic organism (Stockley 2000). Data from prospective studies which have assessed airway bacteria in the stable state and at subsequent exacerbation in the same patient are scant; one study which has followed a cohort of COPD patients with regular sputum sampling and bacterial analysis (Sethi 2000) has shown that observed changes in strain of airway bacterial isolates, which were associated with increased risk of exacerbation. However, although strain changes were associated with a 2-fold increase in risk of exacerbation, only one third of strain

changes observed coincided with an exacerbation. Therefore, strain change may be one of many factors which trigger exacerbations but it is likely that other factors play a role in modulating their severity or response to therapy.

The interplay between bacteria and host immune systems in COPD is complex. Whilst bacteria may colonise the lower airway in the stable state, due to defective host defences, there does appear to be an appropriate immune response to bacteria at exacerbation at least in terms of humoral mechanisms. Bakri and colleagues have shown that after exacerbation associated with *Moraxella catarrhalis*, new serum IgG antibodies and sputum IgA antibodies specific to the infecting strain developed after the majority of exacerbations (Bakri 2002).

Whilst bacterial pathogens may trigger a percentage of exacerbations, they may also play a role in modulating their severity or response to therapy. Different species of isolate have a differential inflammatory effect in the airway; for example, *Haemophilus influenzae* is associated with higher levels of airway inflammation than exacerbations not associated with this pathogen (Sethi 2000). Furthermore it is known that exacerbations are associated with higher airway bacterial loads (Monso 1995) than the stable state but to what degree changes in load modulate inflammatory and physiological responses at exacerbation requires investigation.

1.6.3 VIRAL INFECTIONS AND EXACERBATION

The role that respiratory viral infections play in exacerbations has been discussed and studied for some time. The marked seasonality of exacerbations has been known to both clinician and epidemiologists alike and the association of peak exacerbation incidence with the winter epidemic of respiratory viral illnesses is well described (Donaldson 2006).

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Early studies used viral culture and serology as methods of detection, but these are relatively insensitive and virus detection rates were consequently low (Gump 1976, Buscho 1978, Smith 1980). Therefore these early studies tended to underestimate the true incidence of viral infection due to the insensitivity of detection techniques, particularly as human rhinovirus the most commonly detected viral pathogen in modern studies is fastidious and difficult to culture on common media (Tyrell 1970). With the advent of PCR and other molecular diagnostic techniques used in more recent studies much higher detection rates for common viral pathogens during COPD exacerbations have been reported.

One of the first such studies using PCR was performed by our group (Seemungal 2001). This study confirmed that respiratory viruses can be detected by PCR in 39% of exacerbations with human rhinovirus the most commonly identified pathogen. This study used naso-pharyngeal sampling which has subsequently been shown to be a less sensitive technique than sputum analysis for detection of viruses (Seemungal 2000) suggesting the overall incidence of viral infection may be higher if the lower airway itself is sampled. The relative prevalence of viral pathogens detected is illustrated in Figure 1.3.

These findings have been confirmed in a population of COPD patients admitted to hospital, with viral pathogens identified in 56% of patients using a combination of sputum and nasal lavage techniques (Rohde 2003). Very similar results to the *Seemungal* study were found in further study by Greenberg et al. in the US (Greenberg 2005). It is likely that even using sensitive PCR techniques that observed detection rates are underestimating the true incidence of viral infection. The incidence of common cold symptoms was 65% in the *Seemungal* study and detection rates for viruses commonly associated with colds was less than 40%. One reason for

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this may be that patients do not present with exacerbations until the onset of lower airway symptoms and often some days past the onset of lower airway symptoms, therefore at a time when the peak viral load may have subsided.

These studies of association between viral detection and exacerbations does not prove causality however additional evidence that viruses are important in the aetiology of these events comes from the finding that virally associated exacerbations are more severe than non virally associated exacerbations. This has been shown in terms of exacerbation recovery time (Seemungal 2000) and airway inflammation in terms of higher IL6 (Seemungal 2001). Recently exposure models of HRV infection in COPD patients have been developed (Mallia 2005).

The evidence from studies which have focused on individual types of pathogens suggest that both bacteria and viruses may play a role in triggering and modulating the nature of COPD exacerbations; however, to date there have been very few studies which have looked at the combined effects of both type of pathogens in a particular individual at exacerbation.

In the normal population there is an established association between respiratory viral infection and consequent or secondary bacterial infection (van der Sluijs 2004, Lowenberg 1975.). The mechanisms for this association have been investigated and current evidence suggests that respiratory infection enables bacterial adherence to airway epithelial cells (Ishizuka 2003, Fainstain 1980).

Early epidemiological data in populations of COPD patients has suggested a similar association between the incidence of viral and bacterial infection at exacerbations (Smith 1976). Considering that the rates of isolation of bacterial pathogens at exacerbation run from 40 to 70% and PCR detection rates of viral pathogens conservatively estimate the incidence of infection to be 40-50%, it is highly

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probable that a significant proportion of exacerbations involve both types of pathogen. However, to date there has been no direct evidence of additive effects of viral and bacterial infections on airway inflammation or clinical outcomes in COPD.

One study which has attempted to study the effects of bacteria and viruses only sampled (Aaron 2001) 14 exacerbations; furthermore, detection of either bacteria or viruses occurred in only 3 of these events which is at odds with the remainder of the literature on this topic suggesting inadequate sampling or detection techniques for these pathogens. Clearly, further work is required to determine the effects that combined bacterial and viral infection have on the airway in exacerbations of COPD and this is one focus for this study.

FIGURE 1.3 DETECTION RATES OF COMMON RESPIRATORY VIRAL PATHOGENS AT EXACERBATIONS OF COPD EXACERBATIONS FROM EAST LONDON COPD COHORT.

SEEMUNGAL 2001.

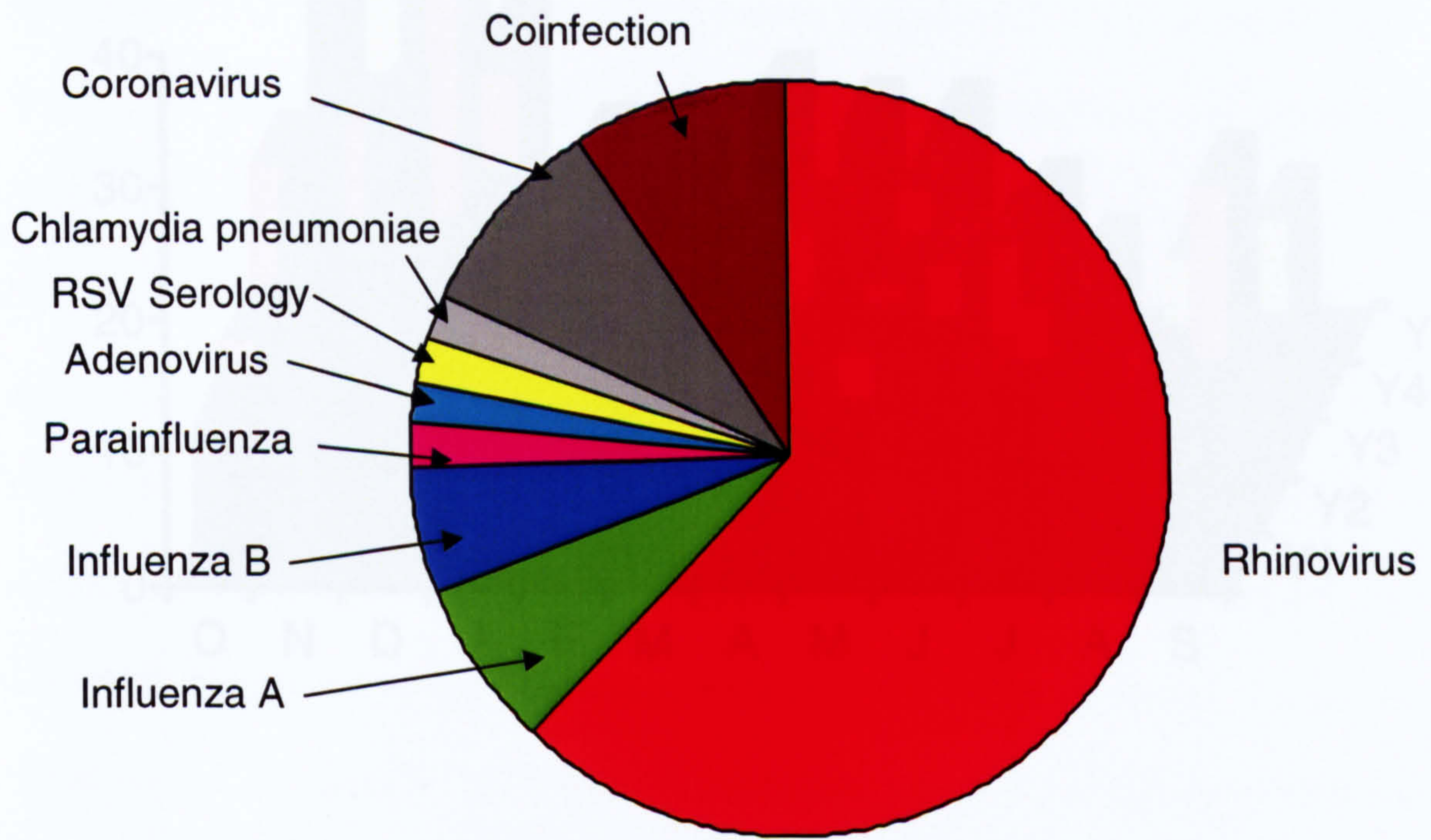
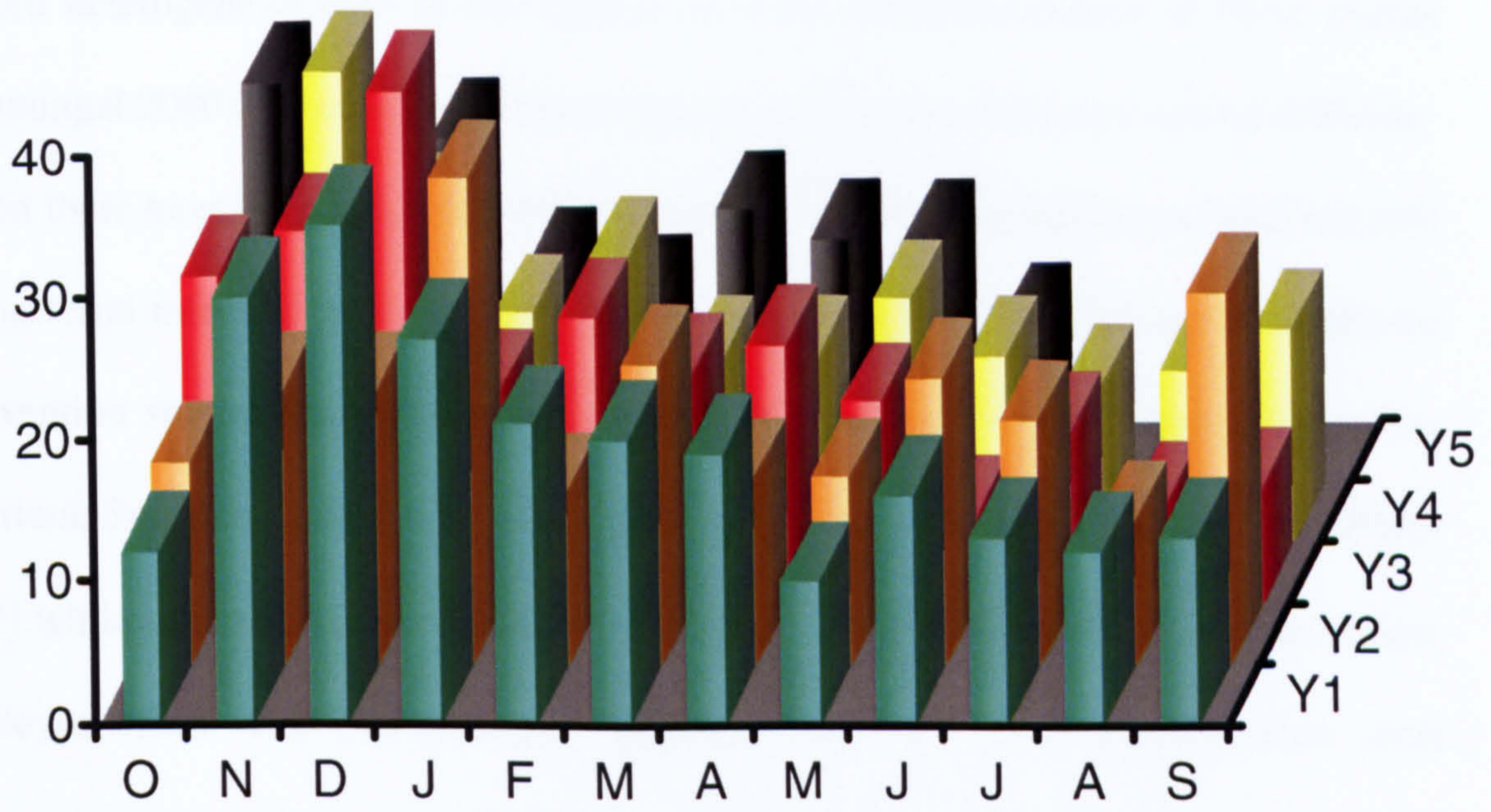


FIGURE 1.4 SEASONALITY OF EXACERBATIONS IN FIRST 5 YEARS OF EAST LONDON COPD COHORT STUDY.



1.6.4 EXACERBATION THERAPY AND IMPROVING OUTCOMES

Considering the spectrum of disease and the array of aetiologies that may trigger exacerbations or modulate their natural history it is no surprise that there is marked heterogeneity both in the clinical severity, and time course of these events (Seemungal 2000). Hence, predicting responses to prescribed therapy can be difficult. Whilst there have been a number of observational studies highlighting a potential role for bacterial infection in the pathogenesis of exacerbations, the findings of antibiotic intervention studies are not consistently positive and the value of their use remains uncertain. Some controlled trials of antibiotics have shown benefit (Berry 1960, Pines 1972) while others have not (Elmes 1965, Nicotra 1982). A recent Cochrane review of eleven trials with 917 patients suggests “that in COPD exacerbations with increased cough and sputum purulence antibiotics, regardless of choice, reduce the risk of short-term mortality by 77%, decrease the risk of treatment failure by 53% and the risk of sputum purulence by 44%; with a small increase in the risk of diarrhoea.”(Ram 2006). Clearly if bacterial infection was the only factor in triggering exacerbations a more marked benefit of antibiotic therapy would be expected. These somewhat qualified conclusions as to the efficacy of antibiotics in this setting would suggest that although bacterial infection plays a role, other host and environmental factors may be important in modulating the occurrence and severity of exacerbations.

Whilst current pharmacological therapies target the inflammation, infection and bronchoconstriction associated with exacerbations, their efficacy even in combination is limited and targeting therapies to presumed aetiologies of particular exacerbations remains largely a matter of clinical judgement. Furthermore the prescription of pharmacological treatments alone does not address the deleterious

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effects on functional performance, psychological and social factors caused by exacerbation.

Predicting outcome of exacerbations is therefore problematic, however patients with more severe disease at baseline are less likely to do well (Garcia-Aymerich 2001, Wouters 2004), particularly those with poor baseline functional capacity (Garcia-Aymerich 2003). Indeed, following severe (hypercapnic) exacerbations requiring hospital admission in over a third of patients may require mechanical ventilation, with an in-hospital mortality of over 10% (Connors 1996). Furthermore, the outcomes following admission remain poor with 80% of patients requiring inpatient management of a severe exacerbation readmitted within a year (Chu 2004). The outcome of less severe exacerbations managed in primary care is less well described. However, observational studies have revealed that a significant proportion of patients experiencing exacerbation symptoms fail to recover back to baseline levels (Seemungal 2000) and that poor outcomes are often related to failure to seek appropriate therapy (Wilkinson 2004).

Despite recognition of the poor prognosis associated with COPD exacerbations requiring hospitalisation and attempts to summarise the evidence base in management guidelines, there are indications that appropriate standards of care for acute exacerbations are not met and simple assessments to identify at risk patients not completed (Roberts 2001). There is obviously considerable scope to improve management of COPD exacerbations, and thus to improve outcomes. It is possible that by careful observation of responses to currently standard therapy that factors which may predict response to treatment can be identified and hence future delivery of treatment improved.

1.7 AIRWAY INFECTION IN STABLE DISEASE

1.7.1 LOWER AIRWAY BACTERIAL COLONISATION

Despite the inhalation of many millions of pathogenic organisms each day the lower airway of healthy individuals is sterile. The mechanisms that protect the airway from infection are complex and include innate and adaptive defences. The production of mucus by the respiratory epithelium and its upward transportation by the ciliary escalator both minimises the opportunity for bacterial adherence and removes pathogens and particulate matter from the lungs. Airway secretions contain a number of anti-bacterial agents such as lactoferrin and lysozyme which further embarrass pathogenic adhesion and growth (Boyton 2002).

Hence any process which affects these host defences can lead to persistence of bacteria in the lower airway, leading to activation of the innate and adaptive immune responses which can lead to airway damage as can the direct effects of bacteria on the epithelium. The integrity of the respiratory epithelium itself is vital to its protective role against infection. Bacteria can affect the airway epithelium directly and via the recruitment of neutrophils (Ras 1990, Noguera 2001) with release of excessive amounts of neutrophil derived proteases resulting in damage to airway epithelial cells (Wilson 1992).

Tobacco smoking directly impairs the innate immune defences of the airway by a number of mechanisms including slowing the ciliary escalator both by long term effects on ciliary clearance (Stanley 1986) and by stimulating mucus hypersecretion which can lead to uncoupling of the surface layer of mucus from that being transported by cilia beneath. Cigarette smoke also excites inflammatory and oxidative responses which can damage the airway and lead to bacterial persistence (van der Vaart 2004).

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Bacterial colonisation is a term used to describe the persistence of bacteria in the airway usually associated with a persisting weakness in airway defences. When colonisation develops a balance is established between bacterial growth and airway immune defences, and bacteria may persist in significant numbers with a low grade, chronic inflammatory response. Studies of subjects with COPD have established that both this form of bacterial colonisation is a common phenomenon (Monso 1995, Soler 1998) and that patients with more severe disease and active smokers are more at risk. The most commonly identified colonising bacteria in COPD patients are similar to those seen at exacerbation *Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pneumoniae*. These pathogens can exist in the lower airway in significant numbers (10^6 - 10^8 organisms ml^{-1}) in the diseased lung, with the patient remaining 'clinically stable'. Their persistence is reliant both on pre-existing impairment of lung defences and pathogen specific mechanisms to prevent eradication such as paralysis of cilia (Wilson 1984).

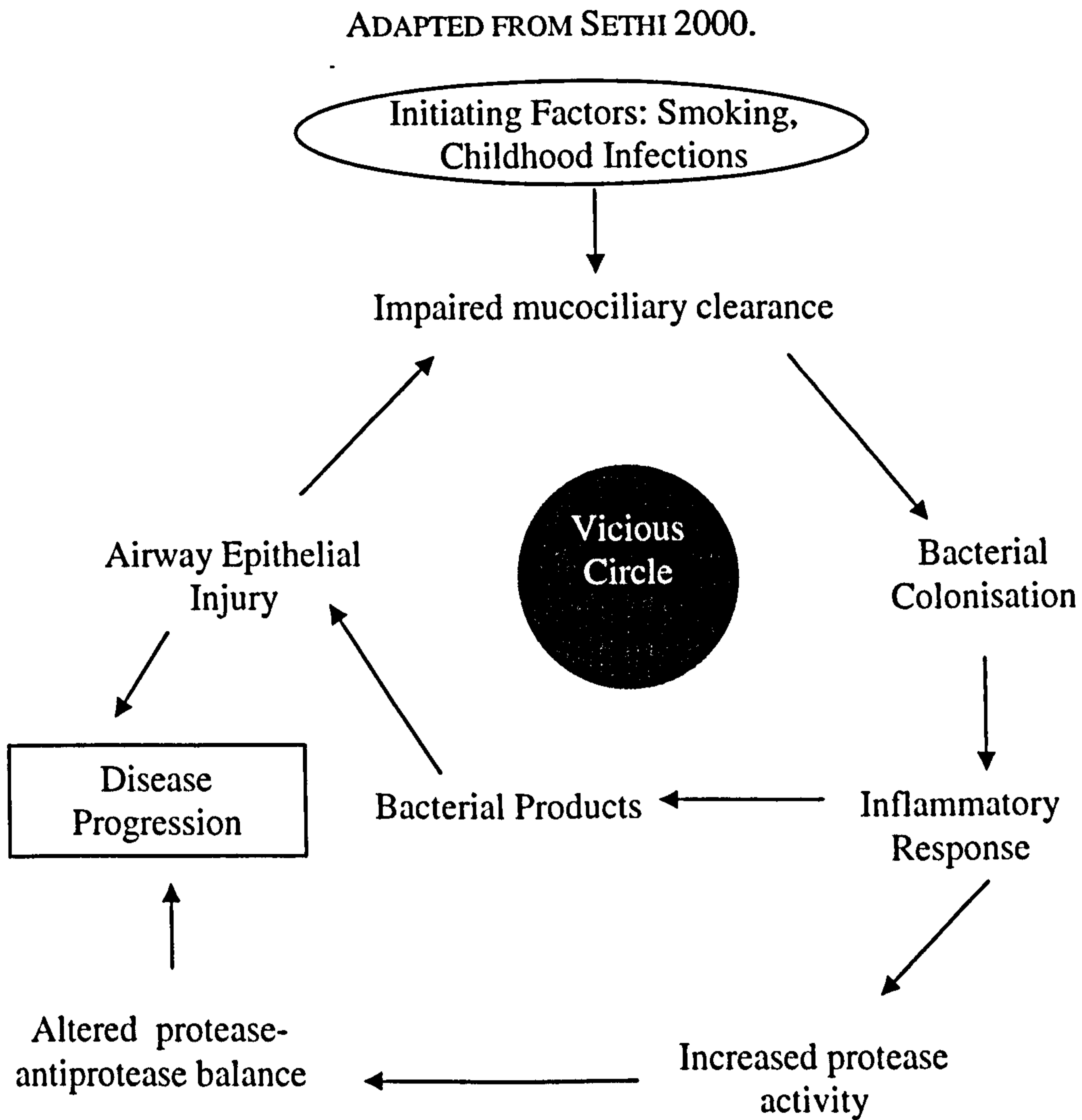
Studies using quantitative bacteriology have demonstrated a relationship between the airway bacterial load (number of bacteria per ml) and the degree of observed airway inflammation (Hill 2000, Patel 2002). This process of neutrophilic airway inflammation persists in COPD patients despite smoking cessation (Rutgers 2000), and is heightened in patients with worse lung function (Stanescu 1996) and as bacterial colonisation is also more marked in patients with more severe disease (Zalacain 1999), a causal relationship has been suggested. Corroborative evidence comes from animal models of chronic bacterial infection in the lung which have shown changes characteristic of those seen in COPD with patterns of inflammatory cells, cytokine expression and pathological changes to both airways and alveoli mimicking those seen in humans with the disease (Vernooy 2002). With differential

pro- inflammatory effects by different bacterial species and indeed strains observed (Adler 1986).

The 'vicious circle hypothesis' attempts to explain the proposed cyclical association between airway infection, subsequent inflammation epithelial cell and collateral lung tissue damage with further weakening of lung defenses with deleterious consequences on airway disease progression: see Figure 1.5 (Wilson 2002, Sethi 2001).

The progression of the observed airway immune response from predominately innate in mild disease to adaptive in more severe disease (Hogg 2004) suggests that responses to airway colonisation may modulate the pattern of airway inflammation to a greater extent as the disease progresses. However, studies to date have been largely cross-sectional in nature and have failed to demonstrate a direct association between bacterial colonisation of the lower airway and disease progression. Furthermore, intervention studies involving antibiotic eradication of airway bacteria are required to determine to what degree the relationship between lower airway bacterial colonisation, airway inflammation and declining lung function in patients with COPD is causal.

FIGURE 1.5 SCHEMATIC DIAGRAM OF THE ‘VICIOUS CIRCLE HYPOTHESIS’



1.7.2 CHRONIC RESPIRATORY VIRAL INFECTION IN COPD

The role of chronic respiratory viral infection in the pathobiology of COPD is less well established than that of bacteria. Whilst the effects of acute viral infections of the lower airway have been described at exacerbations of COPD, to what degree viruses persist in the lower airway when the subject with COPD is in the stable state and if present what their effects on the natural history of COPD are has yet to be described.

There is a small body of evidence to suggest that ‘chronic’ viral infection may play an important role in COPD. There is some evidence that chronic airway infection

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with adenovirus, may play a role in the development of airways obstruction, but studies have been cross-sectional in nature and relationships between viral infection and disease progression have not been demonstrated (Retamales 2001). Furthermore a role for RNA viruses in the pathogenesis of COPD has been little explored. However there is an established literature on the mechanisms underlying the persistence of RNA viruses in other disease states. The measles virus a member of the Paramyxoviridae family can persistently infect neuronal tissue and dendritic cells and can cause severe disease, such as sub acute sclerosing pan-encephalitis, several years after the acute infection (Litvak 1943, Rima 2005, Lamb 1996)

Respiratory syncytial virus (RSV) is a negative strand RNA virus, also of the Paramyxoviridae family, but of the genus Pneumovirus. It is the major cause of acute lower respiratory tract infections in young children, where it occurs in winter epidemics, but is rarely identified in the summer (Gardner 1968, Glazer 1973). Human RSV has no animal reservoir and the source of these winter epidemics remains unknown. Recent studies have also identified RSV as an important pathogen in the elderly and in adults with cardiopulmonary disease (Falsey 2005, Falsey 1995, Falsey 1996, Walsh 1989).

We have previously detected RSV in naso-pharyngeal samples from COPD patients in a cross sectional study (Seemungal 2001). Whilst the other commonly detected respiratory viral pathogens, such as human rhinovirus, were much more prevalent at exacerbation than in the stable state, RSV was detected at similar rates regardless of whether the patient was stable or having an exacerbation. (Seemungal 2001) Using quantitative PCR, these findings have been confirmed in stable COPD patients and at exacerbation, with low viral loads identified in comparison to those seen in children with seasonal bronchiolitis (Borg 2003). However longitudinal

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studies to determine whether RSV is able to persist in the lower airways of stable COPD patients are lacking and the clinical consequences of RSV persistence or that of other respiratory viruses have not been determined.

CONCLUSION

A number of studies of patients with COPD have identified a potential role for bacterial and viral infection in the aetiology of airway inflammation and exacerbation. The contribution of infective processes to disease progression or severity of exacerbations has not been fully described. The next chapter will summarise the specific aims of this study to investigate the role of airway infection in the natural history of COPD.

CHAPTER 2

HYPOTHESIS AND STUDY AIMS

HYPOTHESIS

Previous studies of airway infection in COPD have identified that respiratory bacteria can commonly be found in the lower airway of COPD patients in the stable state and that the presence of bacteria are linked to airway inflammation. However studies to date have been largely cross-sectional in design and have not informed on the associations between bacterial colonisation and disease progression in COPD. Similarly the role of respiratory viruses in stable disease has not been elucidated. Furthermore whilst both bacterial and viral pathogens have been detected at exacerbation there are few data on how these pathogens modulate the nature and severity of exacerbations or what the impact of co-infection may be.

Therefore the studies described in this thesis address the hypothesis that:

In patients with COPD lower airway infection with bacterial and viral pathogens is a common phenomenon, both in the stable state and at exacerbation which has important patho-physiological effects on the airway and hence impacts upon the natural history and clinical outcome of the disease.

HYPOTHESIS AND STUDY AIMS

The specific aims of the study were:

- To prospectively determine the prevalence and chronicity of lower airway bacterial colonisation in a population of patients with stable COPD.
- To determine the prevalence and chronicity of lower airway viral colonisation in a population of patients with stable COPD.
- To determine the relationships between lower airway bacterial and viral infection on airway inflammation and disease progression in these patients.
- To determine the relationships between lower airway infection and systemic inflammation.
- To determine the effects of lower airway bacterial and viral infection at exacerbations on the nature and severity of inflammation and clinical outcomes.

The initial studies identified that respiratory syncytial virus could be identified in airway samples of a sub-population of COPD patients in the stable state. Data from acute infections in children suggested that RSV infection modulates the nature of the immune response to viral infection and we therefore investigated:

- The relationship between RSV detection and responses of peripheral blood mononuclear cells to viral stimulation in an ex-vivo assay.

HYPOTHESIS AND STUDY AIMS

The analysis of the effects of airway infection and inflammation on exacerbation outcomes suggested that the timing of presentation for exacerbation treatment may have important effects on the response to therapy. An analysis of the dataset from the East London COPD cohort was performed to determine:

- The relationship between exacerbation reporting behaviour and clinical outcomes including exacerbation recovery, hospitalisation, and health related quality of life.

The clinical and laboratory methods used to address these specific aims are addressed in the next chapter.

CHAPTER 3

DESCRIPTION AND DISCUSSION OF METHODS USED

3.1 INTRODUCTION

In this chapter the methods used in the work contributing to this thesis are described. The methodology common to all aspects of the thesis are described in this chapter; clinical measurement, cohort follow up, sampling techniques, sample processing and detection of respiratory pathogens. The methodology peculiar to a particular sub-study will be described in the methods section of the appropriate chapter. The rationale for using the methods described and their limitations is discussed.

3.2 ETHICAL APPROVAL

The ethics committee of the East London and City Health Authority approved the studies described in this thesis. All patients gave their written, informed consent prior to recruitment into the study.

3.3 PATIENT RECRUITMENT AND CHARACTERISATION

Patients were recruited from the outpatients department of the London Chest Hospital in Bethnal Green, London, into the East London COPD cohort. This is a long

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running cohort of patients with largely moderate to severe COPD which was first recruited in 1995. A rolling recruitment programme has been used to maintain cohort size. Patients were under the overall clinical supervision of the principle investigator of the research group Professor Wedzicha who co-supervised this work.

Inclusion criteria for the study were as in previous East London cohort studies and first published in 1998 (Seemungal 1998):

1. A history of COPD with an FEV₁ (forced expiratory volume in 1s) less than 70% predicted for age, gender and height.
2. History of reversibility to beta-2 agonist of less than 15% and/or less than 200 ml (ATS Statement).
3. A history of at least 10 pack years of tobacco smoking.

Exclusion Criteria were:

1. Patients were excluded who had a past history of asthma,
2. A clinically significant history of bronchiectasis,
3. A history of carcinoma of the bronchus.
4. A history of significant respiratory disease other than COPD.
5. They were also excluded if they were unable to complete diary cards.

PATIENT CHARACTERISATION

The initial assessment consisted of history, physical examination and assessment of physiological parameters. Patients were asked about their daily stable symptoms: dyspnoea, sputum production, cough and wheeze. Details were obtained of whether patients had a history of cardiovascular or respiratory disease other than COPD and the dose and frequency of use of all drug medications. Upon successful

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recruitment each patient was given two identification labels: a study number and 4-letter code each of which was unique to that patient.

SMOKING HISTORY

Patients were classified as ex-smokers if they had not smoked in the past three months and as smokers if they smoked in the last three months. Where participants made their own cigarettes, half an ounce of tobacco was considered equal to one pack of commercial cigarettes (Jarad 1991).

3.4 PATIENT FOLLOW UP AND CLINICAL MEASUREMENTS

LUNG FUNCTION

At recruitment baseline measurements were made of height, weight, FEV₁, forced vital capacity (FVC) and peak expiratory flow rate (PEFR) by rolling seal spirometer (Sensor Medic Corp, Yorba Linda, USA). Predicted values were assessed using equations which took into account age, sex and height (Cotes 1978). Lung function measurements on all patients were taken between 0930 and 1130 hours, At least three serial spirometry readings were taken at each visit and the best performance recorded. FEV₁ reversibility to 400 µg inhaled salbutamol, and arterialised ear lobe blood gases (Pitkin, 1994) were also determined. Postero-anterior chest radiographs were assessed for evidence of other lung pathology.

All patients were shown how to record on daily diary cards, PEFR (Mini-Wright peak flow meter, Clement Clarke International Ltd., Harlow, UK) measured indoors after their morning medication they recorded the best performance of three sequential readings. At recruitment patients were instructed how to record any increase over their chronic (stable) respiratory symptoms during the last 24 hours and to record these on their diary

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card. These symptoms are classified into major and minor a symptom developed from the original work of Anthonisen (Anthonisen 1987) and form the basis of the validated symptom based diagnostic criteria for exacerbations used in all previous studies from the East London COPD group. These are as follows:

(a) 'major' symptoms

- increased dyspnoea,
- increased sputum purulence,
- increased sputum amount

(b) 'minor' symptoms

- nasal discharge/congestion,
- increased wheeze,
- sore throat,
- increased cough

When well or stable the patients were instructed not to record any of the symptom letters on the diary, but when they perceived an increase over their normal, stable condition in symptoms they noted the corresponding letter code. There was no numerical grading system for each individual symptom and patients recorded symptom letters if a symptom was perceived as worse eg dyspnea or of new onset eg. a sore throat (as the latter is not usually present). For example a patient notes that he is developing symptoms of a Cold, and notes C, the next day he is more breathless and still has cold symptoms; recording A and C. The patient experienced deterioration in his symptoms and recorded this change as present or absent for each individual symptom above. Patients therefore judged if a particular symptom is worse than their own perceived normal level. The validated diagnostic criteria of the presence of at least two major or one major and one minor symptom for at least two days was then

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used in the analysis of diary card record. Patients also recorded the time they spent outdoors on a daily basis as a measure of activity.

Patients were reviewed every three months when stable, in the study clinic. Here a review of diaries was performed, a history taken inclusive of any change in medication, smoking behaviour, and of previous exacerbations not reported to the study team. Lung function was recorded before sampling was undertaken. Spirometric measurements were taken 1 hour after the patients' usual bronchodilator medication was taken inclusive of 200 ug of salbutamol via metered dose inhaler. Lung function measurements on all patients were taken between 0930 and 1130 hours, At least three serial spirometry readings were taken at each visit and the best performance recorded.

3.5 EXACERBATIONS

REPORTING EXACERBATIONS

Patients were asked to contact the clinical team by telephone to arrange a clinic visit if their symptoms worsened. Patients were then seen within 48 hours of the call in the outpatient's department of the London Chest Hospital. If a patient was seen acutely at clinic then this was called a reported exacerbation visit. If however the exacerbation was diagnosed from the history or the diary card retrospectively during subsequent follow up then this was called an unreported exacerbation.

DIAGNOSIS OF EXACERBATION

Exacerbations were identified by symptoms recorded on the diary cards, or from the history when patients presented to the physician:

1. any two 'major' symptoms or one 'major' and one 'minor' symptom
2. on two consecutive days

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3. the first of the two consecutive days was taken as the day of onset of exacerbation
4. symptoms present continuously for more than 5 days prior to the possible onset of an exacerbation were discounted.

EXACERBATION TREATMENT

The prescription of treatment for all exacerbations was at the discretion of the attending physician and included prednisolone and/or antibiotic therapy. Exacerbation treatment in the study therefore represented the usual practice of the study physician attending the patient or that of the primary care physician in the case of exacerbations treated by the patients' GP. Records were kept of the date of initiation and type of treatment prescribed to patients for each exacerbation, both at our clinic and also therapy prescribed by the patient's primary care physician.

SEVERITY OF EXACERBATION IN TERMS OF SYMPTOMS EXPERIENCED

The calculation of exacerbation severity in terms of symptoms experienced was assessed by counting up the number of individual symptoms recorded at the onset of an exacerbation eg. A,B1,C= 3 symptoms or A,B1,B2,D, E1,E2= 6 symptoms etc. Symptom severity at exacerbation was calculated as the difference in total daily symptom count at baseline and at exacerbation onset but not from the severity of individual symptoms themselves as these were only recorded as increased (=1) or not (=0).

EXACERBATION TOTAL RECOVERY TIME

This was calculated as the time from exacerbation onset for the 3-day moving average of the exacerbation daily symptom count to return to this baseline (Seemungal 2000). The use of a three-day moving average minimised the effect of day to day symptom variation without biasing the results. Treated recovery time was taken as the time between consultation and recovery. Exacerbation non-recovery was taken as recovery taking longer than 35 days.

3.6 SPUTUM SAMPLING AND PROCESSING

When sputum samples were required these were collected after lung function measurement, patients were asked to blow their noses and rinse their mouths out with water before expectorating sputum into a sterile pot to minimise oro-pharyngeal contamination. Patients unable to produce a sample of sputum spontaneously underwent sputum induction as described below. First, oxygen saturation was recorded (Minolta Pulsox 7; DeVilbiss Healthcare, Middlesex, UK). This was continuously monitored during nebulisation with 3% saline using the DeVilbiss UltraNeb2000 ultrasonic nebuliser. This nebuliser produced an aerosol output of approximately 2 ml/minute with a mean particle size of 0.5–5 µm in diameter. After 7 minutes of nebulization measurement spirometry was performed and nebulisation continued if the FEV₁ had not fallen by more than 20%. After a further 7 minutes the measurements were repeated and the procedure stopped. The collected sputum sample was separated from saliva by macroscopic examination using disposable plastic forceps and divided into two, half was taken for quantitative bacterial culture and the remainder processed and analyzed for inflammatory cytokines. These techniques had

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been established and validated during previous studies by the group and are discussed later in this chapter.

SPUTUM PROCESSING

Sputum was processed within 2 hours of collection. Sputum samples containing < 25 squamous epithelial cells per low power field and > 25 leukocytes per high powered were accepted following processing. The weight of the total sample was recorded. The sputum was then separated from contaminating saliva by macroscopic examination using a pair of disposable plastic forceps. The selected portion of the sputum was placed in a pre-weighed tube and the weight of the selected portion of the sputum recorded. The sputum was then mixed with four times its weight of freshly prepared 0.1% dithiothreitol solution made by mixing the dithiothreitol powder with Hank's balanced salt solution (HBSS). This was vortexed for 15 seconds and then rocked for 15 minutes. A weight of HBSS equal to that of the sputum plus dithiothreitol was then added and the whole mixture was rocked for another five minutes. The suspension was filtered through 50 μ m nylon gauze to remove mucus and debris without removing any of the cells and then centrifuged at 790g (2000 rpm) for 10 minutes. This resulted in the formation of a cell pellet and a supernatant solution. The supernatant was decanted off and stored at -70°C for future analysis and the cell pellet was resuspended in 400-3200 μ l (depending on macroscopic estimation of the size of the cell pellet) of HBSS. The total cell count was determined with a Neubauer haemocytometer using the trypan blue exclusion method to determine cell viability, blue cells being counted as non-viable. The absolute number of non-squamous cells per gram of the original sputum sample was determined and the percentage of viable and non-viable cells obtained. The cell suspension was then

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mixed with HBSS to obtain a count of $0.6-1.0 \times 10^6$ cells/ml of the suspension and cytopins were made using a Cytotek cytocentrifuge. The cytopin slides were stained with Diff-Quik to obtain differential cell counts made by counting 400 cells per slide (Popov 1994, Pizzichini 1996). Cytokines were measured in the supernatant samples using a quantitative sandwich immunoassay (R&D Systems Europe, Abingdon, Oxon, UK) and expressed as pg/ml of the supernatant. The supernatant itself is a tenfold dilution by weight of the original sputum sample minus cells (Bhowmik 2000).

3.7 BLOOD SAMPLING AND PROCESSING

Venous blood was taken from patients at clinic visits using standard techniques, samples were taken for storage of serum and plasma samples. After phlebotomy blood was stored on ice until centrifugation at 4°C for ten minutes. Supernatant was removed and divided into aliquots which were stored at -80°C for subsequent analysis. The specific methodology for extracting and storing PBMCs from whole blood is described later in the thesis.

3.8 SPUTUM BACTERIOLOGY

Part of the sputum sample was sent for quantitative analysis of bacterial colonisation, according to our previous published methodology Samples were processed; ten fold serial dilutions of the homogenized sample were made in Brain Heart infusion broth and 100 μl aliquots plated out onto the surface of a range of different media including blood agar, chocolate agar, MacConkey agar and cysteine lactose electrolyte deficient agar. These were incubated for 18h at 37°C in an atmosphere of air + 5% CO_2 . After incubation, bacterial colonies were diluted and counted and sub-cultured for identification by standard methods (Barrow 1993). *H.*

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influenzae and *H parainfluenzae* were identified and differentiated by their growth patterns on peptone agar on which discs containing NAD or haemin were placed (Unipath, UK). The number of colony forming units/g of sputum were calculated from the number of colonies obtained and the dilution of the sample (Barrow 1993, Patel 2002). Potentially pathogenic micro-organisms (PPM) are bacteria known to be common pathogens of the respiratory tract in subjects with COPD (*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Moraxella catarrhalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and other gram negative enteric bacteria (GNEB).

3.9 VIRUS DETECTION: PCR TECHNIQUES

RNA EXTRACTION AND REVERSE TRANSCRIPTION

Total RNA was extracted from homogenised sputum samples and positive and negative viral controls using a standard silica-gel based kit (Qiagen,UK). Reverse transcription was performed using 1.25ug random hexamers (Promega, USA) and 6.5ul nuclease-free water to each eluted sample, heated to 70c for 10 minutes and quenched at 0c. Followed by addition of 6ul of nuclease-free water (Promega, USA), 10 ul of 5x RT Buffer, 5ul 0.1M DTT, 1.25ul 100mM dNTPs and 400U Superscript RNase H- reverse transcriptase (Invitrogen, USA) in a total volume of 95ul incubated at 37c for one hour. Qualitative PCR was performed using 5ul cDNA solution and hotstart taq polymerase in a mix containing x10 PCR buffer, 25mM MgCl₂, 200uM dNTPs, 294 u/ml Hot Star Taq DNA Polymerase (Qiagen, UK). For all qualitative PCRs products were imaged using 1% agarose gel electrophoresis with ethidium bromide staining and reference DNA ladder (Sigma, USA).

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HUMAN RHINOVIRUS

This was detected according to our previous methodology in our study of viruses at COPD exacerbations (Seemungal 2001). 0.5ul of each of HRV primers at 25mM OL 26 (GCA CTT CTG TTT CCC C) and OL27 (CGG ACA CCC AAA GTA G) (Oswell, UK), 25ul HotStar Taq MasterMix (Qiagen, UK) and 19ul nuclease-free water (Promega, USA) to a final volume of 50ul. PCR was performed using a Flexigene thermal cycler (Techne, UK), product size 380 bp. All the serotypes of picornaviruses were detected with this PCR methodology and subsequent sequencing of PCR product confirmed that HRV was detected.

RESPIRATORY SYNCYTIAL VIRUS (RSV)

A nested PCR was used similar to that used in our previous studies (Seemungal 2001, O'Donnell 1988) using in the first round 5ul of cDNA, 0.5ul 1st round 25 mM primer K1 (ATG TCA CGA AGG AAT CCT TGC), and 0.5ul 25mM primer K2 (TAG CTC TTC ATT GTC CCT CAG C) with 25 ul HotStar Taq Master Mix (Promega, USA) and 19ul nuclease-free water (Promega, USA) to a final volume of 50ul. PCR was performed using a Flexigene thermal cycler (Techne (Cambridge) UK), 1st round product size 360 bp. Second round; 5ul 1st round product 0.5ul 2nd round 25 mM primer K3 (GAG GTC ATT GCT TAA ATG G), and 0.5ul 25mM primer K4 (GCA ACA CAT GCT GAT TGT) with 25 ul HotStar Taq Master Mix (Promega, USA) and 19ul nuclease-free water (Promega, USA) to a final volume of 50ul. Final product 259 bp. (van Elden 2003).

Both RSV and HRV PCR detection assays were highly sensitive and could detect as few as 1×10^2 viral particles per ml of sputum. Figure 3.1.

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DETECTION OF OTHER VIRUSES

These were detected using established qualitative PCR techniques in the department of Professor Sebastian Johnston, NHLI, Imperial College, London.

CORONAVIRUS Sputum was analysed using a nested PCR method with coronavirus-specific outer and inner primers for the two coronavirus types 229E and OC43. The copy DNA after reverse transcription will have two rounds of PCR using primers 0637 and 0647 followed by 1A and 1B. (van Elden 2003).

INFLUENZA A AND B were detected with an established PCR detection method that has been published (Templeton 2004).

PARAINFLUENZA PCR was performed according to a recently published protocol (Templeton 2004).

METAPNEUMOVIRUS PCR was used to detect metapneumovirus according to a protocol that has been recently published (Maertzdorf 2004).

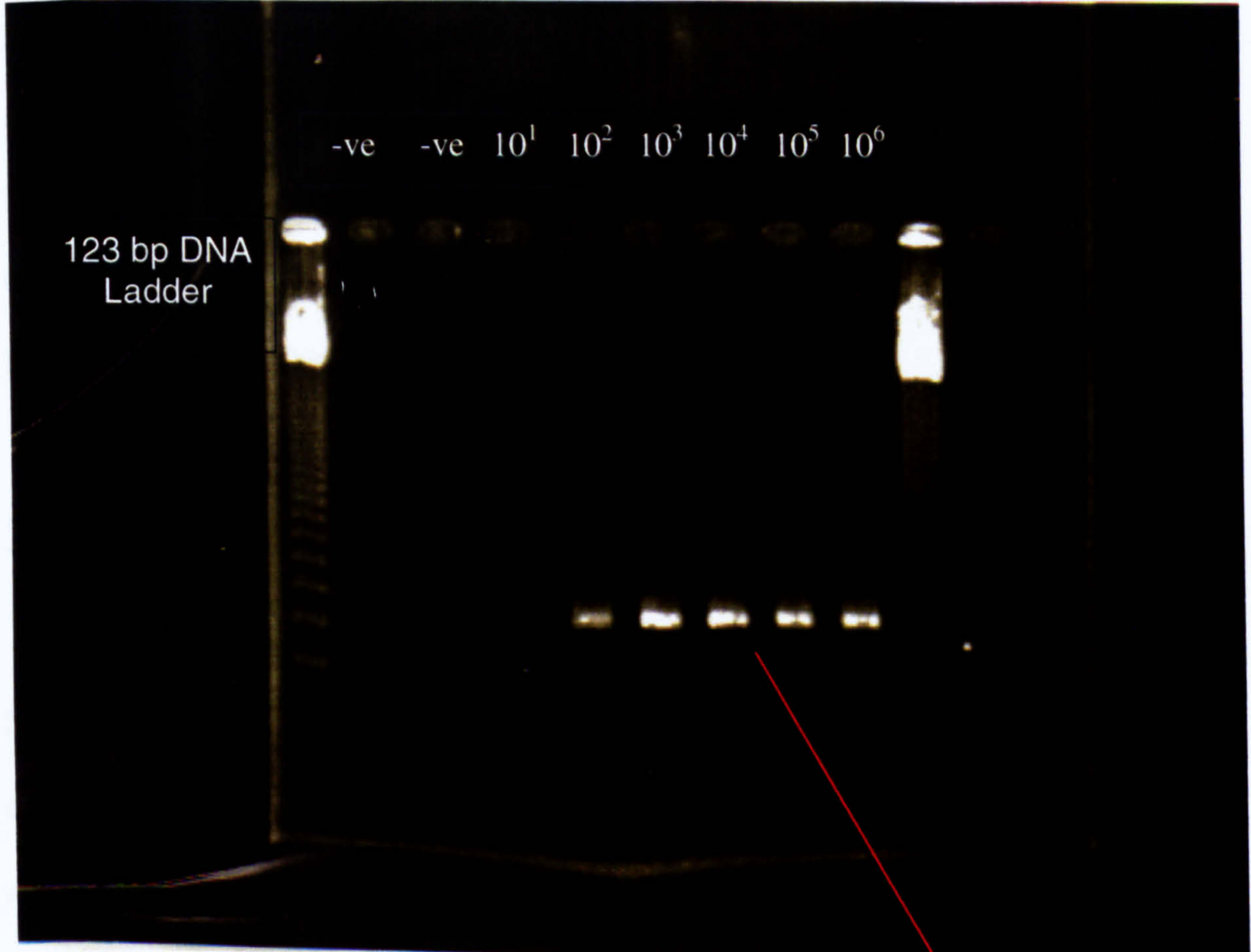
ADENOVIRUS A PCR method was used, employing hexon-specific primers that have been shown to detect all 51 known adenovirus serotypes. The method has been adapted from a published technique (Heim 2003) and has been fully established and validated.

CHLAMYDIA PNEUMONIA and MYCOPLASMA PNEUMONIA were detected using clinically validated PCR protocols (Cunningham 1998).

PCR PRODUCT SEQUENCING In order to confirm and validate the PCR detection assay's results sequencing of both strands of selected positive PCR products using standard methods was performed and compared to reference strains using Clone Manager (Scientific and Educational Software, UK) to align the sequences.

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PLATE 3.1 ETHIDIUM BROMIDE STAINED AGAROSE GEL REVEALING PCR DETECTION (2ND ROUND) FOR RSV AT 259 BP ALONG WITH DNA LADDER AND NEGATIVE CONTROLS.



Positive results

3.12 STATISTICAL ANALYSIS

Normally distributed data are reported by mean (SD) and skewed data by median (interquartile range (IQR)). Correlations were determined using the Pearson or Spearman correlation coefficient (two-tailed). Categorical variables with normal distributions were compared by χ^2 test, whereas those with non-normal distributions

3.10 MEASURING AIRWAY AND SYSTEMIC INFLAMMATION

Sputum and plasma inflammatory cytokines and chemokine were measured in the supernatant samples using a quantitative sandwich immunoassay (R&D Systems Europe, Abingdon, Oxon, UK) and expressed as pg/ml or ng/ml of the supernatant. Sputum myeloperoxidase (MPO) was also measured using ELISA (Calbiochem, Nottingham, UK). All assays had been previously validated during previous studies in our department (Bhowmik 2000, Bhowmik 2002, Seemungal 2000, Wedzicha 2000, Hurst 2005, Hurst 2006).

3.11 MEASURING HEALTH RELATED QUALITY OF LIFE

Indices of Health Related Quality Of Life (HRQOL) were obtained using the St. Georges Respiratory Questionnaire (SGRQ) (Jones 1991, Jones 1992). Three component indices were calculated using empirically derived weightings: Symptom, Activity and Impact scores from which a Total score was computed. Scores vary from 0 (no disability) to 100 (maximum disability). Patients were also asked to complete the modified Medical Research Council (MRC) dyspnoea scale questionnaire. These questionnaires were completed by the patients annually at clinic visit without directions from the researchers.

3.12 STATISTICAL ANALYSIS

Normally distributed data are reported by means (SDs) and skewed data by medians (interquartile range [IQR]). Correlations were assessed using the Pearson or Spearman correlation coefficient (two tailed). Continuous variables with normal distributions were compared by *t*-test, whereas those with non-normal distributions

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were compared by the Mann-Whitney U or Wilcoxon signed ranks test. During the analysis, patients were divided into groups dependent on exacerbation frequency during the study. Patients with an exacerbation frequency that was higher or lower than the median were termed "frequent" or "infrequent" exacerbators, respectively (Seemungal 1998); p values of 0.05 or less were regarded as significant. The SPSS version 10.0 (SPSS Chicago, IL) statistical package and STATA-5 software (Stata Corporation, Texas, USA) were used for data analysis. Further statistical analyses are described in the appropriate chapters.

3.13 DISCUSSION OF STUDY METHODOLOGY

PATIENT SELECTION

The inclusion and exclusion criteria for the cohort and hence the studies described in this thesis were developed and established during the conception of the study. The specific criteria were developed in order to ensure that the patient cohort was representative of a group of patients with COPD that could exist in any clinic population whilst excluding those patients in whom other respiratory diseases may impact on the nature and interpretation of clinical and laboratory findings.

COPD is a common condition and is likely therefore to co-exist with other pulmonary pathologies in any population. Indeed it has been suggested that certain other lung pathologies may predispose the smoker to COPD. For example the entry criteria into the study preclude a previous history of asthma. There has been continued debate in the literature about the importance of asthma or airway hyper-reactivity in the aetiology of COPD. The 'Dutch hypothesis' supports a relationship of asthma with COPD (Orie 1961). The evidence for this comes from a number of disparate studies; bronchial hyper-reactivity, blood eosinophilia, and serum IgE levels are higher in smokers compared to nonsmokers and peripheral eosinophilia was also shown to correlate with airway obstruction (Burrows 1980). Eosinophilic inflammation of the airways in patients thus has been clearly defined in a subset of COPD patients. This is also the group of patients who are likely to show reversibility of airway obstruction with therapy with corticosteroids (Brightling 2000). It is possible that by excluding patients with marked airway reversibility therefore that we are in fact biasing the study population by excluding the phenotype described above. Indeed there is evidence that reversibility testing in COPD is poorly reproducible (Calverley 2003),

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however when considering the role of airway infection in COPD it is important to study disease population in which the contribution of other pulmonary pathologies is minimised as the prevalence and responses to pathogen are likely to differ between subjects with COPD and other diseases (Message 2004). Furthermore, patients were not excluded from the study on grounds of non-pulmonary co-morbidities as is the case in many intervention studies. Co-morbidities are common in patients with COPD and impact significantly on clinical outcomes (Holguin 2005) and may impact upon upon the natural history of COPD. Therefore, the data collected and conclusions made by this study can be considered generalisable to a general COPD population of similar disease severity but additional studies of patients with significant concurrent pulmonary conditions other than COPD are indicated.

TECHNIQUES OF AIRWAY SAMPLING AND SAMPLE PROCESSING

Any clinical study which aims to determine the role of lower airway infection must utilise techniques which enable the investigator to determine not only which pathogens are present in the lower airway but also how many are present, and what the effects of these pathogens on measured indices of airway inflammation are as well as the clinical and physiological consequences of infection. For this purpose, the ability to sample the lower airway to inform the researcher as to the nature of infection and inflammation in the lung is fundamental. Furthermore, the sampling techniques used must be safe, well tolerated and provide reliable and reproducible data which if it is not completely representative of the in vivo airway at least provides a close approximation.

There is now a body of work which has established sputum sampling and processing as an appropriate method to achieve the aims described above. In order to

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provide meaningful data for clinical studies, all aspects of sampling and sample processing must be optimised, including sample collection, sample processing and techniques of laboratory analysis.

SPUTUM INDUCTION TECHNIQUES

Whilst a significant proportion of COPD patients expectorate sputum on a regular basis a substantial proportion do not or only do so intermittently. The use of sputum induction techniques to obtain lower airway samples have been standardised and can aid the researcher in obtaining sputum specimens from patients with an array of conditions including COPD (Pin 1992). In the ERS guidelines on sputum induction published in 2002 (Djukanovic 2002), the section on sputum induction states that ‘The aim of sputum induction is to collect an adequate sample of secretions from lower airways in subjects who do not produce sputum spontaneously in order to study the features of airway inflammation in asthma and other respiratory disorders’ (Paggiaro 2002)

The technique of sputum induction has been well validated since the early finding that inhalation of small amounts of hypertonic saline may stimulate the production of airway secretions which can be collected following expectoration. The mechanisms underlying the production of sputum in response to inhalation of saline are not fully understood. However, a number of studies have confirmed that this technique has the potential to produce high quality, reproducible samples for analysis from patients with COPD (Peleman 1999, Keatings 1996).

When considering what information is being provided by the analysis of induced sputum it is necessary also to determine which areas of the lung are providing the infective or inflammatory ‘signature’ contained within the sputum

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sample obtained. A number of studies have compared the results of analysis of sputum samples with those of bronchoscopic sampling including washes, bronchoalveolar lavage and biopsies (Lensmar 1998, Pizzichini 1998, Grootendorst 1997, Fahy 1995, Keatings 1995). These studies performed in asthmatic subjects and demonstrated variable relationships between eosinophil and neutrophil percentages between induced sputum and bronchial wash and BAL fluid. Furthermore sputum eosinophil counts were related to the number of eosinophils in bronchial biopsies. This suggests that in asthma, induced sputum techniques can produce airway samples that are representative of the inflammatory processes detectable by other means, however sputum from a small study of chronic bronchitics produced differing results, with closer correlations between cell numbers from different sample types only seen in exacerbating bronchitis. (Maestrelli 1997). In COPD, neutrophils were the predominant cell type in induced sputum samples from COPD subjects but not from bronchoscopic specimens. Furthermore sputum differential cell counts were not significantly related to those in wash or BAL. However levels of soluble markers such as eosinophil cationic protein (ECP) and interleukin-8 (IL-8) did relate to those seen in bronchoscopically derived specimens. (Rutgers 2000). Hence induced sputum would appear to be sampling a different airway component than bronchoscopic techniques but results between techniques are comparable especially with respect to measurement of soluble inflammatory markers.

SPONTANEOUS SPUTUM; COMPARISONS WITH INDUCED SPUTUM

A significant proportion of patients with COPD spontaneously expectorate sputum on a regular basis and an even higher proportion do so at acute exacerbation (Seemungal 2000). Early studies comparing the composition of spontaneous and

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induced sputum in asthmatics demonstrated that both sample types contained similar cellular and cytokine profiles, however cell viability was lower in spontaneous samples (Pizzichini 1996, Bartoli 2002). This finding has been reproduced in a comparative study of spontaneous and induced sputum in patients from the East London COPD cohort (Bhowmik 1998). Here in a population of patients with moderate to severe COPD, FEV₁, 38.2% predicted, that is nearly identical to those sampled in the studies described in the thesis, Bhowmik demonstrated that there was no significant difference in total and differential cell counts or in interleukin 8 levels between spontaneous and induced sputum samples. However, cell viability was lower in the spontaneous samples. One may conclude from this study that the use of spontaneous sputum is highly comparable to that of induced sputum for the measurement of cytokines, chemokines and cellular components but not for assays which rely upon cell viability.

SPUTUM SAMPLING AND THE STUDY OF AIRWAY INFECTION

A key feature of this thesis is the study of airway pathogens both viral and bacterial and their effects on airway inflammation and clinical outcomes. Hence the techniques utilised to sample the airway to assess airway inflammation must also be appropriate for assessing airway infection. A body of work exists which has validated the use of sputum in this role exists. A number of studies over a number of years that have assessed airway bacterial infection in COPD phenotypes have shown that sputum samples can be useful in identifying and quantifying airway pathogens (Brown 1954, Lapinski 1964, Smith 1976, Bartmann 1984, Hill 2000, Sethi 2002, Patel 2001). A number of these studies have used spontaneous sputum samples, or a combination of both induced and spontaneous techniques. Previous work by our

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group has shown that both sampling techniques are comparable in estimating airway bacterial load and type (Patel 2000). Indeed obtaining sputum samples from patients with severe COPD, particularly during acute exacerbations with induction techniques can be difficult (see below) and therefore the utility of including analysis of spontaneous sputum samples allows not only additional sampling points to be assessed but also allows more severe patients with spontaneous sputum production to be included in the study.

Any sampling technique of lower airway pathogens is, because of the relative anatomy, open to possible contamination by pathogens colonising or infecting the upper airway. Some techniques are able to minimise this by directly removing samples from the lower airway, for example bronchoscopic techniques, especially with the use of the protected specimen brush. However, bronchoscopies are not without associated risk to patients and are not suitable for long term cohort studies where repeated sampling is required or larger numbers of patients are to be followed up by a single researcher or during acute exacerbations. Contamination of sputum which must be expectorated through the upper airway by bacteria present in the nasopharynx can be minimised but not prevented by the sampling techniques utilised; including nose blowing and mouth rinsing before expectoration. However, to significantly affect the results of sputum studies, large loads of upper airway bacteria would need to contaminate the lower airway samples obtained. Work involving direct sampling of upper and lower airways in our cohort has shown that the airway bacterial load in sputum both in the stable state and at exacerbation is several orders of magnitude higher than that seen in the upper airway and that the prevalence of potentially pathogenic micro-organisms is also significantly greater in the lower airway (Hurst 2005, Hurst 2006). Therefore, the effect size of upper airway

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contamination using sputum techniques is likely to be minimal in terms of quantifiable parameters of infection but may impact on qualitative assays of airway infection such as viral PCR detection.

SAFETY AND TOLERABILITY OF SPUTUM INDUCTION

The use of sputum sampling in studies of pulmonary diseases has numerous advantages, not least in the tolerability and convenience of this approach in comparison to more invasive techniques. However, sputum induction, although generally well tolerated by COPD patients can cause adverse side effects and it is of vital importance that steps are taken to minimise risks to patients. Published guidelines on this technique highlight the importance of patient selection, premedication and careful monitoring during induction. The ERS document highlights the evidence that sputum induction is safe and well tolerated in asthma and mild to moderate COPD. (Wong 1997, de la Fuente 1998, Hunter 1999, Twaddell 1996, Grootendorst 1999, Fahy 1993, Vlachos-Mayer 2000).

Whilst some studies in patients with more severe COPD have shown the technique to be safe and well tolerated, (Pizzichini 1998), it is well recognised that inhalation of nebulised saline can cause significant broncho-constriction, particularly at concentrations above 3% (Bickerman 1958). Pre-medication with inhaled Beta 2 agonists can limit the induction of broncho-constriction, and is now recommended by established guidelines (Pizzichini 2002). However, it is apparent that bronchodilator therapy does not completely prevent broncho-constriction. In a previous study of patients from our cohort a small but significant fall in forced expiratory volume in one second (FEV₁) of 0.098 (0.111)l and in forced vital capacity (FVC) was 0.247 (0.233)l was observed (Bhowmik 1998). In other studies while the procedure

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was well tolerated, the mean change from prebronchodilator FEV1 during sputum induction was 8.5%, and from postbronchodilator FEV1 was 10.7%, with a small number of the patients (11%) experiencing a fall in FEV1 from the prebronchodilator baseline of >20%, and a further 10 (36%) a fall of 10% (Ryttila 2000).

Therefore in patients with very severe disease (FEV1 < 30% predicted) or during exacerbations when airway hyper-responsiveness is likely to be up-regulated changes of the magnitude seen during induction techniques can carry some risks and close patient monitoring is mandatory. A minimum standard would include continuous monitoring of oxygen saturation, repeated spirometry at regular intervals and close clinical supervision (Pizzichini 2002). Indeed during the prospective follow up and repeated sampling of a cohort study, particular individuals can be identified who react adversely to induction and excluded from further sampling in this manner. The induction techniques utilised in this study and outlined in the methods section above, were established and validated by a series of clinical researchers in previous studies in this group. They have proven to be safe, well tolerated and have by continued use of established protocols produced reproducible results which have allowed longitudinal follow up and analysis of cohort data over a number of years.

SPUTUM PROCESSING METHODS

A standardised methodology for processing sputum is necessary in order to produce samples which are suitable for subsequent laboratory analysis, to ensure maximal reproducibility and to exclude samples of poor quality. The processing techniques used in this study have been established and validated in a number of previous studies by our group (Bhowmik 1998, Bhowmik 2000, Seemungal 2000, Patel 2002, Patel 2004, Hurst 2004, Donaldson 2005, Hurst 2005). Indeed in order for

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data from this cohort study to be consistent from one investigator to the next the techniques of sampling and processing have been standardised to ensure the validity of longitudinal analysis. This is reliant upon the reproducibility of techniques and of sample quality over time.

There are two established methods for processing sputum for analysis, the first is reliant upon removing sputum plugs from the sample to minimise the dilutional effects of saliva on the sample the selected sputum technique (Pizzichini 1996). This was performed in this study using macroscopic examination and disposable plastic forceps. The alternative technique involves processing the entire expectorate, comprising sputum plus variable amounts of saliva, which may lead to variable dilutional effects of saliva on the sample and increases in squamous cell contamination (Efthymiadis 1995).

Once sputum was selected it was divided into a portion for bacterial analysis, cell counts and preservation of the liquid phase for later analysis of mediators. The reasons for the parallel processing of samples lie in the realisation that DTT itself can affect the measurement of mediators studied downstream of processing. Indeed whilst use of DTT can improve cytospin quality (Louis 1999) it may also reduce the detectable concentration of markers such as TNF α , LTB $_4$ and MPO (Woolhouse 2002). Therefore, two sub-samples were processed: one with DTT and an additional one with PBS and the use of subsequent aliquots dependent on the analysis required.

The use of sputum to assess pulmonary disease processes will only give one aspect of the processes involved in the pathology of the disease, namely that involving the airway lumen. Indeed, sputum is not produced in the smallest distal airways nor in the alveolar compartment where significant pathology develops in patients with COPD (Hogg 2004). The techniques developed and utilised in this study for the

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assessment of airway inflammation and infection in patients with moderate to severe COPD are however safe, well tolerated and suitable for the repetitive sampling required. Further studies using bronchoscopic techniques including biopsy and histological analysis of surgically obtained tissue samples are required to determine the complex relationships between pulmonary infection and inflammation throughout the lung and whilst these will inform on the mechanisms underlying the development of COPD they are of limited immediate clinical utility, thus further development of non-invasive sampling techniques are required to increase the value of measuring airway pathology in predicting physiological and clinical outcomes.

CONCLUSION

The methods used in this study have been established and validated for the follow up and investigation of infective and inflammatory parameters in patients with moderate to severe COPD. As in any study the limitations of the methods used will affect the ability to interpret the data in the context of the disease as a whole and to extrapolate findings to a wider population. The studies performed have been largely observational in nature and therefore care has been taken to limit conclusions about causality when appropriate. However the findings described in subsequent chapters do represent those from a real patient population and are not open to issues of relevance raised by many in vitro or animal studies. The following chapters describe the findings of the studies designed to address the aims outlined in chapter 2.

CHAPTER 4

LOWER AIRWAY BACTERIAL COLONISATION, INFLAMMATION AND FEV₁ DECLINE IN PATIENTS WITH STABLE COPD

Airway bacterial colonisation is an established phenomenon in COPD. However the majority of studies to date have not been prospective in nature and determining the role of bacteria in disease progression for example has not been possible. In this chapter, the patterns of airway bacterial colonisation over time are described and the relationships with inflammation and disease progression determined.

4.1 INTRODUCTION

Smoking is the most important factor in the aetiology of COPD and is known to cause inflammation in the lung (Di Stefano 1998). However, smokers exhibit a variable rate of decline in lung function, suggesting that other may contribute to the progression of COPD.

Patients with stable COPD exhibit increased airway inflammation (Riise 1995, Keatings 1996) and the degree of airway inflammation is positively related to the

severity of airway obstruction with more bronchial inflammation in patients with a lower FEV₁ (Di Stefano 1998). Furthermore, higher levels of airway inflammation, as evidenced by high sputum neutrophil counts, were associated with a greater rate of decline in FEV₁ (Stanescu 1996). The stimulus for increasing airway inflammation as lung function declines has not yet been determined.

The lower airways of healthy individuals are sterile, but bacteria have been isolated in significant numbers in patients with clinically stable COPD, indicating the presence of lower airway bacterial colonisation (LABC) (Monso 1999, Zalacain 1999, Monso 1995). The presence of bacteria in the lower airway can result in a range of important effects on the lung, including activation of host defences with release of inflammatory cytokines and subsequent neutrophil recruitment, mucus hypersecretion, impaired mucociliary clearance, and respiratory epithelial cell damage (Murphy 1992). Animal models of chronic bacterial infection in the lung have shown changes characteristic of those seen in COPD in terms of inflammatory cells, cytokine expression, and pathologic changes to both airways and alveoli (Vernooy 2002). There is evidence that airway inflammation increases with higher airway bacterial loads determined from quantitative sputum cultures in patients with COPD (Hill 2000). Thus, it has been suggested that chronic LABC contributes to progression of airways obstruction (Wilson 1992, Wedzicha 2000).

Airways obstruction and in particular COPD is an established risk factor for cardiovascular disease (Sin 2003, Dahl 2001). In longitudinal studies severity of airways obstruction has been shown to be a more reliable predictor of cardiac cause mortality than more accepted risk factors such as serum cholesterol (Hole 1996). Indeed, cardiovascular disease is the commonest cause of morbidity in patients with COPD (Schuneman 2000, Friedman 1976). As airways obstruction develops in

patients with COPD, the risks of CHD increase. These are associated with elevated levels of inflammation-sensitive plasma proteins which are not explained by smoking alone (Engstrom 2002, Wedzicha 2000).

Plasma fibrinogen and other acute phase proteins are markers of systemic inflammation and have been shown to be elevated in COPD patients in the stable state compared to a normal population (Meade 1993, Sin 2003, Engstrom 2002) and to rise further during acute exacerbations (Wedzicha 2000). Whilst the stimuli to airway inflammation such as cigarette smoking and lower airway bacterial colonisation in COPD patients have been described (Riise 1995, Stanescu 1996, Hill 2000), the role of these factors in the pathogenesis of systemic inflammation is not fully established. Although an analysis of baseline data from an interventional study has suggested that lower airway bacterial colonisation may be associated with higher levels of systemic inflammation (Banerjee 2004), other studies have failed to show this relationship (Hurst 2005).

Fibrinogen is produced by hepatocytes in response to stimulation by systemic inflammatory cytokines chiefly interleukin 6 (IL-6) (Castell 1989, Gabay 1999). Elevated plasma fibrinogen is an established risk factor for coronary heart disease (CHD) (Meade 1993, Ernst 1997, Salomaa 2002). A large meta-analysis of 18 studies yielded an associated relative risk of CHD of 1.8 for patients with fibrinogen levels in the top third of baseline measurements (reference range 2.0-4.5 g/l) (Danesh 1998). In addition to increased risk of CHD, elevated fibrinogen levels are also associated with increased risk of stroke (Smith 1997) and overall mortality (Yano 2001).

Previous studies performed to evaluate the relationship between airway bacterial colonisation, inflammation, and lung function have been cross-sectional in design and have not addressed the important relationship between these parameters

and effects on disease progression. Furthermore whilst lower airway bacterial colonisation is linked to airway inflammation in COPD its contribution to systemic inflammation is unclear. This section of the study addresses the hypothesis that bacterial colonisation leads to increased airway and systemic inflammation and thus contributes to the accelerated progression of airway obstruction and potentially additional cardiovascular morbidity. Therefore, by studying changes in airway bacterial load and type over time and relating these parameters of infection to indices of airway inflammation and decline in lung function we could inform on the role that bacterial colonisation may play in COPD.

4.2 METHODS

The methods of patient selection, recruitment follow up and sampling are described in Chapter 3 of this thesis. The methods specific to the analysis described in this chapter are described below.

PATIENT POPULATIONS

The inclusion criteria and clinical and sampling methodology have been described in chapter 3. Seventy four patients from the COPD cohort were sampled in total to assess the pattern of lower airway bacterial colonisation and the analysis of relationships between bacterial colonisation and airway and systemic inflammation. Patients were followed prospectively and sampled in the stable state as described in Chapter 3. Of this population, thirty patients were sampled at one year after the initial sample for the study of the relationship between changes in bacterial colonisation and FEV₁ decline. Each patient had been clinically stable (exacerbation-free) for at least 6 weeks before both recruitment and sampling at the end of the study by patient interview and review of diary cards. Patients who suffered an exacerbation around the end of the study period were only sampled when they had been clear of exacerbation symptoms and had completed any exacerbation treatment for at least 6 weeks.

MEASUREMENT OF LUNG FUNCTION

Lung function was measured with a rolling seal spirometer (Sensor Medic Corp., Yorba Linda, California). Lung function measurements were taken in the morning, 1 hour after the patient's usual bronchodilator medication inclusive of 200 µg of salbutamol via metered dose inhaler. At least three spirometry readings were taken at each visit, and the best performance was recorded.

SPUTUM SAMPLING

Sputum was sampled at the beginning and the end of the study. Sputum interleukin (IL)-6 and IL-8 levels were measured using ELISA (R&D Systems, Abingdon, UK).

VENOUS BLOOD SAMPLING

4.5ml of venous blood was obtained from an ante-cubital vein without venostasis into vacuum tubes with sodium citrate (0.5ml). Thrombin-clottable plasma fibrinogen was measured in the stored serum samples by the method of Clauss (Clauss 1957). Plasma interleukin 6 (IL-6) were measured using a standard ELISA technique (R&D Systems Abingdon, UK).

QUANTITATIVE BACTERIAL ANALYSIS

Samples were processed by using sputolysin, quantitative bacteriological analysis was performed as described in Chapter 3.

STATISTICAL ANALYSIS

Normally distributed data are reported by means (SDs) and skewed data by medians (interquartile range [IQR]). Correlations were assessed using the Pearson or Spearman correlation coefficient (two-tailed). Continuous variables with normal distributions were compared by *t*-test, whereas those with non-normal distributions were compared by the Mann-Whitney U or Wilcoxon signed-ranks test. Repeat sampling allowed analysis of both intra-patient variability and inter-patient variability of airway bacteriology and inflammatory markers. To analyze the effects of bacterial colonisation independently of patient characteristics (smoking status, exacerbation

frequency, and FEV₁), we used using the general linear mixed model (xtreg) procedure in STATA-5 software (Stata Corporation, Texas, USA). These procedures are designed for panel (cohort) data and are particularly useful where data are correlated, as in repeated-measures designs and where there are complex error structures (Donaldson 2005).

During the analysis, patients were divided into groups dependent on exacerbation frequency during the study. Patients with an exacerbation frequency that was higher or lower than the median were termed "frequent" or "infrequent" exacerbators, respectively; p values of 0.05 or less were regarded as significant.

4.3 RESULTS

4.3.1 PATIENT CHARACTERISTICS

The baseline physiological characteristics of the 74 patients who were recruited for the study are summarized in Table 4.1.

Table 4.1 Baseline Patient Characteristics

	Sampled patients (n=74)	
	Median	IQR
Age (years)	67.4	62.2 to 71.4
FEV ₁ (l)	0.98	0.77 to 1.37
FEV ₁ (% predicted)	39.2	29.6 to 57.8
FEV ₁ (% reversibility)	7.94	0.8 to 13.1
FVC (l)	2.41	1.86 to 2.90
FEV ₁ /FVC (%)	43.9	35.5 to 53.1
PEF (l/min)	158	122 to 238
P _a O ₂ (kPa)	8.94	8.14 to 9.43
P _a CO ₂ (kPa)	5.88	5.32 to 6.29
Smoking (years)	45	39 to 51
Current Smokers	28	
Exacerbation frequency (per year)	2.51	1.28 to 3.83

Further details of individual patient populations involved in any sub-analysis are included with the relevant results section.

4.3.2 STABLE STATE LOWER AIRWAY BACTERIAL COLONISATION IN PATIENTS WITH MODERATE TO SEVERE COPD SPUTUM BACTERIOLOGY

Potentially pathogenic bacterial micro-organisms (PPMs) were identified in 57.3 % of the initial sputum samples with the remaining 42.7 % demonstrating significant non-specific growth (NSG). The relative prevalence of individual bacterial species from all samples is illustrated in Figure 4.1. The most prevalent colonising bacterial organism was *Haemophilus influenzae* present in 19.1 % of samples.

The overall mean airway bacterial load was $10^{7.98(0.83)}$ cfu/ml. Potentially pathogenic organisms (defined in chapter 3), were present at a greater bacterial load of $10^{8.18(0.77)}$ cfu/ml compared to non-specific growth $10^{7.67(0.11)}$ cfu/ml ($p=0.001$) with a trend to a higher load in active smokers; $10^{8.14(0.10)}$ cfu/ml than in ex-smokers; $10^{7.91(0.08)}$ cfu/ml ($p=0.083$). No significant relationship was seen between disease severity and airway bacterial load.

The variation in airway bacterial load between different colonising bacteria are illustrated in Figure 4.2. *Haemophilus influenzae* load ($10^{8.50(0.09)}$, $p=0.004$) and *Moraxella catarrhalis* load ($10^{8.54(0.14)}$, $p=0.033$) were both present at higher bacterial loads than the other PPMs, with no significant difference in airway bacterial load identified for the other species.

4.3.2.1 SEASONALITY OF BACTERIAL COLONISATION

The effects of seasonality on airway bacterial colonisation was analysed by comparing airway bacterial loads and relative pathogen prevalences for samples taken from each season: Spring (March-May), Summer (June-August), Autumn (September-November) and Winter (December-February). The mean stable airway

bacterial load varied with season (anova $p= 0.018$) and was higher in the Summer than in other seasons Figure 4.3, whilst the variations in relative prevalence of airway pathogens isolated did not reach statistical significance (Figure 4.4).

FIGURE 4.1 RELATIVE FREQUENCY OF BACTERIAL ISOLATES EXPRESSED AS A PERCENTAGE OF TOTAL SAMPLES IN 74 PATIENTS.

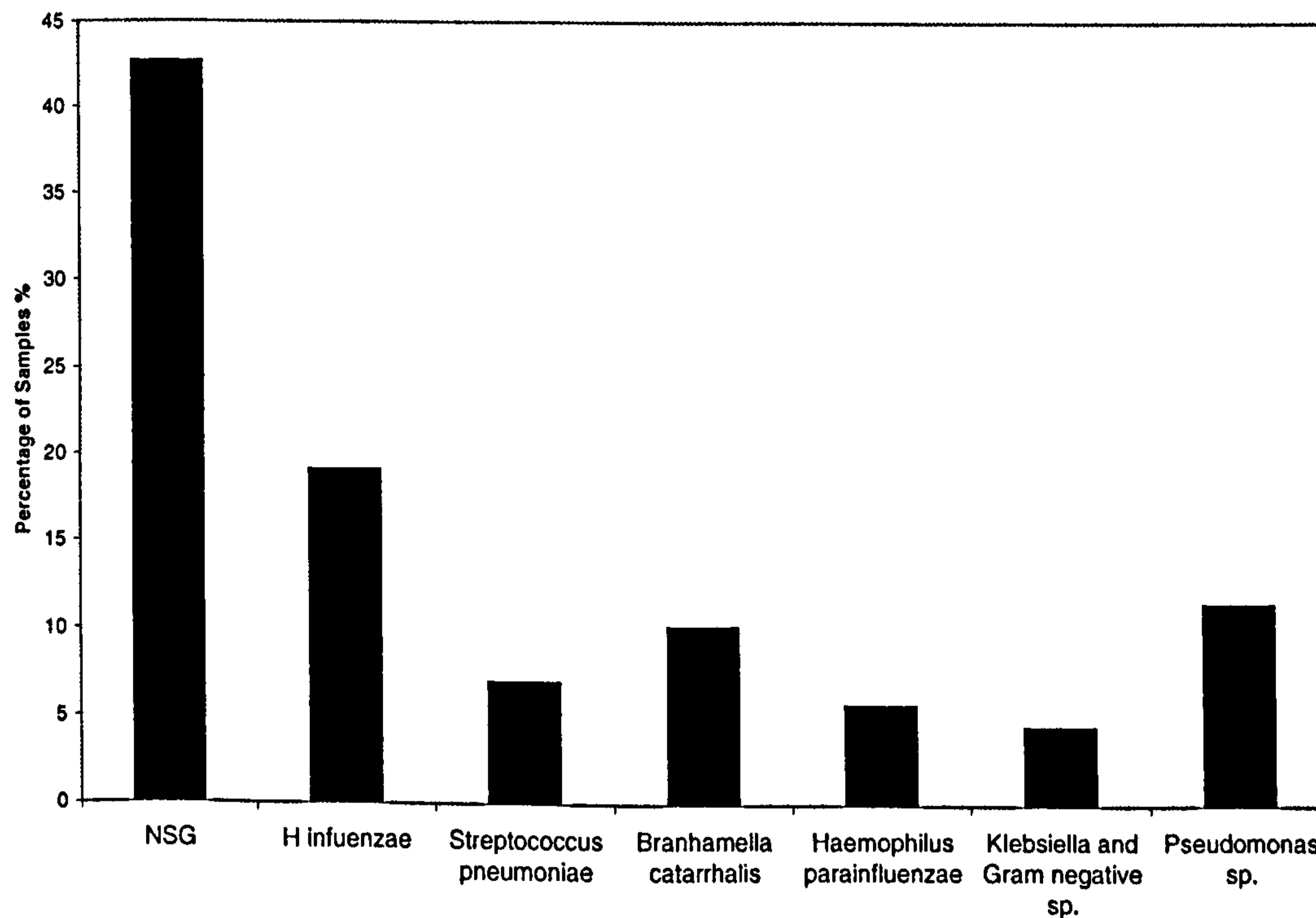


FIGURE 4.2 AIRWAY BACTERIAL LOAD EXPRESSED AS LOG COLONY FORMING UNITS PER MILLILITRE (LOG CFU/ML) FOR DIFFERENT BACTERIAL ISOLATES FROM SPUTUM SAMPLES.

* denotes significantly different airway bacterial load for particular isolate in comparison to load of other potentially pathogenic organisms. *Haemophilus influenzae* p=0.004, *Moraxella catarrhalis* p=0.033. NSG refers to Non-Specific bacterial Growth.

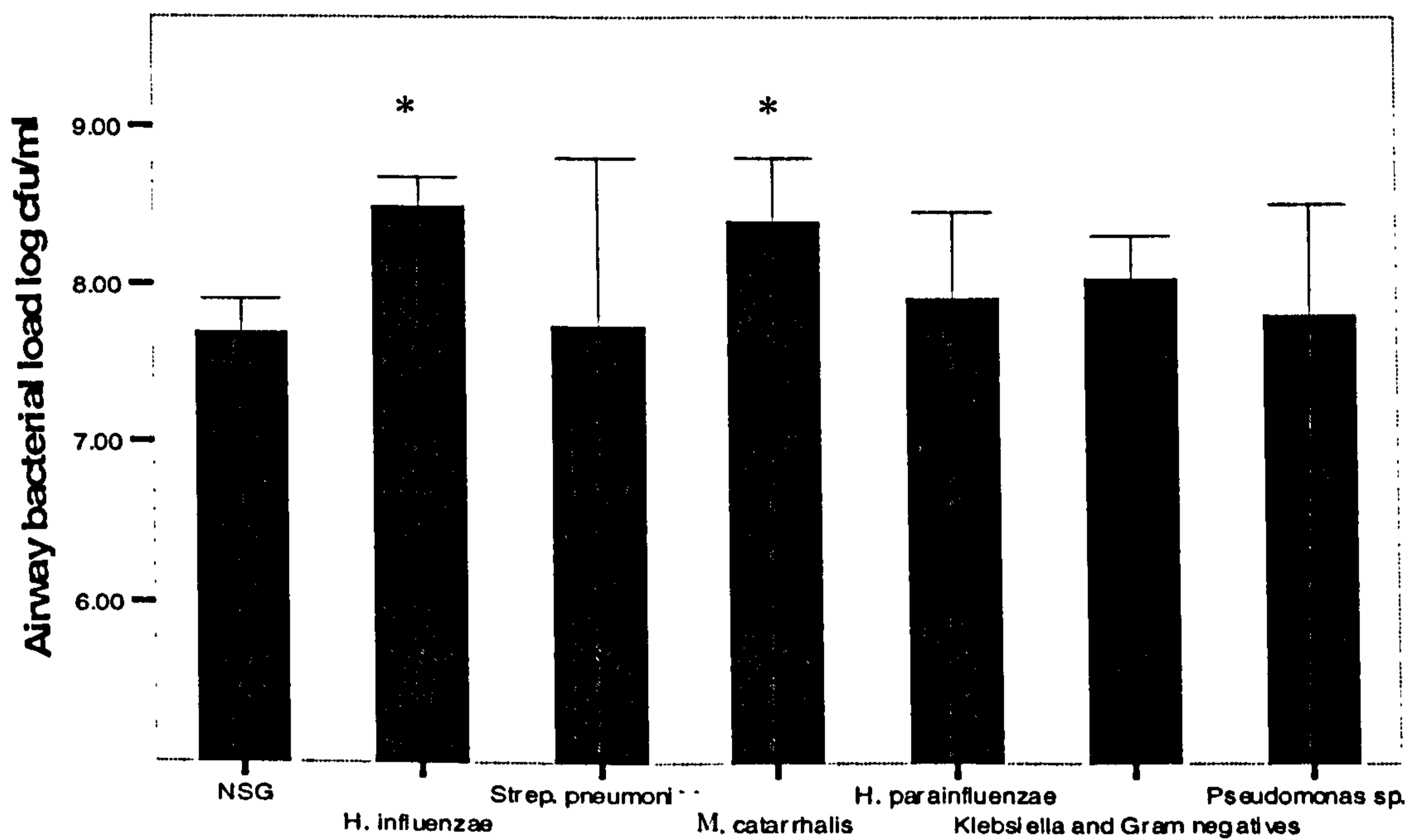


Figure 4.3 SEASONAL VARIATION IN AIRWAY BACTERIAL LOAD log cfu/ml for 1 Spring (March-May), 2 Summer (June-August), 3 Autumn (September-November), 4 Winter (December- February). $p = 0.018$ ANOVA, for difference between seasons.

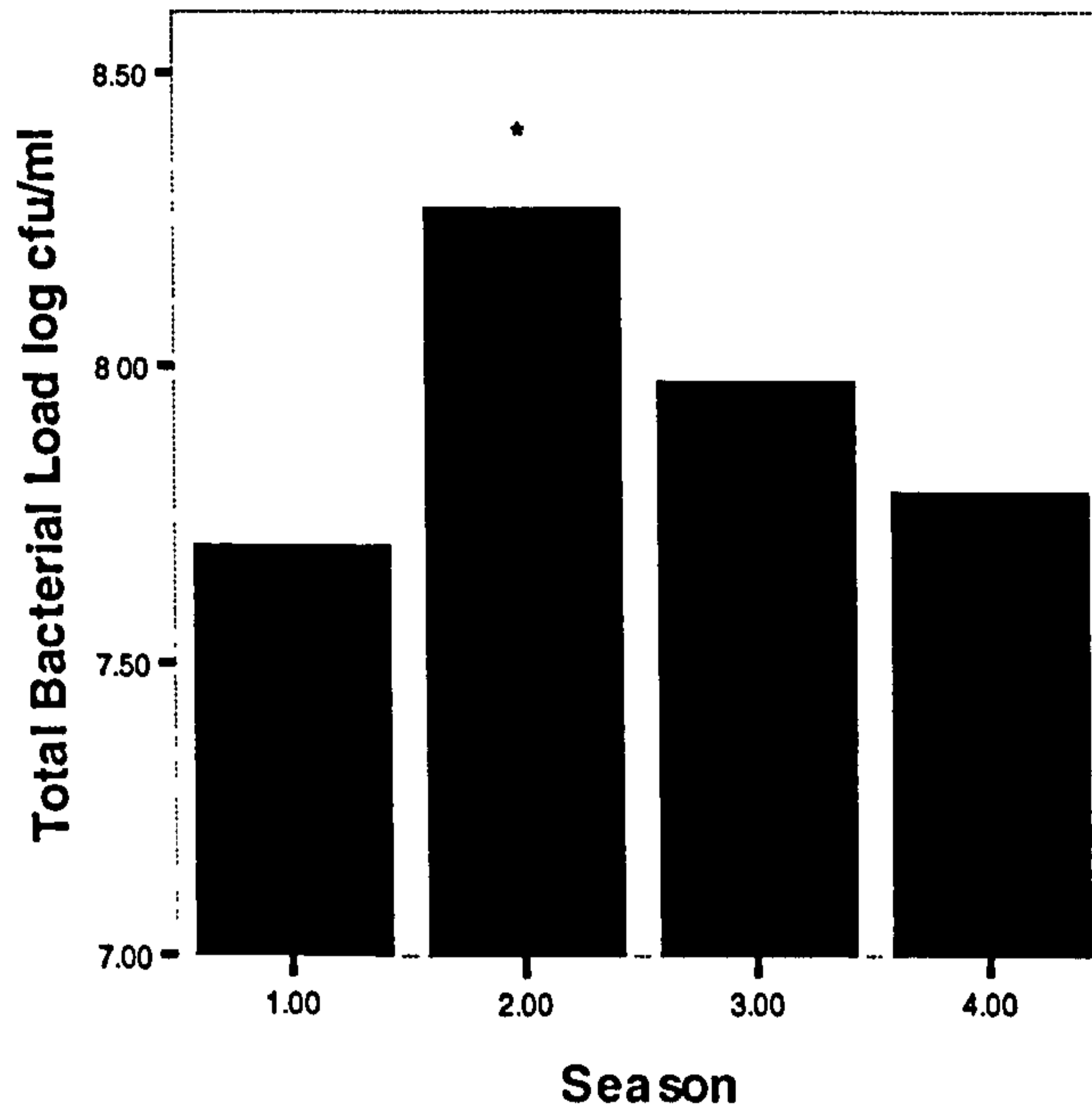
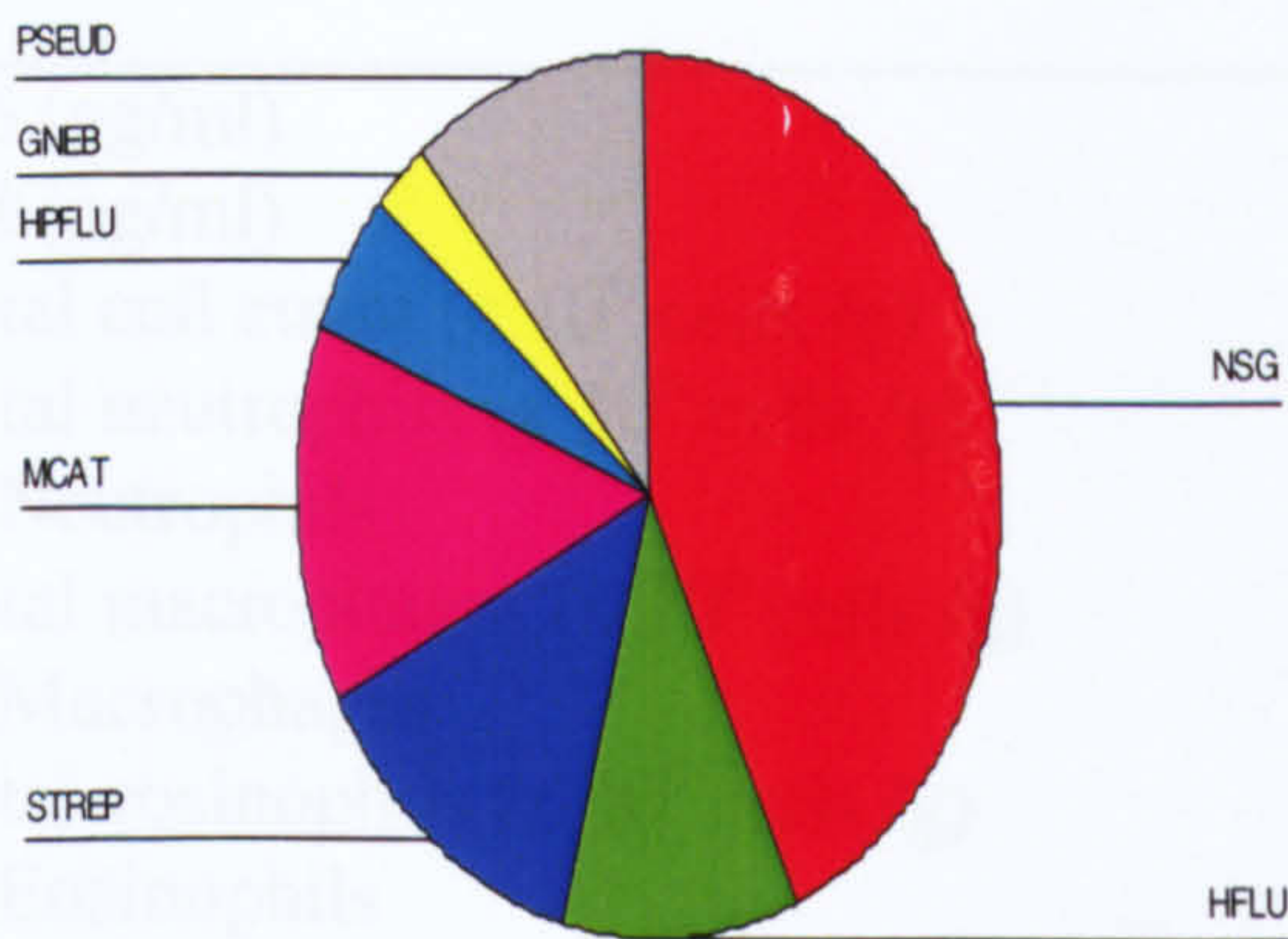
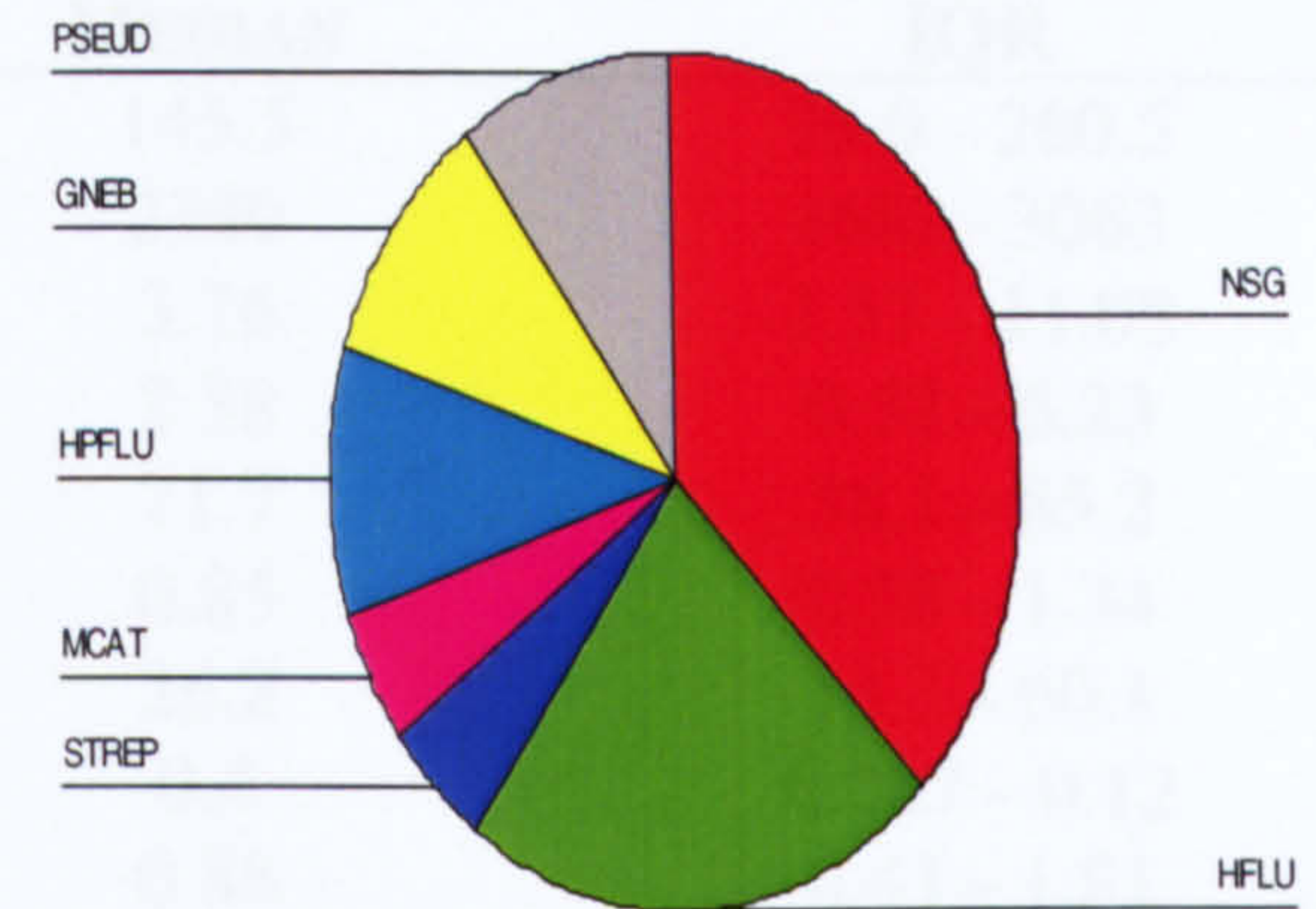


FIGURE 4.4 SEASONAL VARIATION IN RELATIVE PREVALENCE OF SPUTUM BACTERIAL ISOLATES. Spring (March-May), Summer (June-August), Autumn (September-November), Winter (December-February). NS. ($p > 0.12$) ANOVA, for difference between seasons, $n = 74$.

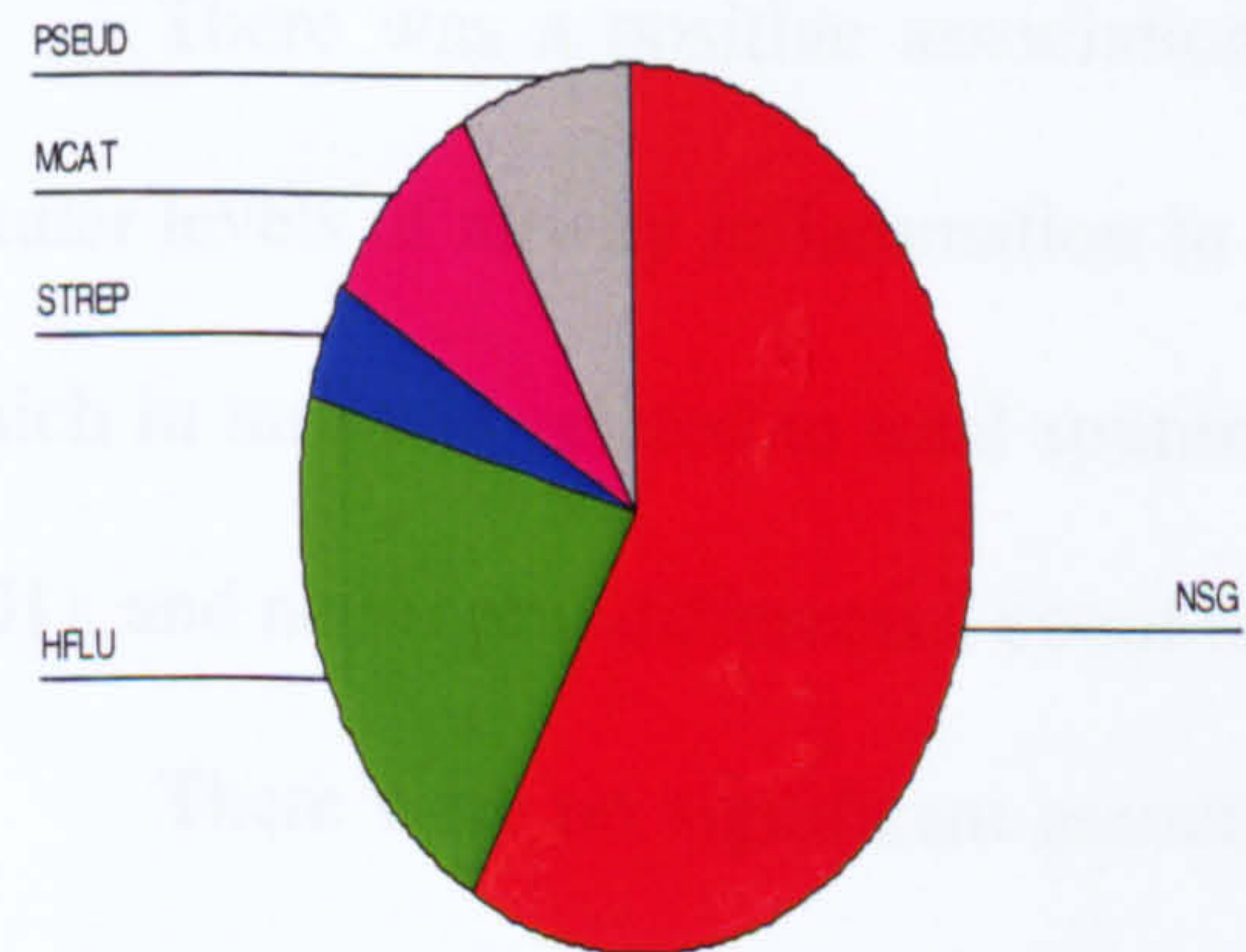
Relative Prevalence of Bacterial Isolates: Spring



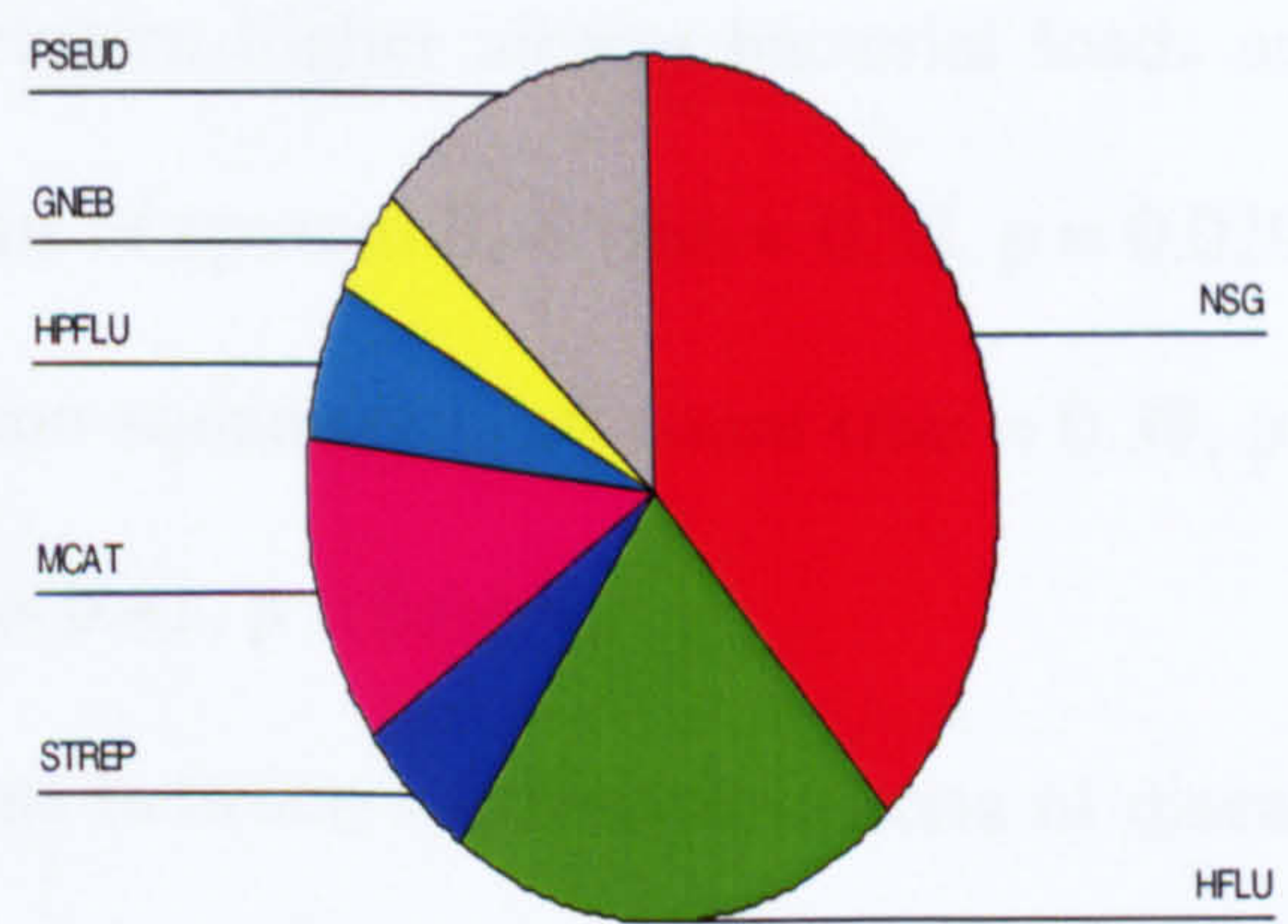
Relative Prevalence of Bacterial Isolates: Summer



Relative Prevalence of Bacterial Isolates: Autumn



Relative Prevalence of Bacterial Isolates: Winter



Key: Red = NSG, Green = *Haemophilus influenzae*, Blue = *Streptococcus pneumoniae*, Pink = *Moraxella catarrhalis*, Yellow = Gram Negative Enteric Bacteria (GNEB), Grey = *Pseudomonas aeruginosa*.

4.3.3 AIRWAY INFLAMMATION

4.3.3.1 INDICES OF AIRWAY INFLAMMATION.

The baseline inflammatory indices from stable state sputum samples, n= 74, are displayed in Table 4.2 below.

TABLE 4.2 SPUTUM INFLAMMATORY INDICES IN STABLE STATE COPD PATIENTS n= 74.

Cell counts expressed as total or percentage of non-squamous cells per sample

	MEDIAN	IQR
Il 6 (pg/ml)	145.5	76.0 – 260.5
Il 8 (pg/ml)	2349	1682 - 3063
Total cell count (x 10 ⁶ cells /g)	3.76	1.57 – 11.08
Total neutrophils (x 10 ⁶ cells /g)	2.38	0.52 – 6.23
% Neutrophils	71.7	38.1 – 85.2
Total macrophages (x 10 ⁶ cells /g)	0.85	0.38 – 1.34
% Macrophages	26.2	14.3 – 60.1
Total eosinophils (x 10 ⁶ cells /g)	0.4	0.087 – 0.12
% Eosinophils	0.88	0.43 – 1.81
Total lymphocytes (x 10 ⁶ cells /g)	0	0 – 0.006
% Lymphocytes	0	0 – 0.44

4.3.3.1 AIRWAY INFLAMMATION, BACTERIOLOGY AND PATIENT CHARACTERISTICS

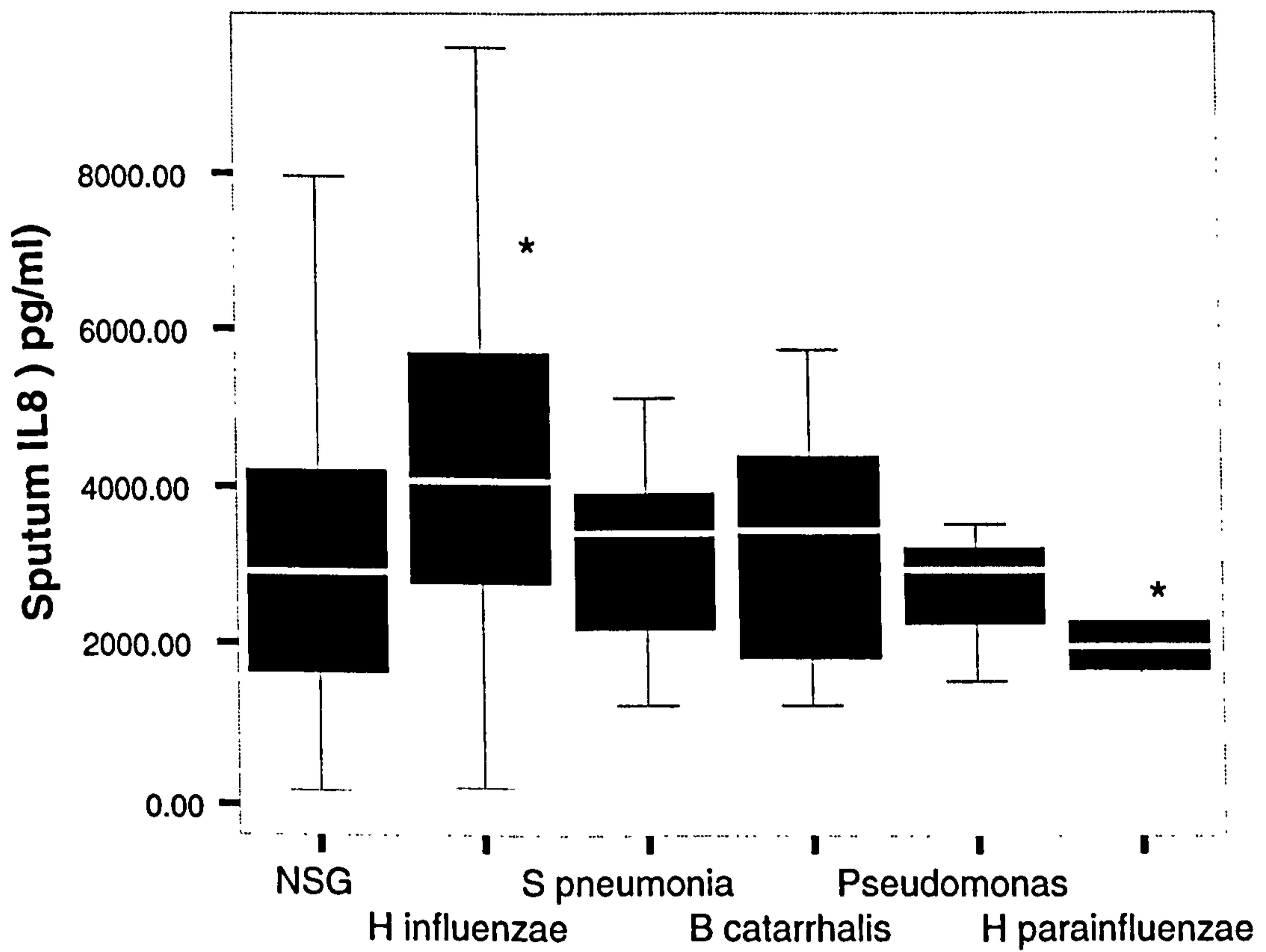
There was a positive association between higher airway bacterial loads and greater levels of airway inflammation in terms of sputum IL-8 (rho = 0.38, p = 0.029), which in turn was related to total sputum (non-squamous) cell count (rho = 0.39, p = 0.01), and neutrophil differential count (rho = 0.41, p = 0.010).

There were no significant associations between spirometric criteria of disease severity and sputum cell counts however, sputum IL-6 was greater in patients with lower percent predicted FEV₁ (rho= -0.347, p= 0.036). Patients with more frequent exacerbations had greater number of total sputum cells (rho = 0.297, p = 0.04), greater percentage of neutrophils; (rho = 0.323, p= 0.027) and higher levels of sputum (IL-8; rho = 0.326, p= 0.043).

Airway inflammation was greater in patients colonised with *Haemophilus influenzae* and lower in those colonised with *Haemophilus parainfluenzae*; see figure 4.5.

FIGURE 4.5 VARIATION IN SPUTUM IL 8 PG/ML WITH AIRWAY BACTERIAL ISOLATE .

* = significant difference from median IL8 for all other samples (p<0.05).



4.3.4 AIRWAY BACTERIA AND SYSTEMIC INFLAMMATION

4.3.4.1 PLASMA FIBRINOGEN AND INTERLEUKIN 6 (IL-6)

The mean (SD) plasma fibrinogen for all samples was 4.23 (0.84) g/l. The median (IQR) serum IL-6 was 6.48 (5.11-10.05) pg/ml. The plasma fibrinogen level was related to the IL-6 level $r = 0.43$, $p=0.001$. Plasma fibrinogen was higher in samples from active smokers than from ex-smokers; 4.47(0.89)g/l vs 4.14 (0.79)g/l respectively, $p = 0.023$,.

There was a trend towards higher IL-6 in active smokers; 5.87 (4.94-9.31) pg/ml than ex-smokers 9.02 (5.75-12.41) pg/ml, $p = 0.078$. Plasma fibrinogen was greater in patients with more severe airways disease (lower % predicted FEV₁) $r = 0.238$, $p=0.003$.

4.3.4.2.RELATIONSHIPS BETWEEN SPUTUM BACTERIA AND PLASMA MARKERS

The plasma fibrinogen and IL-6 levels varied with the nature of bacterial colonisation in the associated sputum sample; fibrinogen levels were greater in samples associated with colonisation with a potentially pathogenic organism; fibrinogen 4.35(0.89) g/l, IL-6 7.31 (5.73-12.17) pg/ml compared to those exhibiting non-specific growth; fibrinogen 4.23(0.83) g/l $p = 0.031$, IL-6 5.4 (4.49-9.02) pg/ml, $p = 0.001$.

The plasma levels of fibrinogen and IL-6 for samples grouped by corresponding bacterial isolate are shown in figure 4.6. Colonisation with *Pseudomonas aeruginosa* was associated with greater systemic inflammation; fibrinogen 4.82(1.04) g/l, IL-6 10.94 (6.50-14.41) pg/ml than all other PPMs; fibrinogen 4.16 (0.77) g/l $p = 0.036$, IL-6 6.58(5.67-10.11) pg/ml $p = 0.003$. Whereas

colonisation with *Haemophilus parainfluenzae* was associated with lower fibrinogen; 3.68(0.47) g/l than with other PPMs; fibrinogen 4.27(0.84) g/l, p=0.039.

IL-6 levels were related to airway bacterial load (r =0.175, p =0.035), but no relation between load and fibrinogen was found. The association between *Pseudomonas* and higher plasma fibrinogen remained if allowance for FEV₁, bacterial load and smoking status was made in the regression model (regression coefficient 0.6, CI 0.21-0.99), p=0.003.

In Table 4.3, the results of a multiple linear regression with fibrinogen and IL-6 as outcome variables are shown. The influence of colonising bacterial species on these plasma markers remains significant even if the possible confounders smoking and factors associated with disease severity, are included in the analysis.

TABLE 4.3 MULTIPLE LINEAR REGRESSION ANALYSIS OF FACTORS ASSOCIATED WITH PLASMA FIBRINOGEN AND IL-6 LEVELS AS OUTCOME VARIABLES

Factor	Fibrinogen			IL-6		
	β	CI	p	β	CI	p
FEV ₁	-0.62	-1.06 to -0.17	0.007	-1.97	-0.42 to 0.25	0.081
Smoking	0.24	-0.05 to 0.54	0.113	0.50	-0.99 to 1.99	0.508
Bacterial Load	-0.02	-0.19 to 0.15	0.813	0.38	-0.50 to 1.27	0.392
Bacterial Species	0.08	0.01 to 0.14	0.021	0.38	0.07 to 0.70	0.019
pO ₂	0.08	-0.05 to 0.23	0.222	-0.39	-1.1 to 0.32	0.281
pCO ₂	-0.16	-0.31 to -0.07	0.049	-0.17	-0.93 to 0.59	0.656

4.3.4.2. AIRWAY AND SYSTEMIC INFLAMMATION

There were no significant correlations found between airway and systemic inflammation.

FIGURE 4.6. RELATIONSHIP BETWEEN PLASMA FIBRINOGEN AND INTERLEUKIN-6 (IL-6)
in 161 blood samples from 74 patients with stable COPD. $r = 0.43$, $p=0.001$.

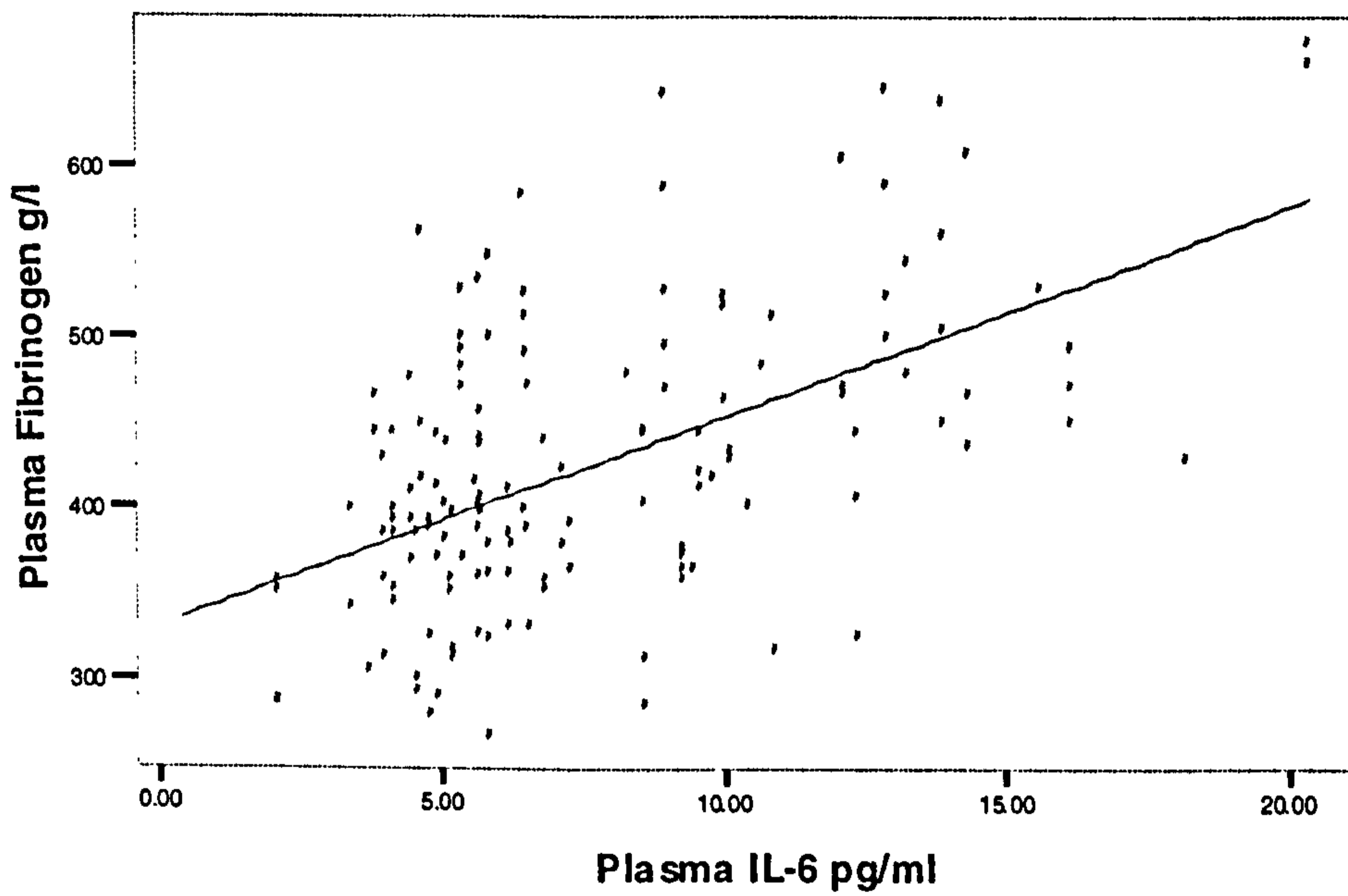
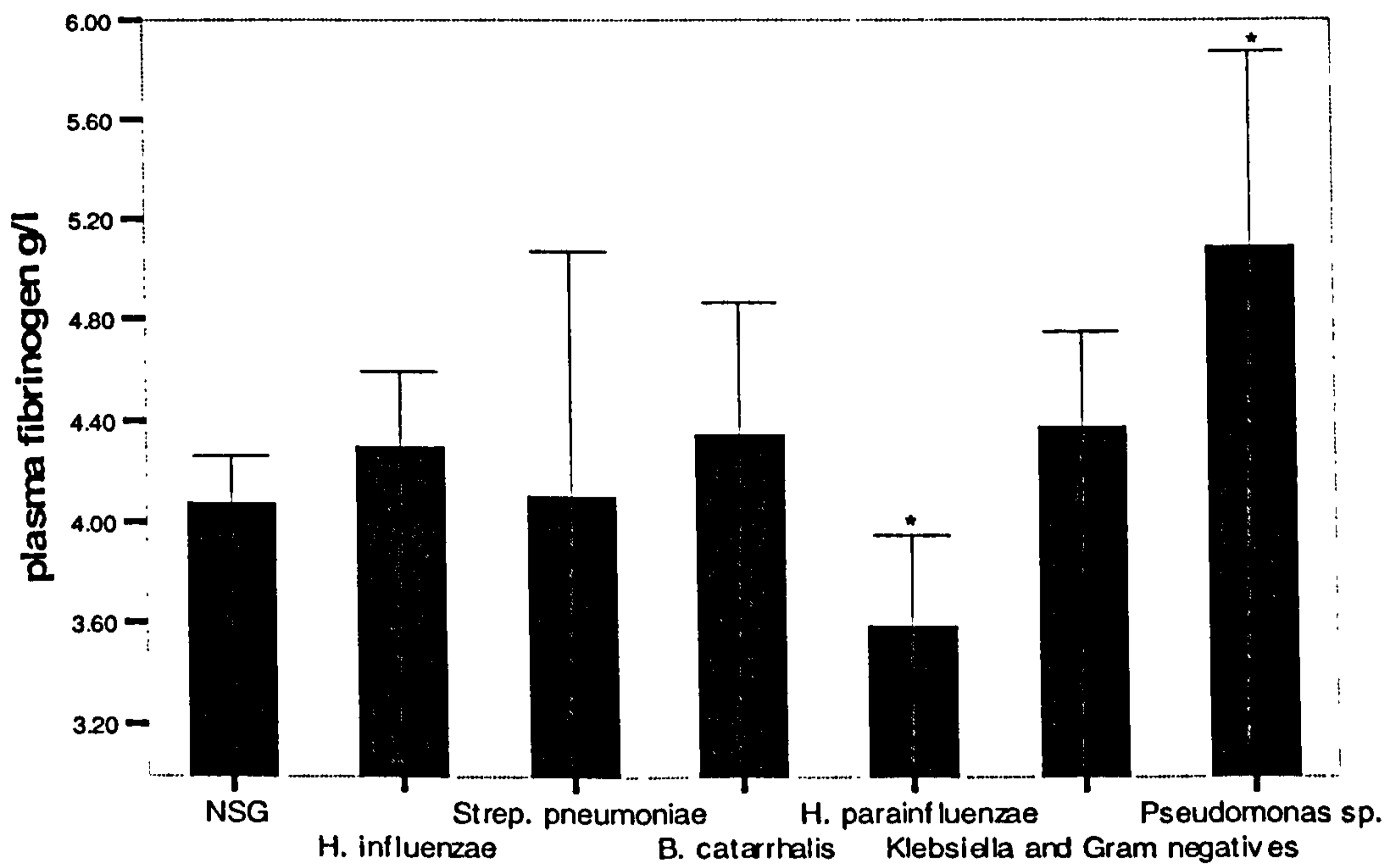


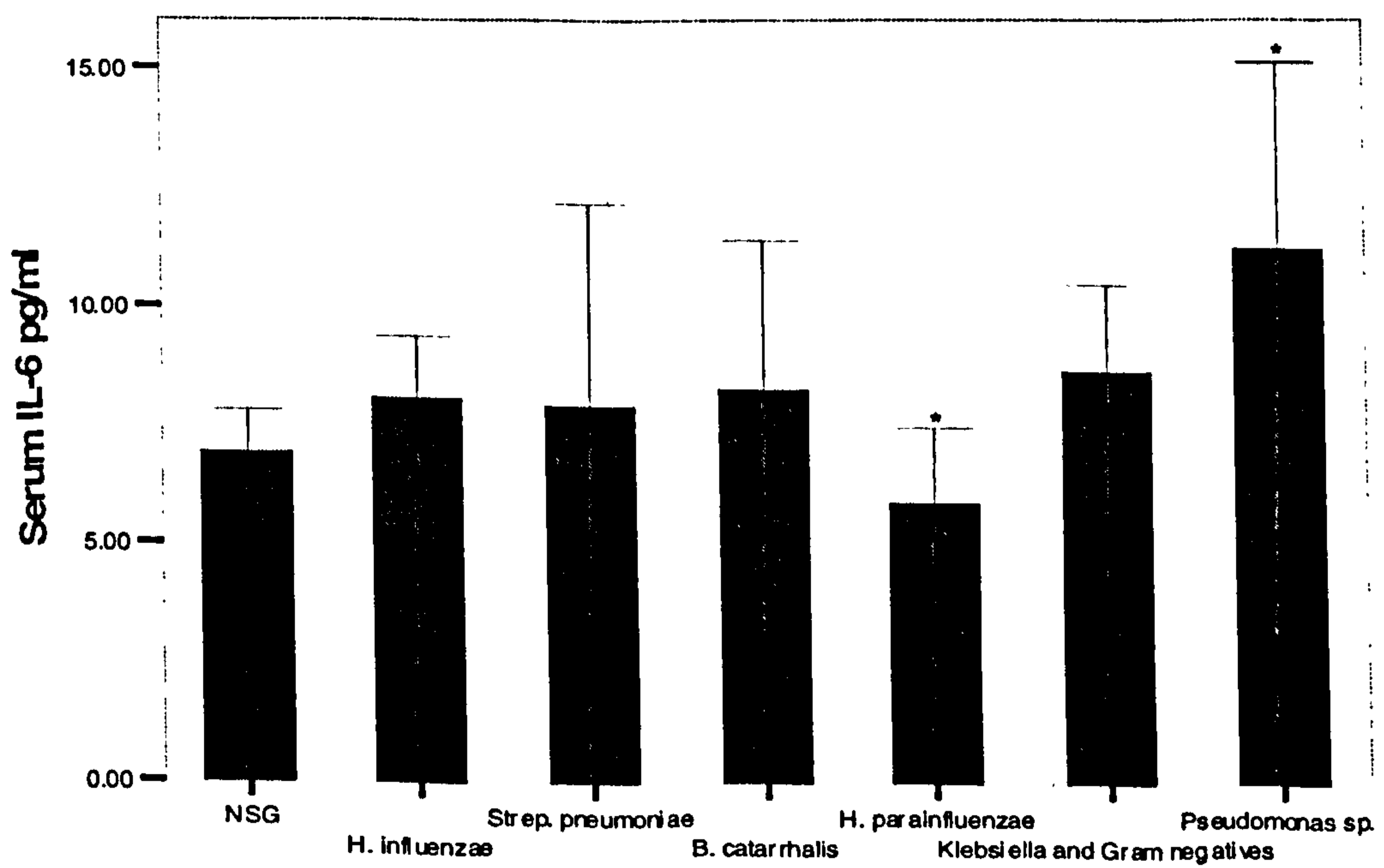
FIGURE 4.7. PLASMA FIBRINOGEN (A) AND INTERLEUKIN-6 (B) LEVELS ASSOCIATED WITH DIFFERENT SPUTUM BACTERIAL ISOLATES. * denotes significantly different level of plasma marker from other potentially pathogenic organisms (see text for values)

NSG refers to Non-Specific bacterial growth.

(a)



(b)



4.3.5 LOWER AIRWAY BACTERIAL COLONISATION AND FEV₁ DECLINE OVER 1 YEAR

As an initial study into the relationship between lower airway bacterial colonisation and disease progression, thirty patients from the cohort who had previously tolerated sputum induction and who were established on optimal inhaled therapy, were sampled and were followed for a median (IQR) period of 1.05 (1–1.22). The mean (SD) FEV₁ for this population was 0.947 (0.329) l, and the predicted FEV₁ was 34.8% (13.6%), with a range from 13.80 to 69.95% predicted, at the start of the study. Eleven of the 30 patients were current smokers at the time of recruitment and did not alter their smoking habits during the study. Twenty eight of the patients were receiving inhaled steroids with a mean (SD) dosage of 1.55 mg (0.92) beclomethasone equivalents; no changes to the dose of inhaled steroid occurred during this sub-study. The baseline data for these 30 patients did not differ significantly from that of the other patients in the cohort. At the first sample point, 24 patients produced sputum spontaneously, the remainder being induced, compared with 22 patients expectorating spontaneously at the second sample point.

FEV₁ DECLINE

The mean (SD) FEV₁ at recruitment was 0.947 (0.329) L and declined to 0.883 (0.367) L at the end of the 1.05 (1–1.22)-year sample interval. The mean annual rate of decline was 57.6 (137.6) ml per year; expressed as percentage of initial FEV₁, this equates to 6.08% of baseline FEV₁ decline per year. The 30 patients had a total of 86 exacerbations during the study period, 40 (46.5%) of which were reported to the study team; the remainder of the exacerbations were diagnosed from diary card review, a proportion of which (17.4%) had been independently reported to a general

practitioner. Fifty-two exacerbations received antibiotic treatment during the study. The median (IQR) exacerbation frequency in this study was 2.39 (1.95) exacerbations

TABLE 4.4. CHARACTERISTICS OF 30 PATIENTS IN THE 1 YEAR STUDY OF BACTERIAL COLONISATION AND DECLINE IN FEV₁.

Characteristic	Mean (SD)
Number, female/male	8/22
Age, years	66.43 (10.25)
FEV ₁ , L	0.95 (0.33)
FEV ₁ percentage predicted	34.81 (13.61)
FVC, L	2.51 (0.70)
FEV ₁ /FVC percentage	38.40 (10.70)
PEF, L/min	218.07 (76.14)
PaO ₂ , kPa	8.55 (1.11)
PaCO ₂ , kPa	6.20 (1.00)
Pack-years of smoking	74.3 (66.5)
Inhaled steroid dosage	1.55 (0.92)
Beclomethasone equivalent, mg/day	

per year. Patients with an exacerbation frequency higher than this median (frequent exacerbators) had more severe airways obstruction with a mean FEV₁ of 0.86 L compared with infrequent exacerbators with a mean FEV₁ of 1.07 (p = 0.05).

BACTERIAL LOAD AND FEV₁ DECLINE

All cultures of sputum samples grew significant numbers of bacteria ranging from 10^{5.4} to 10^{9.6} cfu ml⁻¹. The mean (SD) total bacterial count at sample 1 was

$10^{7.47(0.91)}$ cfu ml⁻¹ and rising to $10^{7.93(0.81)}$ in sample 2 ($p = 0.019$) or a rise from 29,512,092 cfu ml⁻¹ to 85,113,804 cfu ml⁻¹ when expressed without log transformation.

Patients with an increasing airway bacterial load demonstrated a more severe decline in FEV₁ over the study period compared with patients with stable or decreasing airway bacterial load who exhibited less marked decline or slight improvement in FEV₁. This relationship between FEV₁ decline and changes in bacterial load (Figure 4.7) was statistically significant in terms of absolute FEV₁ decline ($r = 0.593$, $p = 0.001$) and decline expressed as a percentage of baseline FEV₁ ($r = 0.633$, $p < 0.001$). The total bacterial count of the second sample was itself related to the absolute rate of decline over the study ($r = 0.560$, $p = 0.001$) (Figure 4.8). The total bacterial count of the first sample was not predictive of the subsequent decline in FEV₁ over the study ($r = -0.125$, $p = 0.369$).

A linear regression analysis of the relationship between bacterial load and FEV₁ decline revealed that a 10-fold increase (10^1 cfu ml⁻¹) in bacterial load is associated with an 82.4-ml decline in FEV₁ over the study period; the regression coefficient was 0.095 (95% confidence interval, 0.032–0.132) ($p = 0.002$). As the mean increase in bacterial load was from $10^{7.47}$ to $10^{7.93}$ cfu ml⁻¹, this represents a decline in FEV₁ attributable to the airway bacterial load of 33.3 ml/year for this patient group.

A multivariate regression analysis of potential factors in the observed decline in lung function (ml/year) was performed: regression coefficient (95% confidence interval), change in bacterial load (log cfu ml⁻¹) 63.1 (34-133, $p = 0.003$), number of cigarettes smoked per day -11 (-110–890, $p = 0.818$), exacerbation frequency -2(-270–220, $p = 0.819$), and baseline FEV₁ 1(-2–5, $p = 0.368$). The change in bacterial load

and the decline in FEV₁ were the strongest and the only significant relationship in this analysis.

FIGURE 4.8 CORRELATION BETWEEN CHANGE IN FEV₁ AND CHANGE IN TOTAL BACTERIAL COUNT OVER STUDY PERIOD. Figure shows decline in FEV₁ expressed as millilitres of loss (*negative values indicate improving lung function*) against log change in total bacterial count (*positive values indicate increasing numbers of bacteria over study*) n=30. ($r = 0.59, p = 0.001$).

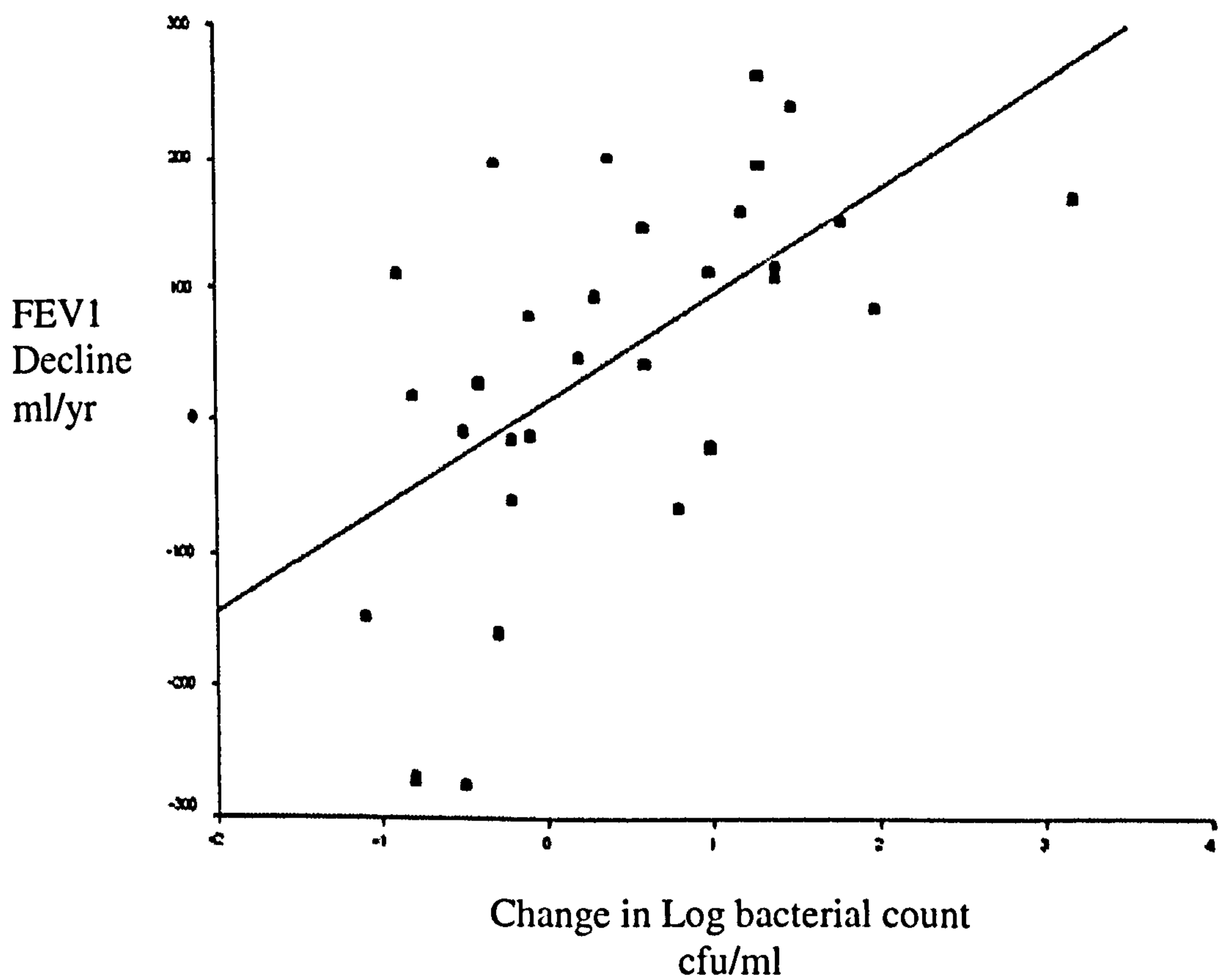
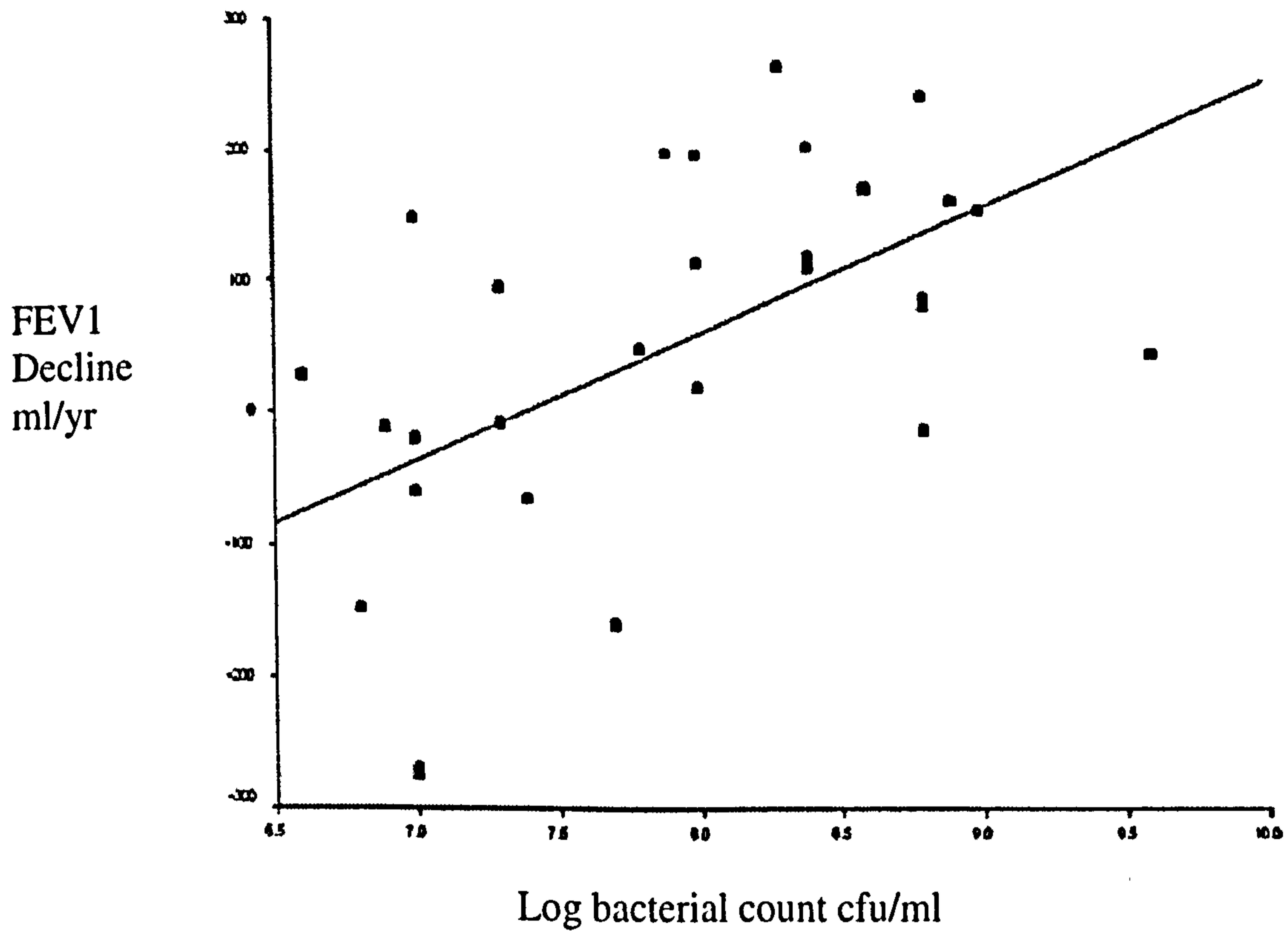


Figure 4.9 CORRELATION BETWEEN RATE OF CHANGE IN FEV₁ OVER STUDY PERIOD (ADJUSTED TO ML/YEAR) AND TOTAL BACTERIAL COUNT AT THE END OF THE STUDY.

Figure shows decline in FEV₁ expressed as milliliters of loss (negative values indicate improving lung function) against log change in total bacterial count from sputum taken at the end of the study n=30. ($r = 0.56, p = 0.001$).



BACTERIAL ISOLATES IN THE 1 YEAR STUDY.

The results of the qualitative bacteriology from the 30 sputum samples taken at the beginning (sample 1) and the end (sample 2) of the study are shown in Figure 4.9. The graph illustrates the relative frequency of each bacterial isolate expressed as a percentage of the 30 samples at each time point. Sixteen (53.0%) and 17 (56.6%) patients were colonised with a potentially pathogenic organism at recruitment and completion of the study, respectively however bacterial colonisation was dynamic with changes in bacterial species identified. The remainder of patients' sputum produced nonspecific growth of bacteria, defined as growth of bacterial species not usually associated as respiratory pathogens in immunocompetent individuals such as *Streptococcus viridans* group, *Neisseria* spp, *Corynebacterium* spp, and coagulase negative staphylococci. The relative frequencies of individual bacterial species are as shown. Five and six subjects respectively displayed colonisation with more than one potentially pathogenic microorganisms at recruitment and completion. At each time point, *Haemophilus influenzae* was the most prevalent individual bacterial type present in 9 (30%) and 7 (23.3%) patients at samples 1 and 2, respectively.

The nature of bacterial colonisation was dynamic with changes in the species of bacterial isolate over the sample interval. Fifty percent of the subjects grew entirely different bacterial species at each sample point, whereas the other 50% demonstrated persistence of a specific bacterial species or nonspecific growth across the sampling interval. Patients who demonstrated changes in colonising bacterial species during the study exhibited higher mean bacterial loads ($10^{8.18}$ log cfu ml⁻¹) than those who maintained the same species in both samples ($10^{7.55}$ log cfu ml⁻¹, $p = 0.03$).

The decline in FEV₁ seen in the study group was significantly greater in those subjects with unstable bacterial type at a 102-ml (IQR 19–196, n = 18) decline in FEV₁ per year compared with a 3.6-ml (IQR -158–112, n = 12) decline per year in the group with persistence of one bacterial type at both time points (p = 0.017); this relationship with FEV₁ decline expressed as a percentage of baseline is illustrated in Figure 4.10. Changes in bacterial species were not related to the number of cigarettes smoked per day (rho = 0.056, p = 0.767), the sputum collection method (induced or spontaneous) (rho = 0.012, p = 0.84), the recorded exacerbation frequency (rho = 0.149, p = 0.44), or the antibiotic usage during the study (rho = 0.048, p = 0.808). An analysis of each of the individual bacterial species and the associated lung function changes did not reveal any attributable significant differences in FEV₁ decline between subjects colonised with different bacterial species, although numbers in each subgroup were too small to draw any valid conclusions from this analysis.

SPUTUM CYTOKINES AND FEV₁ DECLINE

Sputum IL-6 and IL-8 levels were measured on all samples. The median (IQR) IL-6 levels were similar 114 (283) pg ml⁻¹ and 51 (297) pg ml⁻¹ in samples 1 and 2, respectively, and IL-8 levels were 3,183 (1,688) pg ml⁻¹ and 3,012 (1,684) pg ml⁻¹ (p=NS). Levels of sputum IL-6 and IL-8 were related to one another in each patient (rho = 0.378, p = 0.007). The absolute changes in IL-6 between samples 1 and 2 correlated with the changes in IL-8 seen between the two samples (rho = 0.542, p = 0.011). The sputum IL-8 levels were related to pack-years of smoking; those patients with IL-8 higher than the median having smoked for a mean of 100 pack-years and those with lower IL-8 for 42 pack years (p = 0.018). Patients exhibiting a decline in lung function exhibited higher overall sputum median (IQR) IL-8 levels of 3 343

(1,592) pg ml⁻¹ compared with those with stable or improving FEV₁ 2,160 (2,050) pg ml⁻¹ (p = 0.032). Similarly, patients with a higher bacterial load (greater than the overall mean, sample 1 and 2 combined) had higher overall IL-8 levels (2,938 pg ml⁻¹) compared with those with bacterial counts lower than the mean (2,329 pg ml⁻¹) (p = 0.05). There were no significant relationships between sputum IL-6 levels and bacterial counts or lung function decline. Similarly no significant relationships were seen between sputum cell counts and airway bacterial load or lung function decline in the 1 year study.

Figure 4.10 RELATIVE FREQUENCY OF BACTERIAL ISOLATES FROM FIRST (*BLACK BARS*; N = 30) AND SECOND (*HATCHED BARS*; N = 30) SPUTUM SAMPLES. expressed as a percentage of samples. NSG = nonspecific growth.

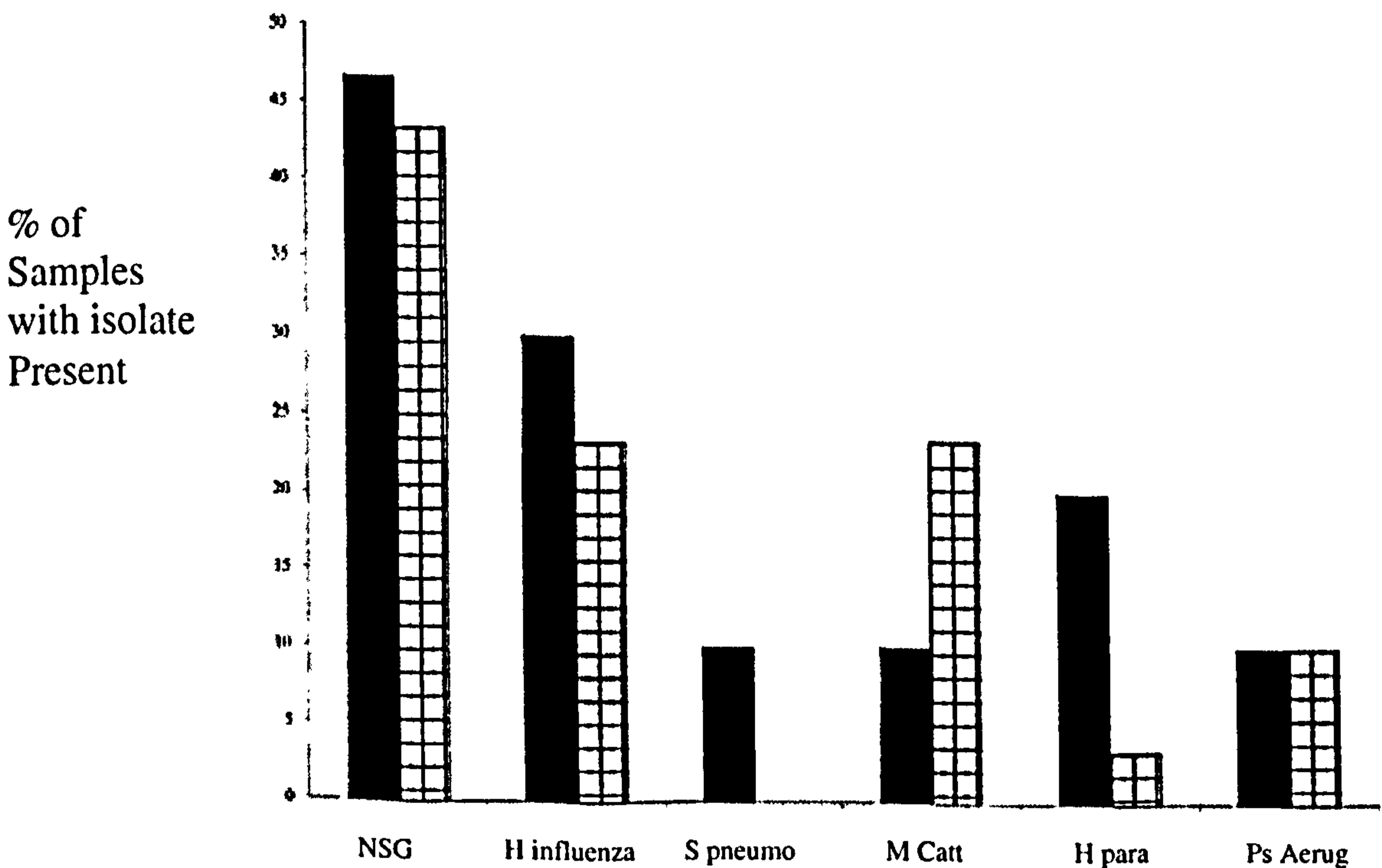
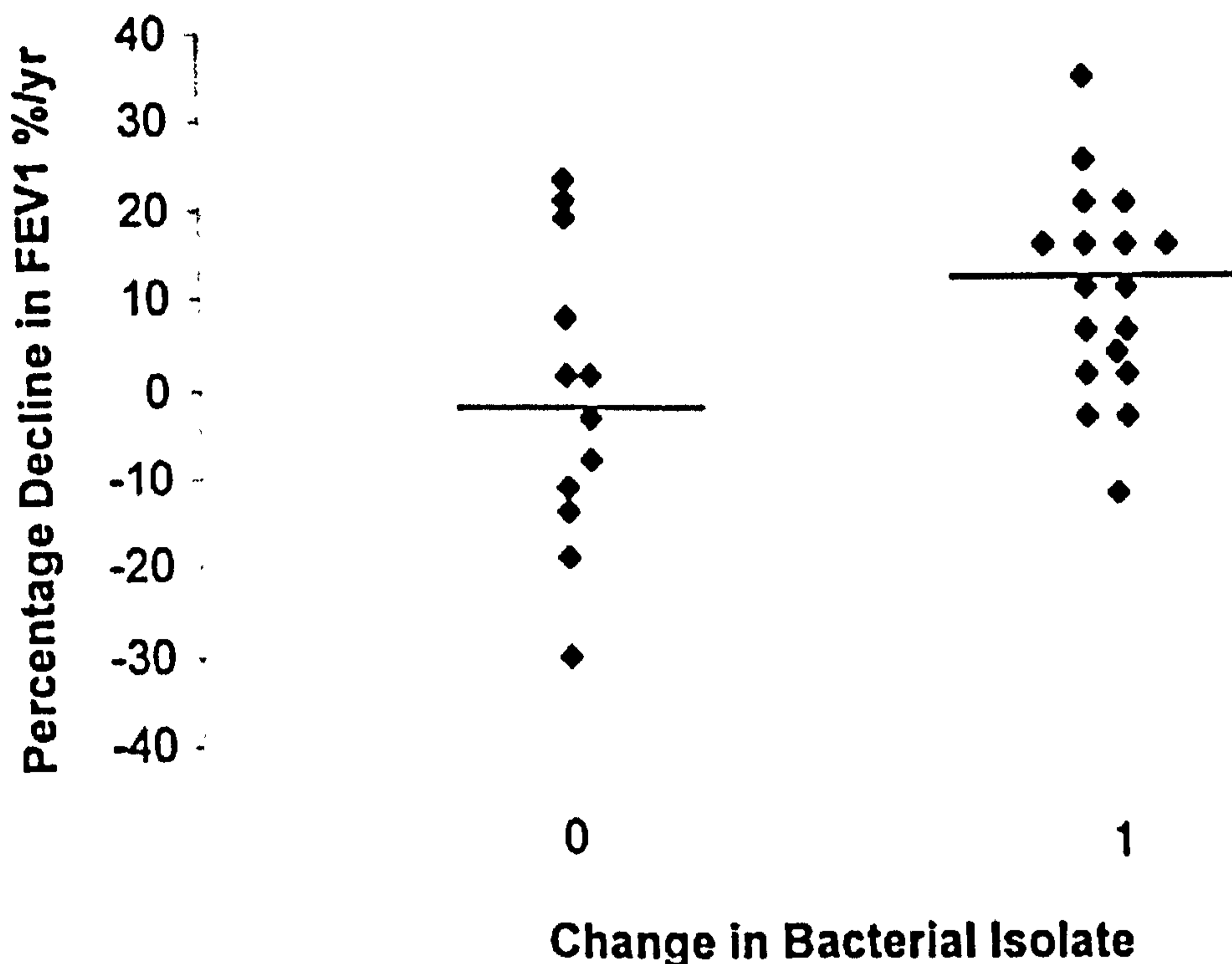


FIGURE 4.11 DIFFERENCES IN FEV₁ DECLINE BETWEEN SUBJECTS WITH PERSISTENT (0) AND CHANGING (1) BACTERIAL ISOLATE. 0 = Same bacterial species identified in sputum at beginning and end of study; 1 = Change in species of bacterial isolate; p = 0.017. Decline expressed as percentage of baseline FEV₁ (negative values indicate improving lung function).



4.3.6 ANALYSIS OF LONG TERM RELATIONSHIPS BETWEEN AIRWAY BACTERIAL COLONISATION AND DISEASE PROGRESSION

The analysis shown above of the relationship between airway bacterial colonisation and disease progression was performed over a limited time period of one year. It is possible that the relationships elicited reflect reversible changes in lung function associated with fluctuations in airway bacteria rather than irreversible disease progression due to lung matrix destruction or airway remodelling. Therefore in order to determine if high airway bacterial loads are associated with accelerated decline in lung function over the longer term a retrospective analysis of the relationships

between FEV₁ decline and bacterial load was performed using data collected over the duration of the East London Cohort Study. This analysis included data from 148 patients who had completed at least one year in the cohort since its inception. Over this time-period the cohort was maintained at between 70 and 80 patients with a programme of rolling recruitment and maximum of 7.3 years of data was available for any one patient for this analysis. The baseline characteristics of these patients at recruitment are shown in Table 4.4.

Table 4.5 Baseline Initial Characteristics of the 148 COPD Patients in the analysis of Airway Bacteriology and FEV₁ Decline

Patient Characteristic	Median	IQR
Age, yr	68.5	62.6 to 73.7
FEV ₁ , L	0.98	0.73 to 1.30
FEV ₁ , % predicted	38.5	27.7 to 50.3
FEV ₁ , % reversibility	5.45	0.00 to 12.6
FVC, L	2.44	1.81 to 2.93
FEV ₁ /FVC, %	42.3	33.5 to 53.1
Peak expiratory flow, L/min	174	137 to 250
Pao ₂ , kPa	9.0	8.23 to 9.58
Paco ₂ , kPa	5.8	5.40 to 6.35
Smoking history, yr	42	32 to 50

The annual change in FEV₁ over this time period was 40.2(CI 36.0 to 44.5) ml/yr or 1.5 (1.3 to 1.7) %FEV₁ predicted loss per year. The airway total bacterial count rose from an initial median value of 10^{7.7(7.0-8.25)} log cfu.ml at an annual rate of 0.15 log units per year. Patients with a bacterial load greater than the median level demonstrated a faster decline in FEV₁ of an additional 0.51 (0.13 to 0.88) % FEV₁

predicted loss per year, over and above that of 0.76 % FEV₁ predicted loss per year seen in subjects with a low bacterial load, $p < 0.01$.

Subjects with rapidly rising airway bacterial loads (rise in TBC above the median for the group) also demonstrated a faster decline in FEV₁ by 0.5% FEV₁ predicted loss per year, greater than those subjects with stable bacterial colonisation or rises below the median for the group ($p = 0.006$).

4.4 DISCUSSION

This analysis has demonstrated that lower airway bacterial colonisation is common in this population of COPD patients with moderate to severe disease. The findings confirm that higher bacterial loads are associated with greater airway inflammation in the stable state and for the first time demonstrate a relationship between airway bacterial colonisation, airway inflammation and disease progression in terms of FEV₁ decline. The data also suggests that certain pathogens particularly *Haemophilus influenzae* are present in greater numbers and are associated with greater levels of airway and systemic inflammation than other pathogens.

The prevalence of lower airway bacterial colonisation in this study was high at 57%, in comparison to some other studies of COPD patients. In previous studies, the prevalence of lower airway bacterial colonisation was found to be greater in subjects with more advanced airways obstruction (Zalacain 1999, Monso 1999). The mean FEV₁ of less than 40% predicted of our group, represents more severe COPD than the majority of previous studies (Sethi 2002) and may explain the higher prevalence of LABC found.

The finding that the airway bacterial load was greater in the summer months is a novel one. Seasonality is a common phenomenon in COPD with the incidence of exacerbations being much higher during the winter months due to seasonal variation in respiratory viral infection. The relative frequency of exacerbations and therefore of prescribed courses of antibiotics was lower in the summer than the winter months in this study and this may explain the lower bacterial numbers during the summer months. This would suggest that airway bacterial load alone is not the key factor in exacerbation susceptibility but that other mechanisms are involved (Sethi 2002, Seemungal 2000). An alternative explanation would be that during the winter,

exacerbations are common due to viral infections and higher airway bacterial loads increase susceptibility to these. Therefore patients with higher loads in the winter are likely to exacerbate and hence will not be sampled in the stable state. During the summer months, the patients with high airway loads may be stable enough to be sampled in the 'stable state' and hence a confounding effect is observed.

This work included the first longitudinal prospective study to assess directly the relationship between lower airway bacterial load and decline in lung function in patients with moderate to severe COPD. The findings demonstrate a significant relationship between the sputum bacterial load and disease progression in COPD, showing that the rate of decline of FEV₁ was proportional to the rise in colonising bacterial load over the initial 1-year study period and that this effect was still observed over a longer follow-up period of up to 7 years.

Individuals who exhibited changes in the nature of bacterial colonisation suffered from faster declines in lung function than those with persistence of one or more bacterial species. These falls in FEV₁ were also associated with elevated levels of the potent neutrophil chemoattractant IL-8.

As discussed previously the presence of bacteria in the lower airway can result in a range of pathologic effects that are deleterious to lung function, including mucus hypersecretion and embarrassment of mucociliary clearance (Adler 1986).

Bacteria can affect the airway epithelium directly or via the recruitment of neutrophils (Ras 1990, Noguera 2001) bacteria may cause release of excessive amounts of neutrophil derived proteases resulting in damage to airway epithelial cells. Airway inflammation has been shown to increase as airway obstruction worsens, but bacterial colonisation was not originally considered as an explanation for this finding (Di Stefano 1995). Bacterial colonisation has been shown to be detrimental to lung

function in a number of pathological conditions, including cystic fibrosis and bronchiectasis (Packer 1992, Angrill 2002). Previous evidence that LABC contributes to worsening lung function in COPD can be found in data from a study which found that *H. influenzae* colonisation was associated with increased airway inflammation in patients with chronic bronchitis and airflow obstruction compared with patients with chronic bronchitis but without airflow obstruction, where airway inflammation was less marked (Bresser 2000).

The finding that higher airway bacterial loads are associated with greater airway inflammation in terms of sputum IL-8 levels has been shown by a number of groups previously, most notably in a large study of 160 patients with mixed airway obstructive diseases including COPD (Hill 2000). Those patients exhibiting a decline in FEV₁ during the study had higher levels of IL-8 than those with a stable or improving FEV₁. This finding suggests a mechanistic link between airway bacterial load, airway inflammation, and the associated deleterious effects on FEV₁.

Our group has previously reported that some patients with COPD develop exacerbations frequently, year on year and this group has increased stable airway inflammatory cytokines (IL-6 and IL-8) compared with those with a history of infrequent exacerbations (Bhowmik 2000). Patients with frequent exacerbations also demonstrate a faster decline in FEV₁ than infrequent exacerbators, with exacerbations contributing to approximately 25% of the observed excess lung function decline seen in COPD (Donaldson 2002). Analysis of data from the Lung Health Study (Kanner 2001) revealed that lower respiratory illnesses (exacerbations) in smokers are deleterious to FEV₁ and lends further support to the hypothesis that lower airway infection and associated inflammation contribute to lung function decline. However, this relationship was not significant in ex-smokers. This may appear at odds with the

analysis from our group; however, the Lung Health Study included patients with milder disease than our cohort. Therefore, in these patients with better lung function and preserved lung defences, airway bacterial colonisation is likely to play a lesser role in up-regulating inflammation and down-regulating resolution of inflammation. Therefore, the effects of exacerbations on disease progression are only seen in milder disease in active smokers. Whether this is due to direct effects of smoke on the airway or to effects mediated via bacterial infection is not known.

Previous longitudinal studies of the mechanisms of lung function decline have used possible surrogate markers of airway infection such as mucus hypersecretion. The Copenhagen City Heart Study found that chronic mucus hypersecretion was associated with an excess FEV₁ decline of 22.8 ml/year in men and 12.6 ml/year in women together with increased risk of hospitalization (Vestbo 1996). As bacterial colonisation is associated with mucous secretion (Stockley 2000), and patients with mucous secretion have more airway inflammation (Stanescu 1996); this again provides support for the role of LABC in the accelerated decline of FEV₁.

It has previously been reported that the presence of bacterial colonisation is directly related to exacerbation frequency, and patients with colonisation with *Haemophilus influenzae* have longer and thus more severe exacerbations (Patel 2002). As patients with a past history of frequent exacerbations have increased airway inflammation (Bhowmik 2000), the effects of lower airway bacteria on disease progression may be in part due to increased susceptibility to exacerbations or via modulation of inflammation at exacerbation.

In this study, the relationships between FEV₁ decline and features of LABC were strongest in the analyses, which included measures of "instability" of LABC such as changes in bacterial load and type. It is possible that such changes generate a

renewed stimulus to inflammation in the airway, in turn causing a more rapid decline in lung function. Indeed, there is increasing evidence that bacterial colonisation is a highly dynamic process and that changes in bacterial type are associated with the etiology of exacerbations (Sethi 2002). Bacterial colonisation itself is likely to be affected by exacerbations and their treatment, but to what degree this is remains uncertain. The interrelationships between host defences and bacterial infection in the stable state and at exacerbation are highly complex.

The multivariate regression analysis of the data from this study did not find a significant influence of active cigarette smoking on FEV₁ decline over 1 year, but this was apparent in the longer term follow up analysis. Indeed, although it is established that cigarette smoking is a risk factor for bacterial colonisation and itself leads to increased airway inflammation, the effects of smoking cessation on airway inflammation in severe COPD may not be as clearly defined as in a milder patient group. Indeed, study of the direct effects of smoking cessation on inflammation reveals that the pro-inflammatory effects of cigarette smoke may persist for several years (Lapperre 2006, Rutgers 2000). In a potentially colonised COPD population with severe airways disease the most significant modifiable factor affecting airway inflammation and consequent FEV₁ decline may be the airway bacterial load.

Many previous studies of lung function decline have largely been performed using patients diagnosed with bronchitis or airflow obstruction (Vestbo 1996, Fletcher 1976). Therefore, understanding of the natural history of lung function decline in more severe COPD is based largely on an extrapolation of observations from patients with milder COPD. The quantitative assessment of airway bacterial load and the related sputum markers of inflammation suggests a threshold level of colonisation in the order of 10^5 – 10^6 cfu ml⁻¹ above which LABC is a persisting drive to airway

inflammation (Hill 2000). However, the degree of airways obstruction at which the clinically significant effects of LABC occur and how bacterial colonisation affects the natural history of COPD in the longer term remains uncertain and requires further study.

It is possible that the findings of changes in total bacterial count and FEV₁ may represent changes associated with unrecorded exacerbations. However, diary cards were used to record all changes in symptoms on a daily basis and could therefore be used to detect all exacerbations both reported and unreported as previously described (Seemungal 1998). Furthermore, direct questioning of patients at each study visit was used to clarify symptomology and use of rescue medication during the previous 3 months. Therefore, exacerbations both reported to the study team or primary care requiring extra medication and those unreported but recorded on diary cards were included in assessment of baseline status. Indeed this is a fairly unique strength of longitudinal studies performed using daily diary card data collecting as well as routine clinic follow up, other studies rely on patient recall to ensure stable state lung function measurements and to exclude those juxtaposed to a recent exacerbation. In this study, assessment of diary cards for the 6-week symptom-free period effectively assured that patients had returned to baseline before sampling.

There was no identifiable relationship between the changes in FEV₁ observed and the frequency or timing of exacerbations in the one year study analysis. However, this study was not powered to investigate the relationship between exacerbation numbers and lung function decline demonstrated previously by our group (Donaldson 2002).

The results of this study demonstrate an association between airway bacterial colonisation and systemic inflammation and in particular the elevated plasma

fibrinogen found in patients with COPD. The finding that lower airway colonisation with a potentially pathogenic organism is associated with higher plasma levels of fibrinogen and IL-6 confirms the findings of an analysis of baseline data of a recent intervention study suggesting a role of airway bacteria in driving the systemic inflammatory response in COPD (Banerjee 2004). The finding that individual colonising bacterial species were associated with differing levels of plasma markers is a novel one. This suggests that variation in pathogen-host interaction results in a differential inflammatory response dependent on bacterial species.

The factors influencing systemic inflammation such as smoking and severity of disease are complex and also affect the nature of bacterial colonisation itself. The finding therefore that the type of colonising bacteria modulated plasma fibrinogen even when these co-factors were included in the analysis suggests an important and independent role for airway bacteria in the stimulation of the systemic inflammatory response.

Plasma fibrinogen is an important independent risk factor for coronary heart disease and stroke and a marker of systemic inflammation (Meade 1993, Ernst 1997, Danesh 1998, Smith 1997). The level of plasma fibrinogen is related to the severity of airways obstruction (Dahl 2001) in patients with COPD, suggesting that systemic inflammation and its associated consequences become more marked as disease progression occurs. In a similar manner, airway bacterial colonisation is related to disease severity with the prevalence of colonising PPMs greater in subjects with a lower FEV₁ (Zalacain 1999). The relationship therefore between airway bacteria and systemic inflammation found in this study provides a possible mechanistic link between these two findings.

Our group has previously described the changes in plasma fibrinogen and IL-6 at acute exacerbation in a similar population of patients in our cohort (Wedzicha 2000). Indeed a number of patients in that study were still present in the cohort four years later for this investigation into the role of bacterial colonisation. The initial study found a mean (SD) plasma fibrinogen level of 3.90 (0.67) g/l and median (IQR) IL-6 of 4.3 (2.4 – 6.8) in the stable state in patients with a mean FEV₁ of 1.06 (0.44)l. Four years on, in this study plasma levels measured in an identical manner were 4.23 (0.84) g/l and 6.48 (5.11-10.05) pg/ml respectively with a mean FEV₁ which had fallen to 0.96 (0.37)l. Thus the degree of systemic inflammation can be seen to have increased in association with airways disease progression in this group. This finding has been confirmed in a specific longitudinal analysis of changes in inflammatory indices in the cohort over time (Donaldson 2005).

An increase in bacterial load was found to be associated with the rate of decline of FEV₁ in the one year study. The inter-relationship between bacterial colonisation, airway inflammatory markers, fibrinogen, other systemic inflammatory markers and disease progression remains poorly understood. Further longitudinal studies are required to determine the complex relationships between these factors and the natural history of COPD and interventions such as antibiotic therapy required to determine causality.

The bacterial species which commonly colonise the lower airway of COPD patients, vary in their pathogenicity and ability to invade the respiratory epithelium. *Pseudomonas aeruginosa* can exist in an extraordinary range of environments and has evolved an array of mechanisms to evade innate and acquired immune defences. Secretion of specific factors such as Exo U by the Type III protein secretion system (Finck-Barbancon 1997) enables invasion through the respiratory epithelium and may

reduce the host's ability to limit the infection by this organism to remain within the airway itself, potentially leading to activation of systemic as well as airway inflammatory responses.

In comparison, *Haemophilus parainfluenzae*, a less invasive and virulent pathogen (Middleton 2003), is associated with lower levels of plasma markers in this study. The level of systemic inflammatory response to other PPMs such as *Haemophilus influenzae* or *Streptococcus pneumoniae* was intermediate between that associated with colonisation with *Haemophilus parainfluenzae* and *Pseudomonas sp.* *Haemophilus influenzae* is the most prevalent colonising bacterial species in this patient group (Hill 2000, Patel 2002) and plays an important role in modulating the nature of airway inflammation (Hill 2000). However different strains of this organism exhibit differing degrees of pathogenicity largely related to differing abilities to invade the respiratory epithelium (Bandi 2001, Ahn 2002). It is plausible that different strains of *Haemophilus influenzae* and indeed other bacteria stimulate the systemic inflammatory response by differing degrees and further studies involving typing of individual bacterial species are required.

Although plasma levels of fibrinogen and IL-6 were affected by the nature of colonising bacterial species in this analysis, the relationship between airway bacterial load and systemic inflammation was weak. The degree of neutrophilic airway inflammation has previously been shown to be closely related to the number of airway bacteria (Hill 2000), and activated neutrophils and their products play an important role in airway inflammation (Stanescu 1996) and have been demonstrated in peripheral circulation in patients with COPD (Noguera 1996, Noguera 2001, Noguera 2004, Koenderman 2000). The analysis of relationships in this study between airway bacterial load and airway IL-6 are not as close as those described for

cytokines more typically associated with neutrophilic inflammation. This suggests that other factors apart from bacterial colonisation modulate different aspects of the inflammatory response in the airway. Similarly the weak relationship between airway and systemic inflammation in this study suggests that the relationships between the two compartments are complex.

Indeed, there has been considerable recent debate about the links between airway and systemic inflammation in patients with COPD. It is now recognised that COPD is a multisystem disorder with evidence of widespread systemic inflammation (Agusti 2005). The clinical consequences of systemic inflammation are well described and include; muscle wasting and weakness, loss of fat free mass and in the most advanced cases cachexia (Schols 2000), in addition to the additional cardiovascular morbidity already discussed. The mechanisms leading to these complications are not fully understood. These are likely to be multifactorial and include oxidative stress (Rahman 1996), increased metabolic rate (Creutzberg 1998), hypoxia (Howes 1995) and inactivity associated with severe disease (Donaldson 2005), as well as direct effects of pulmonary inflammation on the systemic compartment.

These systemic effects of COPD contribute significantly to the excessive morbidity and early mortality seen in this patient group and further studies are required to determine how airway bacteria may modulate potential mediators of these extra-pulmonary manifestations of COPD, such as TNF- α (Li 1998), in a similar manner to fibrinogen or IL-6 as demonstrated in this study.

There has been controversy in the literature about the possible association between other causes of chronic infection, elevated plasma proteins and increased risk of CHD (Koenig 2003). Reports that *Helicobacter pylori* (Murray 1995) and *Chlamydia Pneumoniae* infection (Saikku 1988) diagnosed serologically were

associated with increased risk of CHD and that antibiotic eradication therapy resulted in a lower plasma fibrinogen (Torgano 1999) contrast with meta-analyses (Danesh 1998) and other studies (Regnstrom 1998) which contradict these conclusions suggesting no association between *H pylori* and systemic inflammation. This study, all be it in a distinct population of patients with moderate to severe COPD, lends weight to the argument that chronic infection does contribute to elevated fibrinogen and hence increased risk of CHD.

Studies of patients with chronic obstructive pulmonary disease have demonstrated elevated levels of not only plasma fibrinogen but also C reactive protein, tumour necrosis factor- α and soluble cytokine receptors (Schols 1996). This study did not assay CRP levels and this is an obvious limitation. This molecule may not only be a useful marker of systemic inflammation in COPD (Mannino 2003) but may also be directly involved in the pathogenesis of atheromatous plaques and hence cardiovascular disease (Lagrand 1999, Zwaka 2001). Furthermore, CRP levels may be modified with the use of cortico-steroids, as shown in a recent intervention study (Sin 2004), although the mechanisms by which this occurs are not understood and are likely to be complex as this form of therapy has limited effects on airway inflammation (Culpitt 1999).

The study has a number of drawbacks which warrant discussion. It has been performed in a group of patients with moderate to severe airways disease. It is likely that as airways obstruction progresses the contribution of bacteria to airway and systemic inflammation becomes more important and therefore the reproducibility of these findings in more mild disease needs to be determined. This study has concentrated on patients in the stable state. COPD exacerbations are an important stimulus to airway and systemic inflammation and whilst we have already described

the rise in fibrinogen at exacerbation, the role of bacterial infection in further stimulating airway inflammation warrants further study.

CONCLUSIONS

This study has demonstrated that airway bacterial colonisation may also play an important role in driving systemic inflammation in a group of patients with moderate to severe stable COPD. Plasma fibrinogen and IL-6 are modulated in a differential manner dependent on airway bacterial species and to a lesser extent bacterial load. *Pseudomonas* colonisation in particular is associated with heightened systemic inflammation and further studies to determine whether this results in greater morbidity and mortality are required. If the novel findings of this study can be shown to relate to clinical outcomes, the concept that bacterial species differentially stimulate an inflammatory response may inform on potential targets for future eradication studies. Such interventions could alter the natural history of this highly prevalent multi-system disease.

This study has also shown that LABC is an important determinant of decline of lung function in this group of COPD patients with moderate to severe disease. These findings suggest that appropriate antimicrobial therapy in colonised patients may have an important therapeutic effect, offering an opportunity to alter the natural history of this highly prevalent disease. Studies performed over a longer period are required to investigate further the interactions between LABC, smoking, and exacerbations and their effect on the accelerated decline in lung function, which is characteristic of COPD. Whilst a role of airway bacterial colonisation in COPD has been suggested for many years, understanding of viral infection of the airway in stable disease is poor. The next chapter presents the findings of the studies aimed at determining the role of chronic viral infection in COPD.

CHAPTER 5

VIRAL DETECTION, AIRWAY INFLAMMATION AND DISEASE PROGRESSION IN PATIENTS WITH STABLE COPD

In the previous chapter the findings that lower airway bacterial colonisation was associated with airway inflammation and lung function decline contribute to the hypothesis that airway infection may be an important factor in disease progression. This chapter describes the findings of prospective studies to determine the prevalence and associations of respiratory viral pathogens.

5.1 INTRODUCTION

It has been recognised for some time that viral infection is important in the pathogenesis of exacerbations (Smith 1976, Smith 1980, Greenberg 2000, Seemungal 2002). However, the role that viruses play in the aetiology of stable COPD remains uncertain.

There is some evidence in the literature that chronic airway infection with viruses may play a role in the development of airways obstruction. Adenovirus, a DNA virus, has been detected using PCR for genomic DNA, in lungs of COPD

patients (Matsuse 1992) and the virus trans-activating protein (E1A) can be found in both airway and alveolar tissue (Elliott 1995).

Whilst highlighting a role for adenovirus in the development of COPD studies to date have been cross-sectional in nature and relationships between viral infection and disease progression have not been demonstrated. Furthermore, a role for RNA viruses in the pathogenesis of COPD has been little explored.

This chapter describes the results of prospectively sampling the cohort of well characterised COPD patients in the stable state to determine the nature of viral and atypical bacterial persistence in the lower airway and to determine the associations of persistence with airway inflammation and lung function decline.

5.2 METHODS

The methods used in recruiting, follow-up and sampling of patients are common to all work in this thesis and are described in detail in Chapter 2. The work described in this chapter involved follow-up of 74 patients characterised in Chapter 3. Patients were followed up for 2.2 years for the analyses described below and were sampled when clinically stable, at least 6 weeks from a previous exacerbation and not taking oral corticosteroids.

Sputum samples were analysed for viral and atypical pathogens using PCR techniques described in Chapter 3. The remainder of the sputum sample was analysed for inflammatory cytokines (using ELISA); interleukin-6 (IL-6) and interleukin-8 (IL-8) and for myeloperoxidase (MPO) using ELISA. The first sample per patient underwent quantitative bacterial analysis.

PERIPHERAL BLOOD MONONUCLEAR CELL EXTRACTION

Venous blood was taken from 27 COPD patients, randomly selected from cohort volunteers before the RSV PCR results were known. Peripheral blood mononuclear cells (PBMC) were separated from 50 – 60 ml heparinised blood by sedimentation on a Ficoll-Hypaque gradient. PBMCs were resuspended in 90% human serum and 10% dimethyl sulphoxide (DMSO) and cryo-preserved in liquid nitrogen. The RSV PCR status of COPD patients was unknown during the time of blood collection and assay.

PBMC STIMULATION AND ELISPOT ASSAY.

Thawed PBMC were incubated in 96-well Millipore MAHA plates pre-coated with anti-human IFN- γ antibody 1-D1K (Mabtech AB, Nacka, Sweden). Cultures

were performed in 200 µl RPMI 1640 with 5% human serum AB (Sigma), L-glutamine, penicillin and streptomycin supplements and optimal concentrations of antigens determined in previous experiments. Stimulation was performed with : RSV at moi = 5, UV –irradiated RSV equivalent to moi =5 (inactivation was achieved by exposure to 1.2 joules of ultraviolet radiation in a Stratalinker 2400 Stratagene Europe, Amsterdam, The Netherlands). RSV A2 virus was grown in Hep-2 cells. Controls were culture medium without antigens, PHA (10 µg/ml) and Hep2 mock infected cell lysate. Whenever possible cultures were set up at 2.5×10^5 cells/ml, otherwise a correction factor was recorded. All cultures were set up in the same experiment. After 48 hours at 37 C the wells were washed, then incubated at 4 C overnight with 1µg/ml of biotinylated antibody clone 7-B6-1 anti human IFN-γ (Mabtech, Nacka, Sweden), the assay was completed as previously described. Spots were counted using an AID ELISPOT reader, software version 3.0. Raw spot numbers were normalized to cell input, negative control values were subtracted (Hep-2 cells controls for RSV and UV irradiated RSV, medium controls for all other antigens). Negative values (ie: higher numbers of spots in negative controls than in antigen stimulation) and any sample that failed to respond to PHA positive controls were excluded from analysis.

STATISTICAL ANALYSIS

The statistical analysis in common with the other results in this thesis is described in Chapter 3. In summary: baseline recruitment data are presented as medians (inter-quartile ranges (IQR)). The annual exacerbation frequency was calculated by dividing the total number of exacerbations per patient by the number of days the patient recorded data and multiplying by 365. Normally distributed data are

reported by means (standard deviations, SD) and skewed data by medians (interquartile range, IQR). Appropriate comparative statistical tests were performed dependent on the distribution of the data.

Specifically to analyse the associations between viral detection and lung function decline, I hypothesised that a greater frequency of detection of RSV in the stable airway is associated with greater inflammation and thus faster decline in lung function. Studies to date had been cross sectional in nature and hence pilot data on the periodicity of RSV infection was not available; a pre-determined a cut off limit was set of 50% detection rate to divide patients into high and low RSV groups. The rationale behind this categorisation was directed at attempting to ensure adequate sample size in each comparator group, as no pilot data was available an a priori split at 50% detection was made to avoid criticisms of post hoc analysis and to try to ensure a reasonable number of subjects in each group. The analysis of ELISPOT data, which included a sub-population taken at random from the larger cohort differed as these a priori groupings resulted in a very small sample size in the High RSV group. As this distribution made analysis of this data implausible an RSV grouping of PCR positive or negative at any point in the study was used, which allowed for a more even number of subjects in each group.

To analyze the effects of RSV colonisation independently of patient characteristics (smoking status, exacerbation frequency, bacterial load and starting FEV₁) on decline in lung function, we used the general linear mixed model (xtreg) procedure in STATA-5 software (Stata Corporation, Texas, USA). These procedures are designed for panel (cohort) data and are particularly useful where data are correlated, as in repeated-measures designs and where there are complex error structures. Independent variables of (a) time and (b) FEV₁ measurement during the stable state, (c) whether or not the patient was persistently colonised with RSV, which

required 50% of their samples to be RSV positive were included in the analysis. The fourth independent variable (d) was an interaction term obtained as the product of (a) and (c). Allowance for confounders was made by including whether the patient had a bacterial load or inflammatory marker less or greater than the cohort median, exacerbation frequency greater or less than the cohort median and active smoking status together with terms to adjust for the effect of these on FEV₁ decline. Also included in the regression model was the starting FEV₁ to adjust for differences in the rate of FEV₁ decline between patients with high or low starting FEV₁. The magnitude and significance of the effect of RSV colonization on decline were unchanged with inclusion of one or all of these confounders.

As multiple sputum samples were obtained during the study and the unit of analysis was the patient, the first sample obtained from each patient suitable for estimation of inflammatory cytokines was used in the analysis. p values ≤ 0.05 were regarded as significant.

5.3 RESULTS

Table 5.1 shows the baseline spirometric and other characteristics of 74 patients (45 male) who were sampled during the study. There were no differences between the baseline characteristics in terms of gender distribution, FEV₁, FEV₁% predicted, FVC, PEF, FEV₁ reversibility to beta agonists, years of smoking, current smoking status, exacerbation frequency of these patients and the 31 patients in the cohort who were not sequentially sampled (due to recruitment in the latter part of the study, use of long term oral steroids or intolerance of sputum induction). There were no significant differences in these variables between sputum producers and those requiring induction. The 74 patients provided 241 stable sputum samples suitable for processing for analysis. Of these patients 16 (of whom 8 died) of the 74 patients withdrew before the end of the study period. 9 subjects produced 1 sample, 15 subjects 2 samples, 20 subjects 3 samples, 17 subjects 4 samples, 6 subjects 5 samples, 5 subjects 6 samples and 2 subjects 7 samples. Diary card data was collected on a mean of 656 days per person, (maximum possible in study being 762 days), compliance with data collection was thus 86%.

5.3.1 RESPIRATORY VIRUS AND ATYPICAL BACTERIAL DETECTION IN STABLE COPD

The detection rates for all respiratory viral and atypical pathogens in stable state samples are summarised in figure 5.1. The most commonly identified virus was RSV. 59 of the 74 patients sampled had RSV detected in at least one stable sputum sample during the study. Overall RSV was detected in the stable state in 32.8% of the 241 stable sputum samples collected. There were no significant associations between the detection of one type of pathogen and another. Detection of viruses in the stable state was independent of time from the last exacerbation.

FIGURE 5.1 PCR DETECTION RATES FOR RESPIRATORY VIRAL AND ATYPICAL BACTERIAL PATHOGENS IN 241 STABLE STATE SPUTUM SAMPLES FROM 74 COPD PATIENTS.

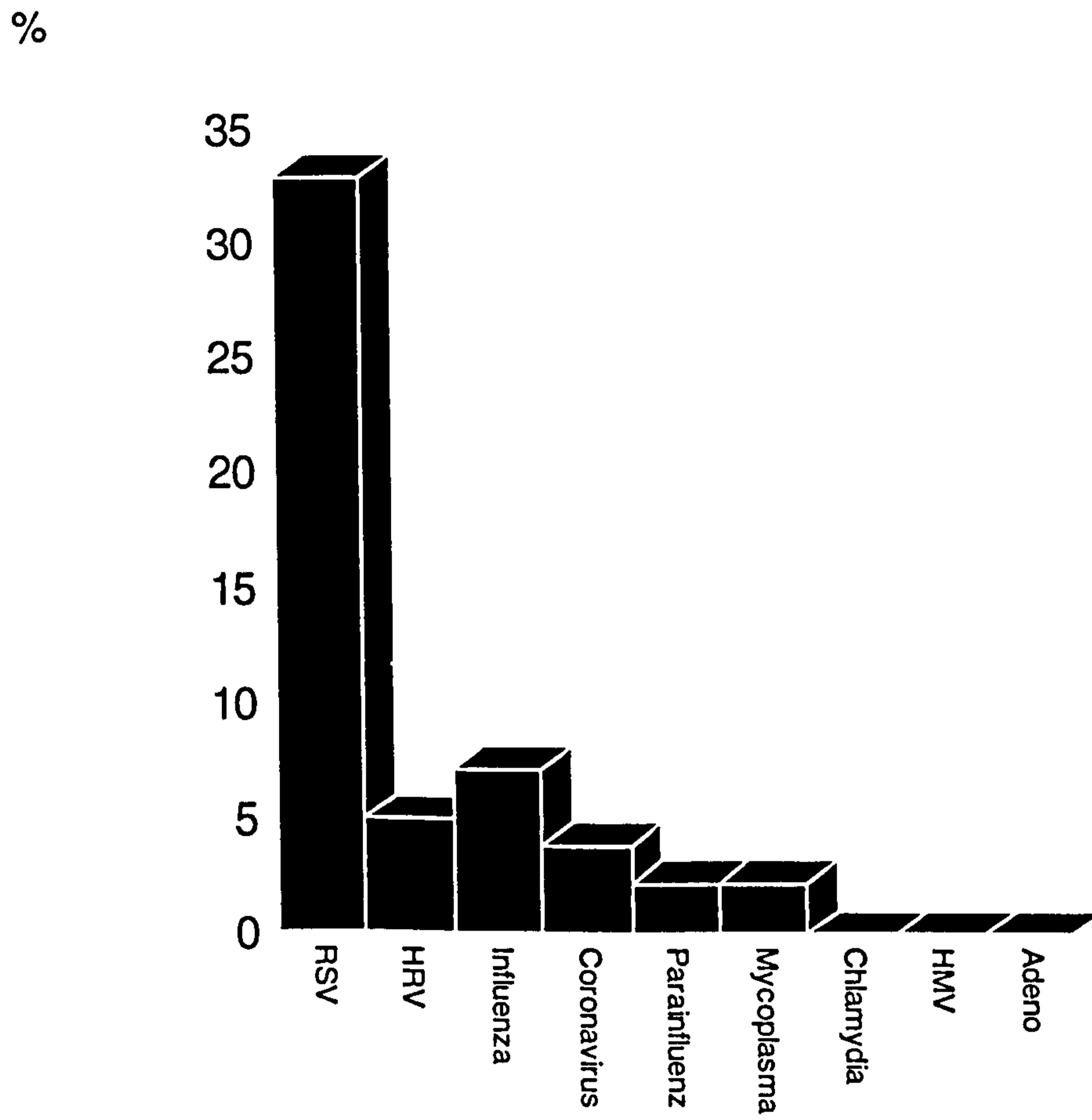


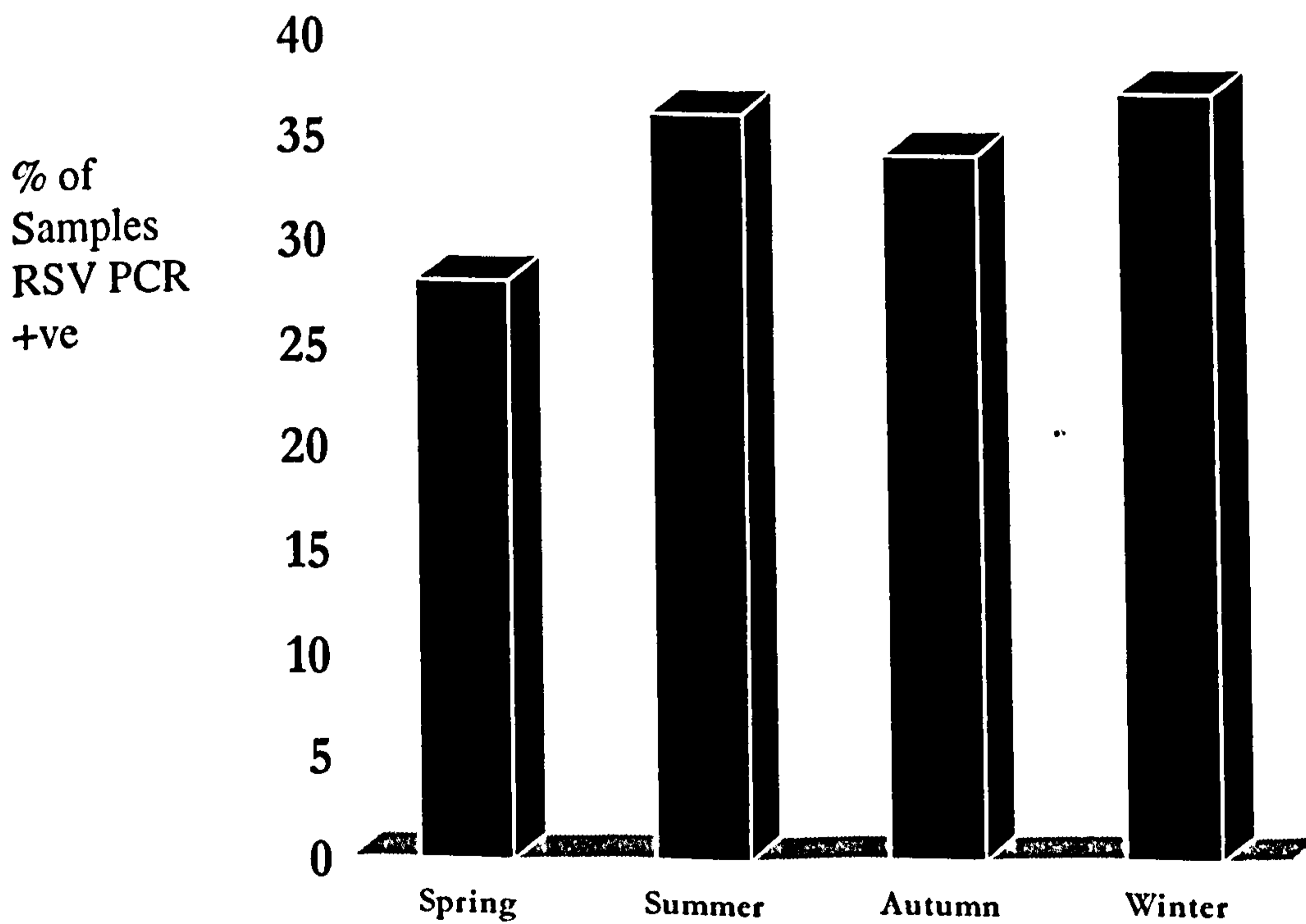
TABLE 5.1. CHARACTERISTICS OF 74 PATIENTS BY PATTERN OF RSV DETECTION

	<i>Low RSV Patients</i>		<i>High RSV Patients</i>		<i>P-value</i>
	<i>(n=56)</i>		<i>(n=18)</i>		
	Median	<i>IQR</i>	Median	<i>IQR</i>	
Age (years)	67.4	61.7 to 71.8	68.7	62.2 to 71.7	0.748
FEV ₁ (l)	0.95	0.77 to 1.37	1.09	0.73 to 1.38	0.735
FEV ₁ (% predicted)	39.2	29.6 to 57.8	42.9	29.0 to 59.4	0.759
FEV ₁ (% reversibility)	9.1	2.1 to 13.1	3.70	0.0 to 12.8	0.231
FVC (l)	2.37	1.68 to 2.90	2.60	2.00 to 3.28	0.297
FEV ₁ /FVC (%)	44.1	35.5 to 53.1	41.3	26.9 to 54.7	0.696
PEF (l/min)	152	120 to 238	186	147 to 230	0.661
P _a O ₂ (kPa)	9.00	8.13 to 9.62	8.47	8.27 to 9.22	0.387
P _a CO ₂ (kPa)	5.91	5.32 to 6.31	5.81	5.32 to 6.11	0.713
Smoking (years)	46	37 to 52	42.5	39 to 50	0.555
Exacerbation frequency (per year)	2.40	1.32 to 3.87	2.76	1.04 to 3.37	0.821
	%		%		P-value χ^2
Sex (Males)	60		60.8		0.976
Chronic dyspnoea	44.8		52.7		0.780
Chronic wheeze	34.3		32.4		0.211
Chronic Cough	41.0		37.8		0.506
Chronic sputum production	41.8		27.8		0.988
History of smoking	98.2		100.0		0.568
Smoking at recruitment	41.8		27.8		0.288

5.3.2 RSV DETECTION IN STABLE COPD

Of the samples, 36.8% of winter samples (December – February), 27.1% of spring samples (March-May), 36.7% summer samples (June-August) and 34.0% in autumn (September-November) were RSV PCR positive. The incidence of RSV detection in stable patients showed no significant seasonality ($p = 0.558$). There was no difference in detection rates between spontaneous and induced sputum samples.

Figure 5.2 RSV PCR Detection Rates from Stable State Sputum Samples According to Season of Sampling.



EVIDENCE FOR RSV PERSISTENCE

To study whether detection of RSV in sputum was due to persistence of the virus in particular individuals or merely as a result of sporadic infection, we compared the predicted probability of RSV detection occurring in all samples from an individual based on the overall detection rate of 32.8%, to the actual prevalence of patients with RSV throughout the study. The probability that an individual would have RSV in 4 out of 4 sputum samples based on a random distribution of the virus in this population is 0.0116 or 1.16%, the actual prevalence of patients with RSV in 4 out of 4 positive samples is 0.2 or 20% suggesting persistence in certain individuals.

Patients were categorized *a priori* using their stable samples RSV status in two groups: 'Low RSV' in which ($\leq 50\%$ of their samples were RSV PCR positive) and 'High RSV' ($> 50\%$ of samples were positive). There were 18 patients in the High RSV group, and 56 patients in the Low RSV group. There was no difference in the number of available samples per patient between the two groups (p-value = 0.424), or in baseline characteristics or the inhaled corticosteroid dosage between the two groups. See Table 5.2. Similarly, there were no differences in exacerbation frequency between the groups; 55% in the High RSV and 48% in the Low RSV group were frequent exacerbations, (with an annual rate of \geq the cohort median of 2.51 (IQR 1.27 to 3.83)).

Sequencing of both strands of 10 randomly selected RSV positive PCR samples confirmed homology in each case with RSV.

5.3.3 RELATIONSHIP BETWEEN VIRUS DETECTION AND AIRWAY INFLAMMATION

Detection of RSV was associated with higher levels of airway inflammation as measured by sputum IL6, IL8 and MPO. The relationships between RSV detection category and airway inflammatory markers are shown in Figure 5.3. Levels of individual airway inflammatory markers above the median were associated with a trend to faster

CHRONIC VIRAL INFECTION IN STABLE COPD

FEV₁ decline. Higher than median levels of IL-6 were associated with an additional FEV₁ decline of 6.7 ml/yr(CI 25.3 to -11.8), IL8 12.2ml/yr(30.9 to -6.4) and MPO 6.9ml/yr (22.1 to 8.3) but these did reach significance $p>0.18$ in all cases.

TABLE 5.2 ANALYSIS OF INFLAMMATORY MARKERS WITH RESPECT TO PERSISTENT COLONISATION WITH RSV; cross-section regression analysis assuming a poisson distribution in the dependent variable to allow for repeated measures.

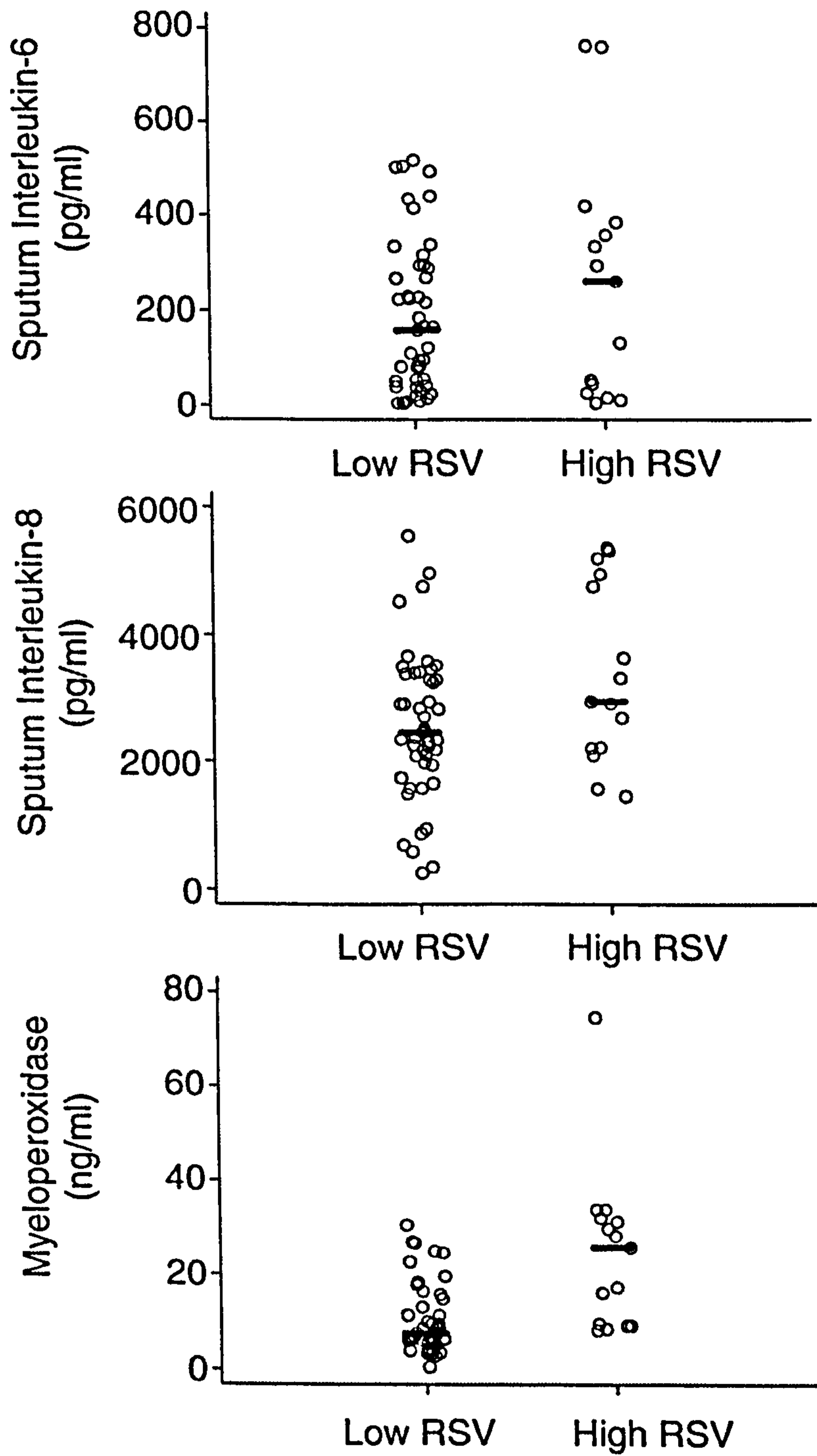
Parameter	Low RSV	Change in comparison in High RSV Group	95% CI	P-value
Sputum IL6 (pg/ml)	181	71	63 to 79	<0.001
Sputum IL8 (pg/ml)	2571	658	629 to 686	<0.001
MPO (ng/ml)	10.3	12.7	7.5 to 18.2	<0.001
Plasma IL6 (pg/ml)	8.81	-1.56	-2.99 to -0.12	<0.001

OTHER RESPIRATORY VIRUSES AND AIRWAY INFLAMMATION

Detection of HRV was associated with higher levels of airway inflammation.

Median sputum IL-8 level was 3177 pg/ml in HRV associated samples versus 2707 pg/ml in non-HRV samples, ($p=0.001$) and median sputum IL-6 was 232.2 versus 198.3 pg/ml, ($p=0.001$). No significant relationships between other respiratory viruses and airway inflammation were found.

FIGURE 5.3. COMPARISON OF SPUTUM IL6 PG/ML, IL8 PG/ML AND MYELOPEROXIDASE LEVELS (NG/ML), FOR LOW RSV AND HIGH RSV PATIENT GROUPS, $p < 0.001$, for difference between groups in all cases using cross-section regression analysis assuming a poisson distribution in the dependent variable to allow for repeated measures.

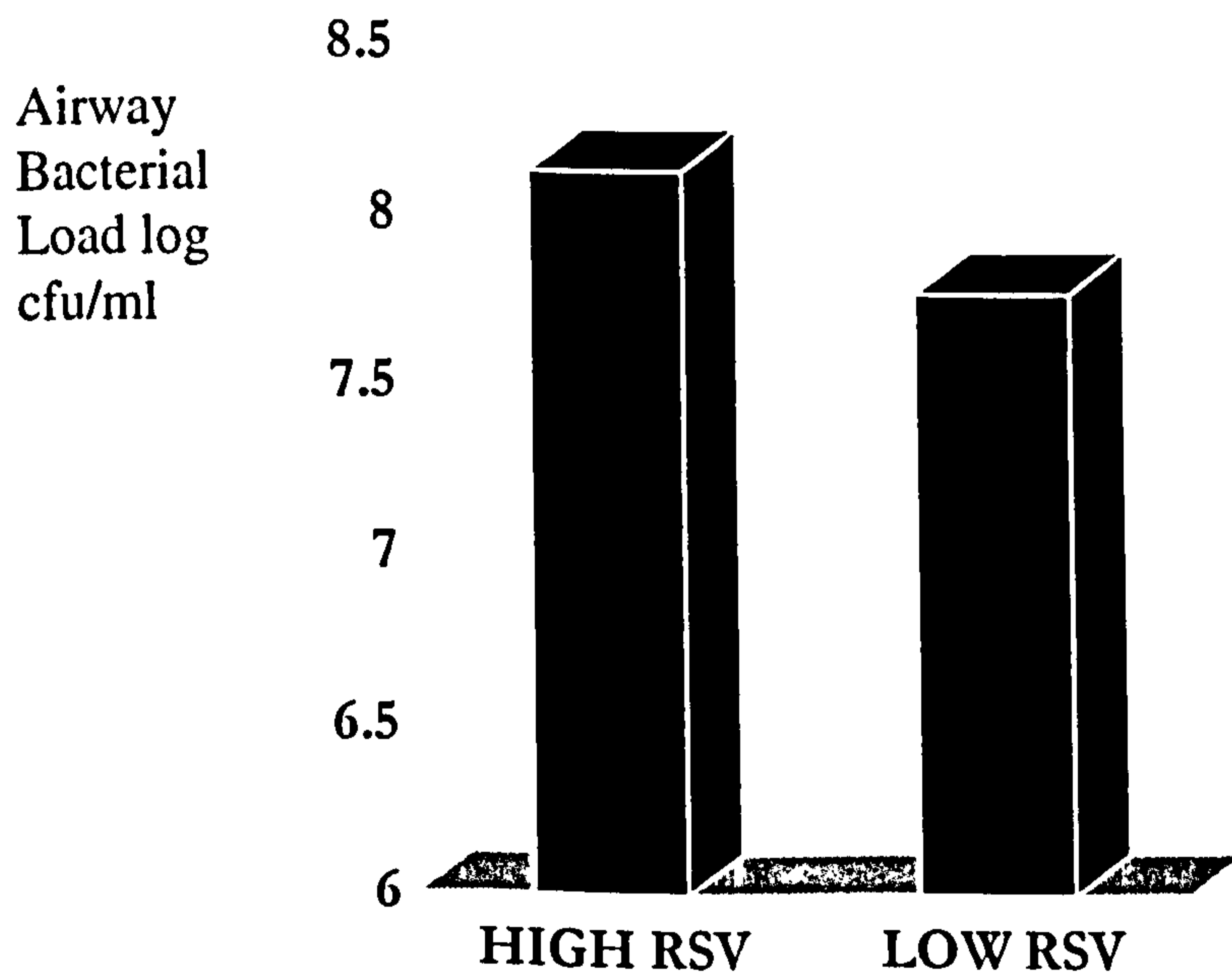


5.3.4 RSV AND BACTERIAL COLONISATION

Total bacterial load was available for 213 of the 241 samples. The overall prevalence of bacterial pathogens (PPMs) was 66.1% . The mean (SD) airway bacterial load was greater in the High RSV patients ($10^{8.12(0.48)}$ log cfu/ml, compared to Low RSV patients ($10^{7.76(0.58)}$ log cfu/ml) (p =0.024). There was no significant association between the detection of RSV and isolation of individual bacterial pathogens, or with all potential pathogenic organisms as a group (p> 0.17 in all cases).

FIGURE 5.4 AIRWAY BACTERIAL LOAD (LOG CFU/ML) IN PATIENTS

DICHOTOMISED INTO HIGH AND LOW RSV DETECTION GROUPS, P=0.024.



5.3.5 RSV AND FEV₁ DECLINE

Between the first and last RSV samples, there were 781 FEV₁ readings on the 74 patients; an average of 8.37 per patient. Those in whom RSV was detected in >50% of samples; High RSV (n=18) showed a decline in FEV₁ of 101.4 ml/year (95% CI 145.8 to 57.1) compared to 51.2 (70.8 to 31.7) ml/year in the Low RSV group (\leq 50% samples RSV PCR positive, n=56), the difference in rate of FEV₁ decline between these two groups was significant; $p = 0.01$, figure 5.4.

Of the 56 patients in the Low RSV group, 20 (35.7%) were active smokers, and of the 18 patients in the High RSV group, 4 (22.2%) were active smokers. If an adjustment for active smoking status was made in the analysis of the relationship between RSV detection and FEV₁ decline, there remained a significantly faster decline in the High RSV group by an additional 47.9 ml/year (87.8 to 0.8; $p = 0.019$). The effect of smoking itself on FEV₁ decline was not significant ($p = 0.205$) in this analysis. In a co-variate analysis adjusting for any effects of exacerbation frequency on differences in rate of FEV₁ decline between RSV groups there remained a faster decline in the high RSV group by 50.6 ml/year ($P = 0.013$).

To ensure that a variation in the number of samples obtained per patient did not affect the relationship between RSV detection and lung function decline, a sub-group analysis of patients with the same number of sputum samples was made using the sample number of 4 per patient. The relationship between RSV detection and FEV₁ decline remained significant $p = 0.009$, with a faster decline in FEV₁ of 52.2 (CI 13.3 to 91.3) ml/year in the high RSV group subset.

FIGURE 5.5 FEV₁ (L) DECLINE OVER STUDY PERIOD FOR: LOW RSV ($\leq 50\%$ OF SPUTUM SAMPLES WERE RSV PCR POSITIVE) N = 56; 51.2 ML/YR (CI 70.8 TO 31.7). HIGH RSV ($>50\%$ SPUTUM SAMPLES RSV PCR POSITIVE); N=18; 101.4 ML/YR (CI 145.8 TO 57.1) PATIENT GROUPS.

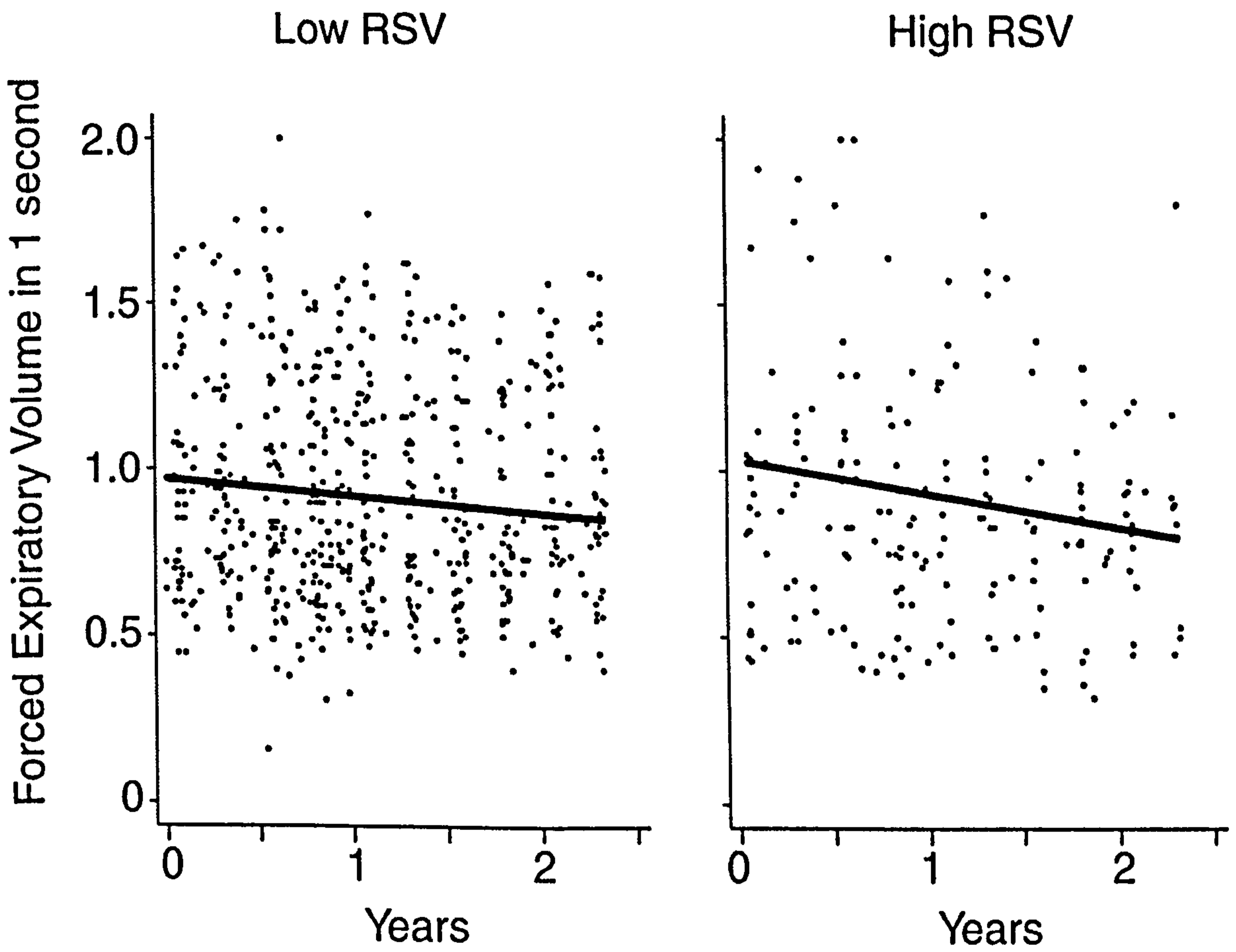
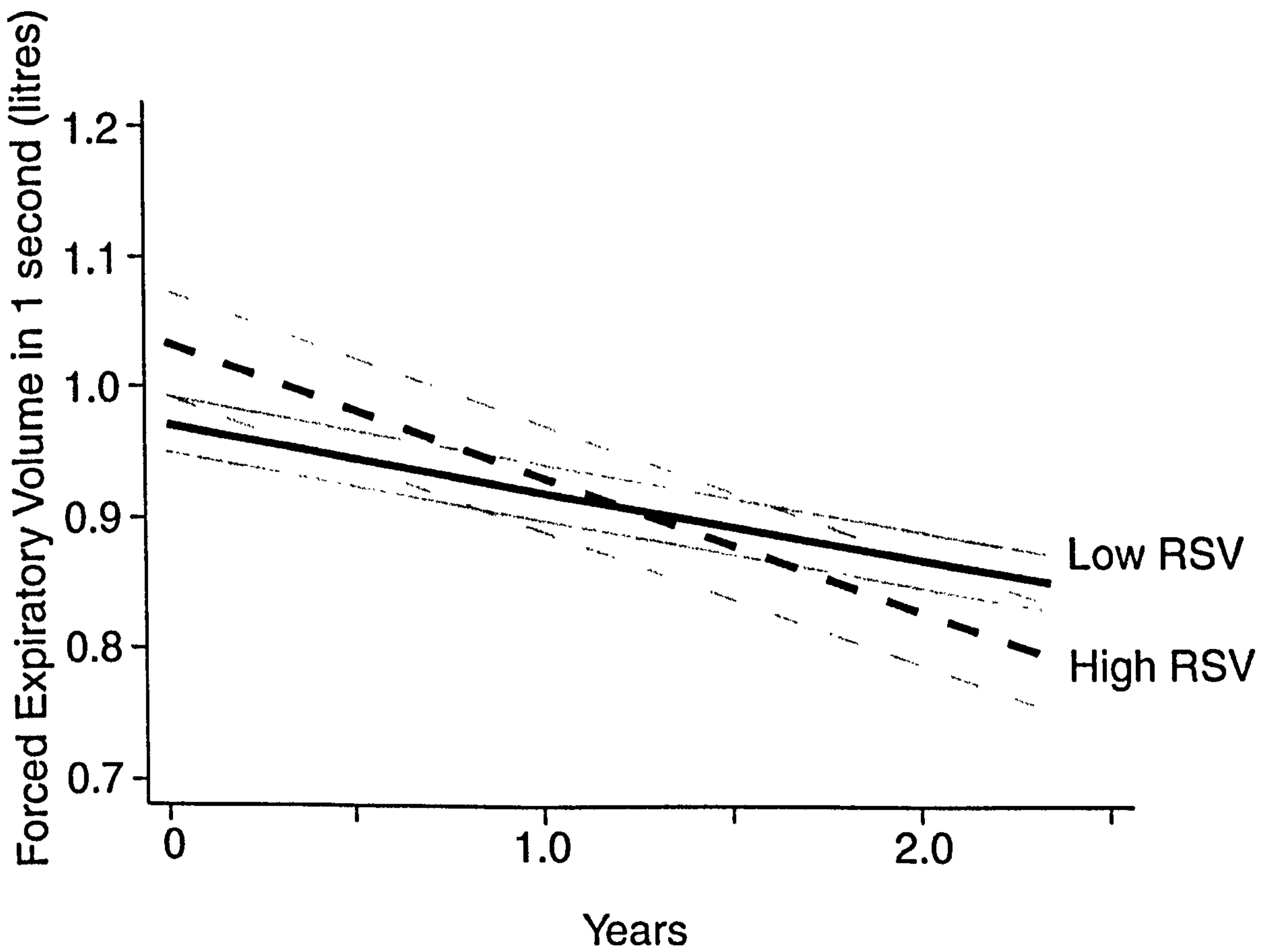


FIGURE 5.6 REGRESSION MODEL OF RSV DETECTION PATTERN AND FEV₁ (L) DECLINE

OVER STUDY PERIOD. Heavy lines represent decline in FEV₁ with standard errors for (a) Low RSV; ($\leq 50\%$ of sputum samples were RSV PCR Positive) $n = 56$; 51.2 ml/yr (SE: 10.1; CI 70.8 to 31.7), intercept 0.97 (SE 0.42; CI 0.90 to 1.05) (continuous lines), b) High RSV($>50\%$ sputum samples RSV PCR positive); $n=18$; 101.4 ml/yr (SE 0.017; CI 145.8 to 57.1) intercept 1.03 (SE 0.08; CI 0.88 to 1.19), (interrupted lines).



Multivariate analysis of RSV and other factors affecting FEV₁ decline

After allowance for total bacterial load, smoking status and starting FEV₁, High RSV patients had an FEV₁ decline of 114.7 ml/year (95% CI 186.8 to 42.4) compared to the decline of 56.6 ml/year (87.0 to 26.2) seen in Low RSV patients; $p < 0.05$ for the difference between the two groups. If the FEV₁ data was expressed as a percentage of the predicted FEV₁, High RSV patients had a significantly faster decline of 1.94% per year (3.63 to 0.2) in addition to the decline of 2.1% per year (3.3 to 0.9) in Low RSV patients ($p = 0.03$). If exacerbation frequency was included in the multivariate analysis, the faster decline in the High RSV group remained significant independent of any effects of exacerbations; 19.3(2.2 to 36.3) ml/year ($p = 0.027$). A multivariate analysis with airway inflammation (MPO), smoking status, starting FEV₁, bacterial load and exacerbation frequency revealed a non significant of an additional 6.9 ml/year (29.2 to -15.4, $p = 0.545$) relative to the low RSV group To determine if the association between RSV detection and lung function decline was independent of airway inflammation, inflammatory markers were included in the model of analysis. The differences between FEV₁ decline in High and Low RSV groups was not independent of associations with airway inflammation; additional decline in High RSV group; with IL-6 as co-variate; 42.9 ml/year (98.4 to -12.6, $p = 0.13$), for IL-8 39.9 ml/year (95.9 to -16.0, $p = 0.16$) and MPO 28.0 ml/year (79.8 to -23.8 , $p = 0.29$).

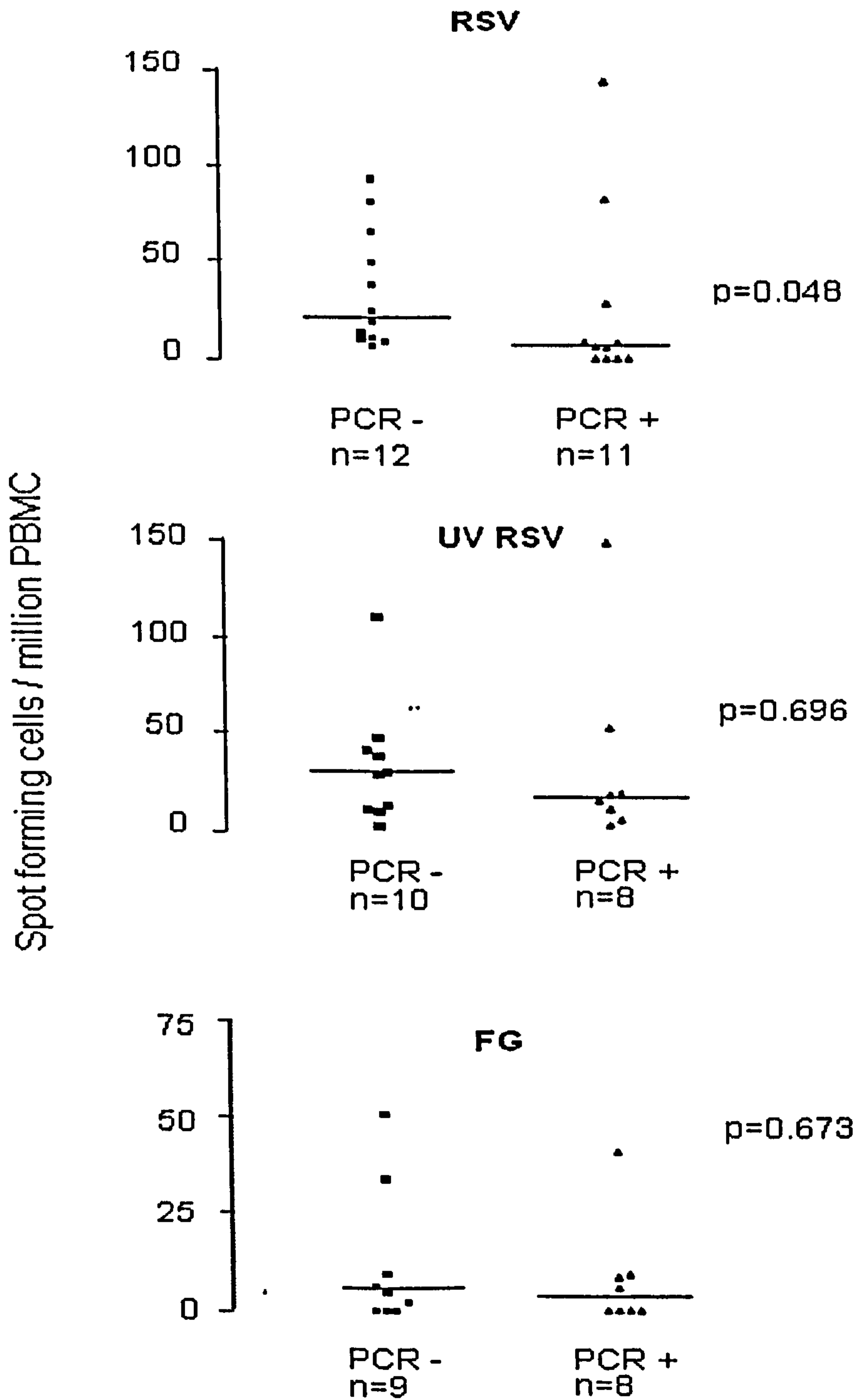
5.3.6 PBMC INTERFERON GAMMA RESPONSES TO RSV

The median frequency of IFN- γ producing cells in response to whole live RSV was significantly lower in COPD patients with RSV detected in one or more sputum samples during the study; RSV PCR +; 60 spot forming cells compared to 215 spot forming cells per 10^6 PBMC, in patients in whom RSV was not detected in any sputum samples, $p=0.048$, see figure 3. No significant difference was observed in responses to UV inactivated whole RSV (Table 4). Similarly no significant difference in IL-4 or IL-10 responses was found between COPD patients with or without RSV infection (data not shown).

TABLE 5.3 IFN- γ RESPONSES OF COPD PATIENTS TO RSV AND UV-INACTIVATED RSV. PBMC from COPD patients characterised for PCR detection of RSV were stimulated with RSV antigens and frequencies of responding cells were determined by ELISPOT. RSV PCR + refers to finding that sputum was PCR positive for RSV at least one time point for that patient. Median numbers of spot forming cells are shown for the two groups. Significant differences ($p<0.05$) are shown in bold characters.

Stimulation	RSV PCR-	RSV PCR+	p value (Mann Whitney U)
RSV (m.o.i.=5)	215	60	0.048
UV-RSV (equivalent to m.o.i.=5)	266	138	0.696

FIGURE 5.7 IFN- γ RESPONSE TO RSV IN COPD PATIENTS CHARACTERISED FOR RSV INFECTION BY PCR. Symbols indicate the numbers of spot forming cells per million PBMC in RSV PCR- (squares) and RSV PCR + (triangles) COPD patients. The horizontal lines and indicate medians. Y axis scale multiplied by 10^{-1} .



5.4 DISCUSSION

This is the first longitudinal prospective study to investigate the relationships between respiratory viral detection may play in the aetiology of inflammation and progression of stable COPD. The findings show that RSV RNA can be detected in the sputum of many COPD patients in the stable state, and its detection is associated with higher levels of airway inflammation, greater airway bacterial loads and an accelerated decline in lung function. Other respiratory viral and atypical pathogens are less commonly detected using the PCR assays described but when present HRV was associated with signals of greater airway inflammation.

- The experimental stimulation of extracted PBMC showed that cells isolated from subjects with RSV detection in sputum had an attenuated interferon- γ response to RSV stimulation, indicative of impaired T cell immunity to RSV which may facilitate viral persistence and explain the frequency of RSV detection.

RSV is an established cause of acute respiratory illness in children, and RSV bronchiolitis is associated with the development of persistent wheeze in later childhood (Stein 1999). It is not clear however, whether this association is causal. Whilst severe RSV bronchiolitis in early life is associated with a subsequent TH-2 type immune response it is not certain whether this is due to an existing predisposition to viral infection due to a pre-existing type -2 phenotype, or two to switch to a TH-2 pattern following RSV infection.

The classical pattern of acute infection in this childhood population is of marked seasonality of infection with narrow and discrete epidemics peaking during the month of January in the UK (Martin 1978). However what drives this marked seasonal pattern of infection has been postulated (Eccles 2002) but to where the virus lays dormant outside the RSV season is not understood.

RSV persistence has been demonstrated in the lungs of guinea pigs (Dakhama 1997) and mice (Schwarze 2004) for up to 150 days after experimental infection. In these animal models RSV persistence was associated with continued infectivity and chronic airway inflammation despite an appropriate systemic humoral and T cellular immune response. Furthermore persistent RSV infection is well known in children with T cell immunodeficiency, but isolation of the virus away from acute illness has not been achieved in healthy humans. This may be explained by the fact that RSV does not usually cause persistent infection in healthy individuals or due to persistence of the virus in anatomically discrete or immunologically privileged sites such as dendritic cells (de Graaff 2005), and neurons (Li 2006), which limit attempts to isolate the virus.

Hence the role of viral infection in the aetiology of airways obstruction in adults is not established. In the introduction the work on adenoviral persistence was introduced. Here a model of chronic viral infection in humans with obstructive lung disease has been described by one group. The concept that viral persistence or latent infection can result in expression of viral proteins by epithelial cells hence altering the cells response to inflammatory stimuli (Duerksen-Hughes 1989) has also been introduced. Not only has the adenovirus been detected but presence of high levels adenovirus protein expression in alveolar epithelium was associated with greater airway inflammation and more severe disease (Retamales 2001).

The studies on adenovirus introduce the distinct concepts of detection of genomic nucleic acid detection of viral proteins and isolation of intact virus. The studies reported in this chapter have used sensitive PCR techniques with primers directed at preserved regions of the viral genome. Interpretation of PCR positive results requires careful consideration. Whilst these techniques are highly sensitive, a positive result

only confirms the presence of the appropriate fragment of the viral genome. Even when contamination can be excluded as a cause of a positive result using sequencing techniques such a result does not confirm the presence of intact virus or indeed actively replicating virus. It remains a possibility that PCR positive results represent persistence of viral RNA following an acute episode rather than ongoing or latent infection. However the significant relationships between detection patterns and inflammation and clinical outcomes that were found suggest that a phenomenon of infection has been identified. Furthermore our PCR technique did not distinguish between RSV type in this study and it is not known whether the virus isolated from an individual is genetically stable and representative of chronic infection rather than recurrent re-infection. Further studies are required to confirm the exact nature of viral persistence in the lower airway.

In both acute and chronic models of infection, the key sites for RSV induced inflammation in the lung are the small airways. This is also the site where epithelial damage and increased mucous production result in small airway obstruction and hyperinflation in COPD (Yanai 1992) and the primary site for the persisting inflammation and airway obstruction which is characteristic of the disease (Hogg 2004, Saetta 1999). Biopsies of COPD subjects have demonstrated that the small airways are infiltrated with inflammatory cells, in particular CD8⁺ cells, neutrophils and airway macrophages (Hogg 2004, Saetta 1999). In particular the presence of CD8⁺ T cells and B lymphocytes organising into follicles, was associated with disease progression (Hogg 2004). The findings of this study may suggest CD8⁺ T cell populations, characteristic of COPD airway biopsies, may be recruited to the lung due to persistent viral infection, but an impaired immune response that is incapable of eliminating virus infection, permits on-going replication at low levels.

RSV detection was associated with heightened airway inflammation in terms of increased levels of IL-6, IL-8 and MPO. It is possible that RSV has direct pro-inflammatory effects on the airway which may contribute to faster decline in lung function. The observed association between RSV detection and FEV₁ decline remained significant if possible confounders such as airway bacterial load, exacerbation frequency, smoking status and baseline FEV₁ were included in the analysis. However, if airway inflammation was included in the covariate or multivariate model no significant effect of RSV on lung function independent of airway inflammation was seen. One explanation is that RSV, inflammation and decline are causally linked however it is also possible that inflammation predisposes the airway to viral persistence and that RSV detection is therefore an epiphenomenon.

The direct relationship between airway inflammation and lung function decline was not significant in this analysis. This may be due to other non-inflammatory processes such as airway remodelling (Hogg 2004), effects on cellular apoptosis which have been demonstrated with RSV infection (Krillov 2000) or via other arms of the inflammatory cascade. An alternative explanation of our findings is that patients with more aggressive COPD and faster disease progression have impaired acquired or innate immune responses, allowing RSV to persist. To determine if there is causal role of RSV infection in disease progression and airway inflammation, it would be necessary to eradicate RSV with vaccines or anti-viral drugs now under development (Zhang 2005).

Whilst RSV may have direct pro-inflammatory effects it is also possible that it acts by modulating the response of lung cells to other inflammatory stimuli, including bacterial lipopolysaccharide (Monick 2003) or by promoting neutrophil adhesion thereby augmenting lung damage (Wang 1998). Bacterial colonisation of the lower

airway in patients with COPD is well described and may provide a stimulus to airway inflammation and disease progression as discussed in Chapter 4. The additional presence of RSV in the lower airway may augment bacterially driven inflammation. It is also possible that chronic viral infection of the lower airway occurs due to increased susceptibility in individuals with existing bacterial colonisation, although we found no direct association between the isolation of PPMs and detection of RSV. It is feasible therefore that eradication of airway bacteria may result in local repair and improvement of local defences against viral persistence.

The relative importance of airway bacterial infection in COPD has been shown to increase with disease severity (Zalacain 1999). Again as discussed in the previous Chapter this study was performed with a group of patients with moderate to severe disease and therefore the findings that RSV can be detected and is associated with accelerated disease progression in this population may not necessarily be extrapolated to patients with milder disease. Further studies across the full spectrum of disease as well as in smoking and non-smoking controls are required to better understand the role of this pathogen in adults.

Acute infection with RSV has been associated with a down regulation of the appropriate anti-viral immune responses seen with infection by other respiratory pathogens (Legg 2003) and that abrogation of these TH1 responses is associated with greater RSV induced inflammation (Boelen 2002). The finding of impaired IFN- γ responses to RSV in the PBMC of COPD patients with PCR positive for the virus suggests that there are fewer circulating RSV specific CD8⁺ T cells in the peripheral blood of these patients would suggest that viral persistence has a comparable effect on immune responses to that seen during acute infection.

The mechanisms underlying the immuno-protective effects of RSV infection are not understood. The switch from a predominant type 1 response may be due to inhibition of RSV specific effector activity as demonstrated in a murine model (Chang 2002). This defect may occur at the level of antigen presentation and T cell activation as there is also evidence to support the role of dendritic cell infection in this process (Bartz 2003). Indeed dendritic cells have been shown to persist in the lung long after acute RSV infection and it is fascinating to postulate that these may be an important focus of viral persistence which also plays a role in orchestrating the anti-viral immune responses. These results on PBMC stimulation may also represent the effects selective recruitment of cells to the lung as a result of RSV infection, or a pre-existing deficiency of RSV specific CD8⁺ T cell mediated responses in certain individuals resulting in persistent RSV infection. Monitoring RSV detection by PCR in COPD patients over time combined with further work to elucidate the mechanisms of immuno-protection are required to improve understanding of this interesting field.

The use of sputum to detect the presence of RSV in the stable state allows repeated sampling of patients with advanced disease that alternative techniques such as bronchoscopic sampling do not. The use of sputum sampling in a longitudinal cohort study; inevitably results in a variable number of samples obtained per patient and this problem is further compounded by the use of quality control in processing to exclude inadequate samples. However, this quality control step is a vital one as issues regarding sampling methodology are of great importance. Recently a study reporting RSV detection in less than 1% of stable state sputum samples from a population of 112 patients with COPD (Falsey 2006). Whilst the PCR techniques utilised were sensitive the patient characterisation, sputum processing and sample quality control were very different from those methods used in the study reported in this Chapter.

Whilst PCR detection assays may appear, on paper, to be highly sensitive it is apparent that the type of biological sample taken; sputum rather than swab, the rapidity of sample processing and storage and the nucleic acid extraction techniques used, all impact upon the likelihood of detection of RSV RNA by PCR as RNA can degrade rapidly at room temperature.

This study did not examine the detection rates for RSV amongst normal controls, but the PCR detection techniques utilised in this study have also been used previously. In a number of these other studies healthy control groups have been studied and low detection rates have been found. Indeed we have studied adult control groups in two recent studies, the detection rates for RSV in both of these was zero (Creer 2005, Green 2002) suggesting that RSV detection in the stable state may be a factor in moderate to severe COPD, but not in healthy controls.

In comparison to the detection rates only relatively low numbers of other respiratory pathogens were detected by PCR in this study. Whilst these findings may suggest that persistent infection with these other pathogens is not common in this patient population there are also a number of explanations why detection rates may underestimate the true prevalence rate. Assay sensitivity is obviously key to interpretation of these findings, the PCR assays used have all been demonstrated to be sensitive in internal validation studies of samples from subjects with acute respiratory illnesses. However the RSV assay used was a nested PCR and the increased sensitivity offered by this system over single round detection assays may be of importance when detecting very low copy numbers of virus associated with persistence rather than acute infection. It is also important to note that the cohort of patients studied had been vaccinated against influenza and this may have had effects on infection with this pathogen. The finding that no adenovirus was detectable is

perhaps surprising when considering the findings of the Vancouver group (Retamales 2001) however this work used lung tissue rather than sputum and it is quite possible that different pathogens play different roles in different compartments of the lung.

When other viruses such as HRV have been detected a relationship with airway inflammation has been present, however the detection rates were too small to determine a clinical effect.

Debate exists over the role that atypical bacterial infection may play in stable COPD. Some reports suggest that persistence of *Chlamydia pneumoniae* in peripheral blood mononuclear cells was both common, and associated with clinical severity of disease (Blasi 2002). However previous work from our group found no *Chlamydia* using PCR analysis of induced sputum and naso-pharyngeal aspirates in the stable state and no associations with inflammatory indices at exacerbation (Seemungal 2002). The data from this study therefore confirms the previous findings of our group but whether *Chlamydia* can persist in a systemic compartment has not been excluded by this study.

CONCLUSION

We have shown that respiratory syncytial virus RNA can be detected from lower airway samples of some patients with COPD in the stable state, with evidence of persistent detection in certain individuals. RSV RNA detection was associated with greater airway inflammation and accelerated disease progression in these patients. Whilst RSV is not unique as a respiratory pathogen able to modulate immune responses (Alvarez 2005) a combination of its prevalence in this population combined with its ability to modulate immune responses may indicate a role not only in persistent infection but also in effects on response to other pathogens. These findings

suggest that RSV may play a role in the natural history of stable COPD, however further investigation into the nature and consequences of viral persistence are required to confirm whether RSV is a potential therapeutic target in this important patient group. The strict seasonality of RSV epidemics is well known and seen in all parts of the globe. In some areas, cases of clinical infection are virtually unknown outside the winter months, and the source of winter outbreaks has not been identified. Our finding that RSV was present outside the RSV season in some patients with COPD therefore is suggestive of chronic colonisation rather than repeated re-infection, but studies to determine the virus isolated from an individual is genetically stable and representative of chronic rather than re-infection are required.

The last two chapters have presented the findings which suggest that chronic infection may be important in stable disease. The role of infection in the aetiology of exacerbations has been studied in some detail; however these studies have focused largely on either viral or bacterial detection. The following chapter describes the finding of studies to determine the effects of each type of pathogen and co-infection at exacerbations of COPD.

CHAPTER 6

EFFECTS OF BACTERIAL AND VIRAL INFECTION AT EXACERBATIONS OF COPD

The findings that airway bacterial and viral infection is associated with increased inflammation and disease progression in patients with stable disease described previously are novel. This chapter presents the study of the role these potential co-pathogens play in modulating the nature and severity of acute exacerbations.

6.1 INTRODUCTION

It has been established that exacerbations of chronic obstructive pulmonary disease are characterised by increased airway (Seemungal 2000, Bhowmik 2000) and systemic inflammation (Wedzicha 2000), and that the marked variability in the nature of the inflammatory response, symptoms, clinical severity and time course of these events (Seemungal 2000) may be modulated by respiratory viral (Seemungal 2001, Seemungal 2000, Rohde 2003) and bacterial infection (Sethi 2000). However, studies to date have largely focused on detection of either viruses or bacteria and therefore interactions between these pathogens and hence the mechanisms which underlie the heterogeneity of exacerbations are poorly understood.

This study has demonstrated that bacterial pathogens are commonly identified in the lower airway of COPD patients in the stable state, in agreement with previously published work (Hill 2000, Monso 1995). The previous analysis has shown that airway inflammation is directly related to the number of bacteria in the lower airway in stable COPD and greater airway bacterial load is itself a stimulus to faster disease progression. Whilst it is known that airway bacterial load rises at exacerbation (Monso 1995) it is not known to what extent these rises modulate changes in airway inflammation and exacerbation severity, or what factors determine changes in bacterial load.

Respiratory viruses have been implicated as important infective triggers of exacerbations (Seemungal 2000, Rohde 2003, Greenberg 2000) with human rhinovirus (HRV) being the most commonly identified viral pathogen. Our group has previously shown that virus associated exacerbations are longer and thus more severe than non-viral exacerbations (Seemungal 2000) but whether this is due to the direct effects of viral infection on the airway or a mechanism involving changes in lower airway bacteria is not known.

Exacerbations are important clinically as they are responsible for a major proportion of primary care and outpatient consultations and inpatient admissions (Pauwels 2001). Whilst it is established that certain patients are at risk of frequent exacerbations (Seemungal 1998) and that the incidence of exacerbations is seasonal (Bhowmik 2005), the clinician remains unable to predict when a particular COPD patient is at risk of exacerbating. Understanding the relationships between baseline markers of infection and inflammation and the risk or timing of subsequent exacerbations would inform on the possibility of targeting treatment and potentially

preventing a proportion of these events and their associated morbidity and healthcare costs.

Our current understanding of the inflammatory milieu in the airway of patients with COPD is limited to a relatively small number of studies which have concentrated on the measurement of a limited range of inflammatory markers related to epithelial, neutrophilic and lymphocytic inflammation. The assays used in this study have concentrated on markers which have been shown to relate to clinical outcomes and trigger factors. Measurement of changes in a broad spectrum of inflammatory proteins and peptides at exacerbation may improve our understanding of the disease process and also identify new targets for therapeutic interventions.

This aspect of the study examined the hypothesis that the heterogeneity of inflammatory, symptomatic and physiological responses at COPD exacerbation is modulated by airway bacterial and viral infection and that a combination of these pathogens would result in greater airway and systemic inflammation and hence clinical and physiological indices of exacerbation severity. The analysis is in two parts; firstly a paired analysis of changes in exacerbation parameters compared to corresponding baseline values in 56 exacerbations in 39 patients. Secondly an analysis of 68 exacerbations in 68 patients without predated baselines. We also determined the relationships between stable state inflammatory markers and time to exacerbation and in a small sample determined the changes in an array of markers at exacerbation using proteomic analysis of sputum.

6.2 METHODS

The cohort recruitment, follow up and sampling was performed as described previously. Patients were reviewed at recruitment and with their diary cards every three months in the study clinic to monitor compliance with data collection, record changes in medication and baseline lung function. Review of diary cards was utilized to ensure that stable sampling was performed when subjects had been clear of exacerbation symptoms and had completed any exacerbation treatment for at least six weeks.

Patients were encouraged to report symptom changes to the study team; they were assessed within 24-48 hours in the study clinic by a respiratory physician prior to initiation of therapy for the exacerbation. The diagnosis of an exacerbation was based on symptomatic criteria previously validated by our group. Exacerbation symptoms were binary coded as present or absent and the sum of these at exacerbation onset was termed the symptom count, which has been validated as a marker of clinical severity. Lung function measurement and sputum and blood sampling was performed on patients prior to the initiation of exacerbation treatment. The treatment given was at the discretion of the attending clinician.

Sputum was sampled if the subject met criteria for the stable state at three monthly review and also at presentation of exacerbation. Immediately following lung function measurement patients were asked to spontaneously expectorate sputum into a sterile container. Patients unable to produce a sample of sputum spontaneously underwent sputum induction.

6.2.1 PROTEOMIC METHODOLOGY

MICROARRAY MANUFACTURE

Glass slides were cleaned with 3-cyanopropyltriethoxysilane. The slides were equipped with a Teflon mask, which divided the slide into sixteen 0.65 cm diameter wells or circular analysis sites called subarrays (Figure 6.1). Printing was accomplished with a Perkin-Elmer Spot Array Enterprise non-contact arrayer equipped with piezoelectric tips, which dispense a droplet (~350 pL) for each microarray spot. Antibodies were applied at a concentration of 0.5 mg/mL at defined positions. Each chip was printed with sixteen copies of one type of array. A set of cytokines, selected for potential biological relevance, was printed with quadruplicate spots in each subarray. After printing, chips were inspected using light microscopy. If the percentage of missing spots observed was greater than 5%, then the batch failed and the slides were discarded immediately. For all print runs described herein, 100% of the antibody features and >95% of the biotin calibrators were printed. Microarray chips were validated in concert with a set of qualified reagents in two ways. First, mixtures of 1-3 different cytokines were prepared so as to provide a high intensity signal and applied to 14 wells of a chip (with each well being treated with a different mixture up to the total complement of detector antibodies) and two arrays were used as blank controls.

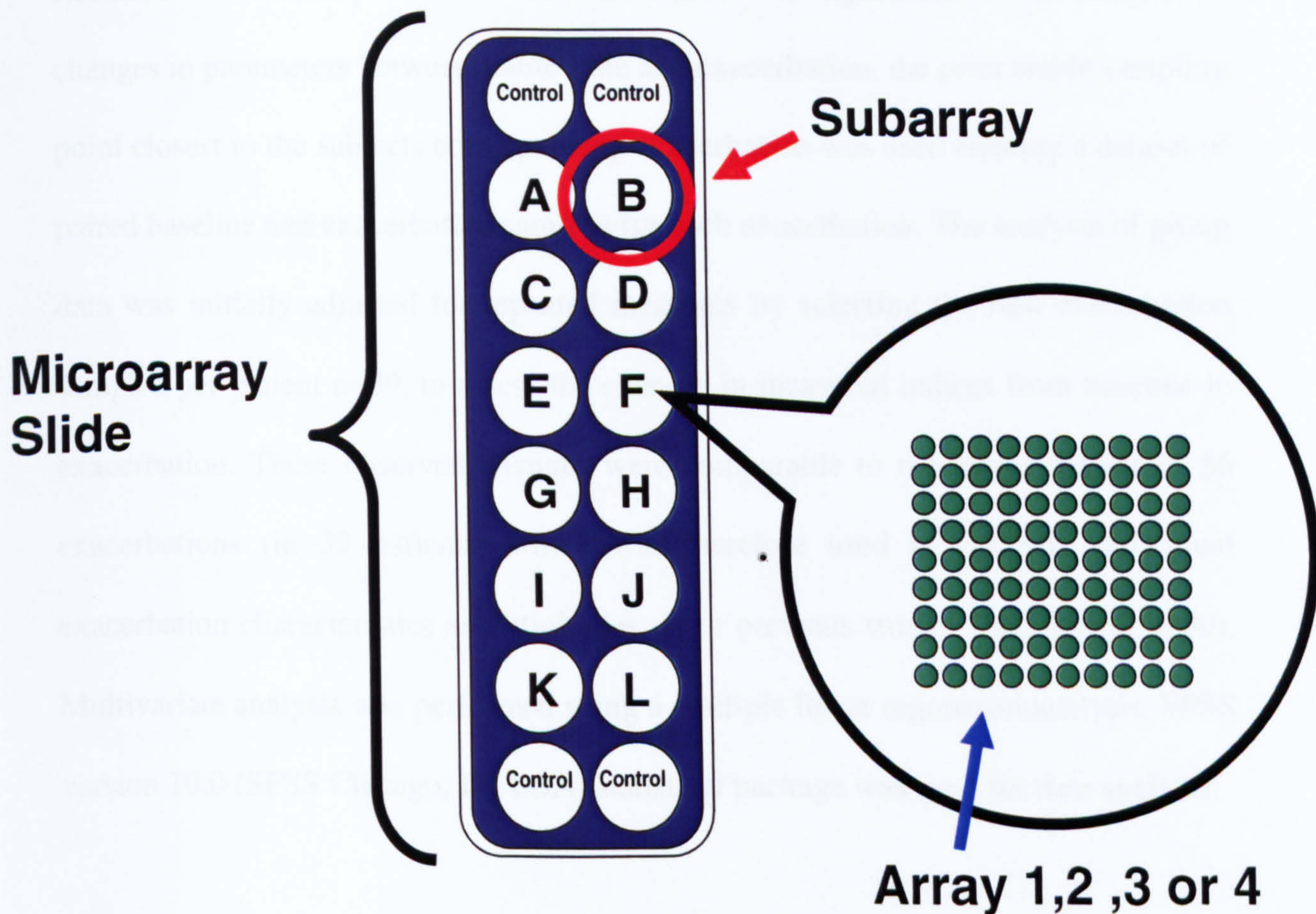
The chips were developed and scanned and the resulting signals were compared to the positional map of the particular array. Second, a titration QC for all analytes of a specified array using known sample matrices was performed. Normal human serum and heparinized plasma were assayed neat or spiked with purified recombinant cytokines representing all analytes in the array. Spiked mixtures were then titrated down the subarrays of a slide from 9,000 pg/mL to 37 pg/mL of spiked

cytokine concentrations along with two subarrays for each un-spiked control sample. Thus the lower limit of sensitivity in plasma being at least 37pg/ml. The data was quantified and for every analyte in the array a titration curve was generated to examine feature intensity behavior as a function of concentration. Taken together, this data was used to confirm the activity of array features and reagent sets.

ROLLING CIRCLE AMPLIFICATION (RCA) IMMUNOASSAY

Prior to assay, the slides were removed from storage at room temperature in sealed containers and opened in a humidity controlled chamber (45-55%). Slides were blocked with Seablock (Pierce Chemical Co.), diluted 1:1 with PBS for 1 h at 37°C in a humidified chamber. Following removal of the blocking solution, they were washed twice with PBS prior to application of sample. On each slide, control serum (Jackson ImmunoResearch Laboratories, Jackson, US) was applied to two subarrays, and a negative control with PBS buffer applied to two subarrays. The test samples were assayed on the remaining 12 subarrays. Twenty μL of the treated sample were then applied to each subarray. The basics of performing immunoassays with RCA signal amplification has been described (Schweitzer 2002) and we used SOPs derived from the protocols used in that study. Slides were scanned (GenePix 4000B, Axon Instruments Inc.) at 10 μm resolution with a laser setting of 100% and a PMT setting of 550 V. Mean pixel fluorescence values were quantified using the fixed circle method in GenePix Pro 4.0 (Axon Instruments, US). Using proprietary software, the fluorescence intensity of microarray spots was analyzed for each feature and sample, and the resulting mean intensity values were determined. Dose-response curves for selected cytokines were examined, ensuring that feature intensity is above background and exhibiting increasing intensity with increasing analyte concentration.

FIGURE 6.1 SCHEMATIC REPRESENTATION OF A SAMPLE PROTEIN MICROARRAY SLIDE WITH 16 SUBARRAYS. Subarrays refer to the 16 wells, or circular analysis sites, on the slide. Array refers to the antibody content printed in a well. Each microarray slide contains only one type of array.



STATISTICAL ANALYSIS

The statistical analysis used for this section of the study was conventional and described in Chapter 3. Changes in parameters from the stable state to exacerbation were assessed using a paired analysis of the stable sample data taken preceding the exacerbation studied. p values ≤ 0.05 were regarded as significant. In the analysis of changes in parameters between stable state and exacerbation, the prior stable sampling point closest to the subjects corresponding exacerbation was used creating a dataset of paired baseline and exacerbation samples for each exacerbation. The analysis of group data was initially adjusted for repeated measures by selecting the first exacerbation sampled per patient $n=39$, to assess the changes in measured indices from baseline to exacerbation. These observed changes were comparable to the larger dataset of 56 exacerbations (in 39 patients) which was therefore used to compare individual exacerbation characteristics and etiologies, as in previous studies (Seemungal 2000). Multivariate analysis was performed using a multiple linear regression analysis. SPSS version 10.0 (SPSS Chicago, IL, USA) statistical package was used for data analysis.

6.3 RESULTS

Table 6.1 shows the baseline characteristics of the 39 patients in the London COPD Cohort sampled during the study of the effects of bacterial and viral infection on physiological and inflammatory indices at exacerbation. Fifty six paired stable and exacerbation samples were obtained from 39 patients.

Of these 39 patients, 15 were on long term oxygen therapy, all patients were receiving long term inhaled corticosteroids (median (IQR) dose 500 (400-1500) micrograms per day beclomethasone equivalents), no patients were on long term oral corticosteroids, and all patients received regular inhaled bronchodilators. The remainder of the patients did not exacerbate during the sampling period (n = 26), did not report an exacerbation to the study team, received antibiotic treatment before sampling or were unable to provide an adequate sputum sample (n =14). The sampled patients did not differ significantly in terms of their baseline characteristics from those who were not sampled.

TABLE 6.1 BASELINE CHARACTERISTICS OF THE 39 PATIENTS SAMPLED AT
BASELINE AND AT EXACERBATION.

	Mean (SD)
Age (years)	68.8 (6.9)
FEV ₁ % Predicted	40.6 (15.6)
Pa O ₂ (kPa)	8.86 (0.89)
Pa CO ₂ (kPa)	5.67 (0.74)
	Median (IQR)
Smoking (pack years)	41.0 (30-52)
Sex (males)	60%
Active Smokers	23%
Chronic Sputum Producers	43%

6.3.1 CHANGES IN LUNG FUNCTION AND INFLAMMATORY MARKERS AT EXACERBATION.

Table 6.2 shows the stable and exacerbation FEV₁, airway bacterial load, sputum IL6 and IL8 and blood IL6 levels for all the 56 sampled exacerbations and on a per patient basis (n=39). In both analyses; the mean FEV₁ fell at exacerbation and the mean airway bacterial load rose significantly. Exacerbations were associated with increased airway inflammation in terms of sputum IL 8. The rises in levels of sputum and serum IL6 did not reach statistical significance.

TABLE 6.2 FEV₁, INFECTIVE AND INFLAMMATORY CHANGES FOR BASELINE (STABLE STATE) AND EXACERBATION. Sample Points and on a per patient basis (n=39) and for all 56 sampled exacerbations (n=56).

	Baseline	Exacerbation	p Value *
N=39	Mean (SD)	Mean (SD)	
FEV ₁ (l)	0.95 (0.36)	0.87 (0.30)	0.012
Bacterial Load (log cfu/ml)	7.47 (0.73)	8.16 (0.76)	0.001
	Median (IQR)	Median (IQR)	
Sputum IL8 (pg/ml)	3647 (2930-4466)	4409 (3983-4787)	0.002
Sputum IL6 (pg/ml)	146.0 (20.4-246.9)	187.6 (49.9-269.1)	ns
Serum IL6 (pg/ml)	4.73 (3.34-7.07)	6.0 (4.25-13.18)	ns
N=56	Mean (SD)	Mean (SD)	p Value *
FEV ₁ (l)	0.96 (0.37)	0.89 (0.32)	0.015
Bacterial Load (log cfu/ml)	7.50 (0.74)	8.09 (0.76)	0.001
	Median (IQR)	Median (IQR)	
Sputum IL8 (pg/ml)	3604 (2913-4390)	4288 (3991-4765)	0.005
Sputum IL6 (pg/ml)	146.7 (29.4-233.0)	185.0 (50.0-280.0)	ns
Serum IL6 (pg/ml)	4.6 (3.1-7.1)	6.6 (4.0-11.7)	ns
	Mean (SD)	Mean(SD)	
Cells (x10 ⁶)/g sputum	4.49(3.81)	13.20(13.87)	ns
% Neutrophils	64.10(24.27)	79.50(23.20)	ns
% Macrophages	19.57(22.48)	34.75(24.12)	ns

6.3.2 AIRWAY BACTERIOLOGY AND VIROLOGY

Airway total bacterial load rose in all samples (n=56) from $10^{7.50(0.74)}$ log cfu/ml in the stable state to $10^{8.09(0.76)}$ log cfu/ml at exacerbation, and also rose significantly in data adjusted for repeated measures (n=39); ($10^{7.47(0.73)}$ to $10^{8.16(0.76)}$ p=0.001). The prevalence of potentially pathogenic organisms (PPM) rose from 48.2% at baseline to 69.6% at exacerbation (n=56) with the remainder of samples demonstrating non-specific bacterial growth (NSG). The most frequently isolated organism was *Haemophilus influenzae* in 14.3% of stable and 37.5% of exacerbation samples, with *Streptococcus pneumoniae* in 8.9% and 14.3%, *Moraxella catarrhalis* in 7.1% and 14.3%, *Haemophilus parainfluenzae* in 10.7% and 0%, *Staphylococcus aureus* in 3.6% and 0%, *Pseudomonas aeruginosa* in 1.8% and 1.8% and *GNEB* in 1.8% and 1.8%, respectively, the remainder demonstrated NSG.

HRV PCR was positive in 11 out of 56 (19.6%) exacerbations and cold symptoms, a measure of putative viral infections, present in 18 (32.1%). The presence of cold symptoms and HRV positive PCR sputum were related; continuity adjusted χ^2 4.11, p =0.04. Exacerbations associated with colds were associated with a greater percentage fall in FEV₁ -14.03 (13.91) than those without colds -3.01(16.38) %, p=0.043.

6.3.3 RELATIONSHIPS BETWEEN INFECTION, INFLAMMATION AND CLINICAL INDICES AT EXACERBATION

Changes in airway bacterial load (n=39) were related to exacerbation severity in terms of changes in lung function and airway inflammation. The rise in airway bacterial load from baseline to exacerbation was related to the percentage fall in

FEV₁; $r = 0.35$, $p = 0.048$. The magnitude of the rise in airway IL8 at exacerbation was related to the rise in airway bacterial load, $\rho = 0.37$, $p = 0.022$.

The changes in airway and serum IL6 observed from stable state to exacerbation were not related to changes in bacterial load; $\rho = -0.76$, $p = 0.649$, $\rho = 0.144$, $p = 0.482$, respectively. However the rise in systemic inflammation was related to that of airway inflammation; change in sputum IL6 correlated with the change in serum IL6; $\rho = 0.435$, $p = 0.023$, $n=39$.

Changes in airway and systemic markers of inflammation at exacerbation were modulated by existing disease severity. The observed change in sputum IL8 at exacerbation per patient was inversely related to the baseline FEV₁ (percent of predicted); $\rho = -0.298$, $p = 0.05$, as was the change in sputum IL6; $\rho = -0.358$, $p = 0.02$ and the change in serum IL6; $\rho = -0.392$, $p = 0.03$. Thus patients with more severe COPD exhibited greater rises in inflammation at exacerbation compared with those with more mild disease.

6.3.4 EFFECTS OF INDIVIDUAL PATHOGENS AND THEIR INTERACTIONS

HAEMOPHILUS INFLUENZAE

H influenzae related exacerbations were associated with higher airway bacterial load ($n=56$); $10^{8.52(0.39)}$ log cfu/ml compared to those where it was not isolated; $10^{7.85(0.81)}$ log cfu/ml, $p=0.001$. There was a trend towards more severe drops in FEV₁ (expressed as a percentage of baseline); $-11.91(15.32)\%$ with *H influenzae* present, than without $-1.20(15.09)\%$, $p = 0.057$. Where 'de novo' *H influenzae* infection occurred (ie *H influenzae* present at exacerbation but not present in stable sample), there was again a greater exacerbation bacterial load $10^{8.56(0.40)}$ compared to where *H influenzae* was not isolated $10^{7.81(0.84)}$ log cfu/ml, $p = 0.001$, and the

percentage fall in FEV₁ was significantly worse in this group -14.60(12.39) compared to the non-*H influenzae* exacerbations -1.17 (15.99) %, p= 0.027.

FIGURE 6.2 EFFECT OF AIRWAY PATHOGENS AND PATHOGEN COMBINATIONS ON PERCENTAGE FALL IN FEV₁ AT EXACERBATION. Columns represent mean values with error bars as standard error of mean. * represents significant (p<0.05) difference between this category and cold and bacterial pathogen category. n=56.

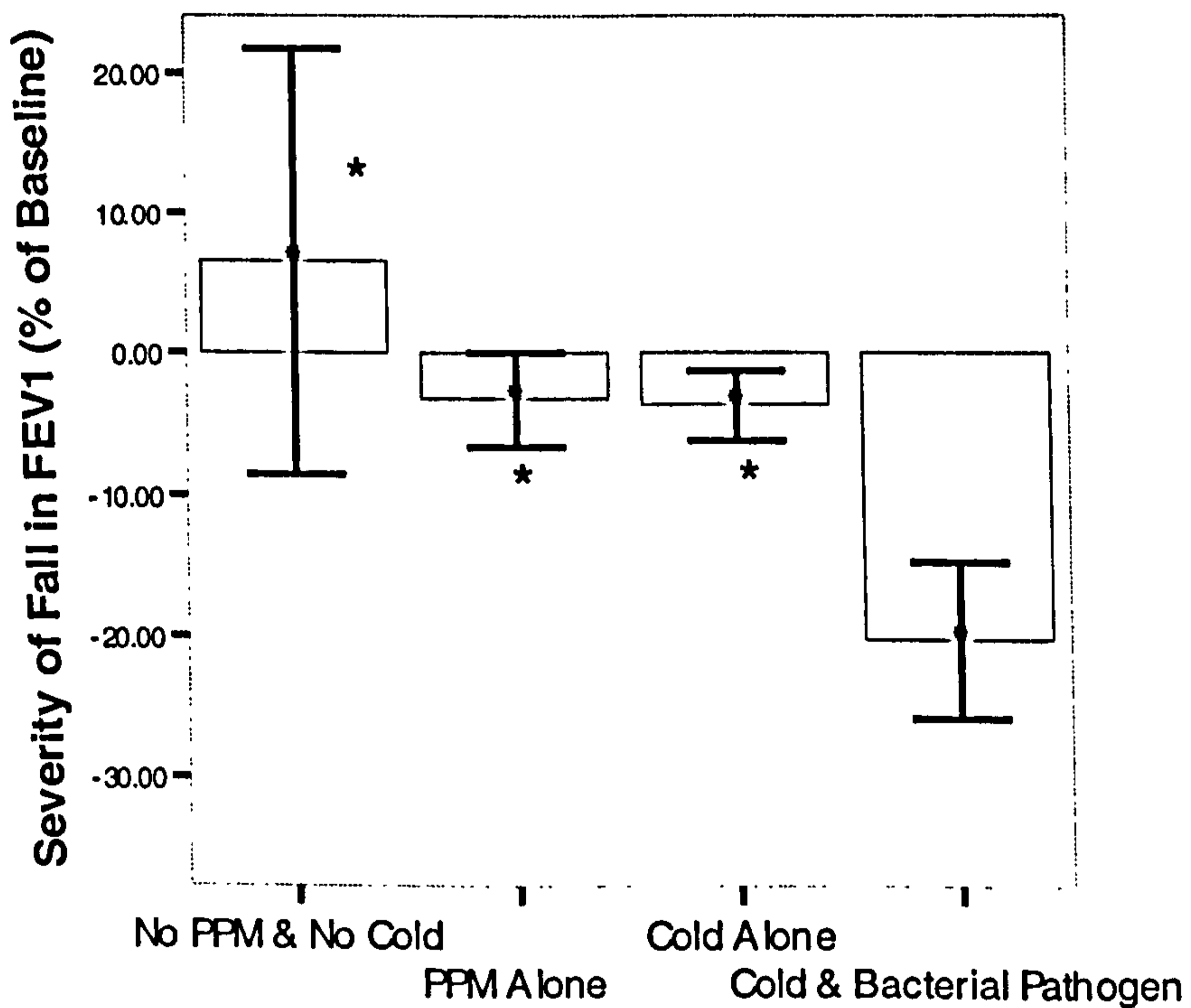


FIGURE 6.3 EFFECT OF AIRWAY PATHOGENS AND PATHOGEN COMBINATIONS ON SYMPTOM SEVERITY. (median symptom count at exacerbation onset), columns represent median values, bars interquartile range * and + denote statistical significant ($p < 0.05$) difference between corresponding labelled categories. $n = 56$. * $p = 0.029$. + $p = 0.019$.

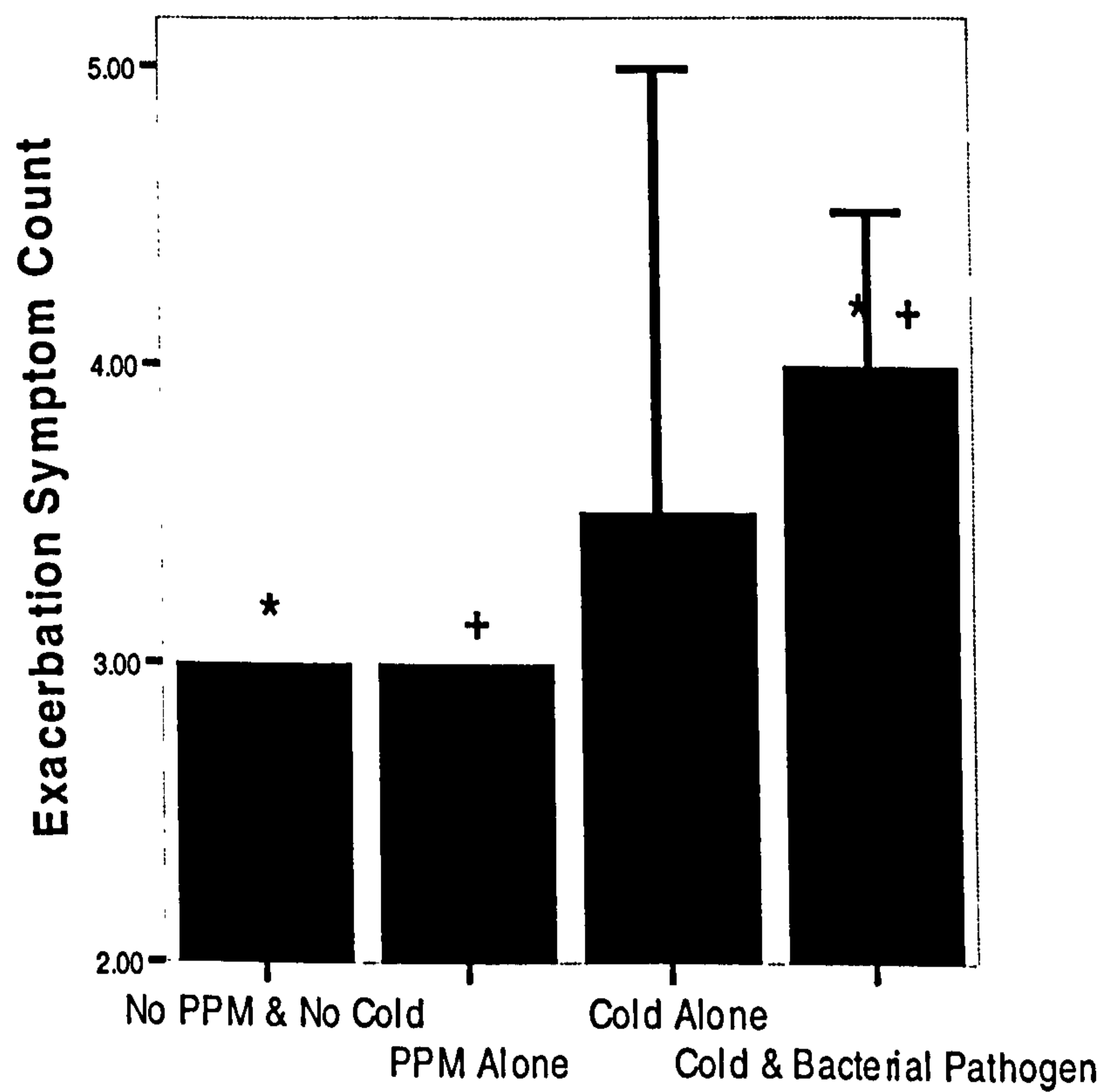
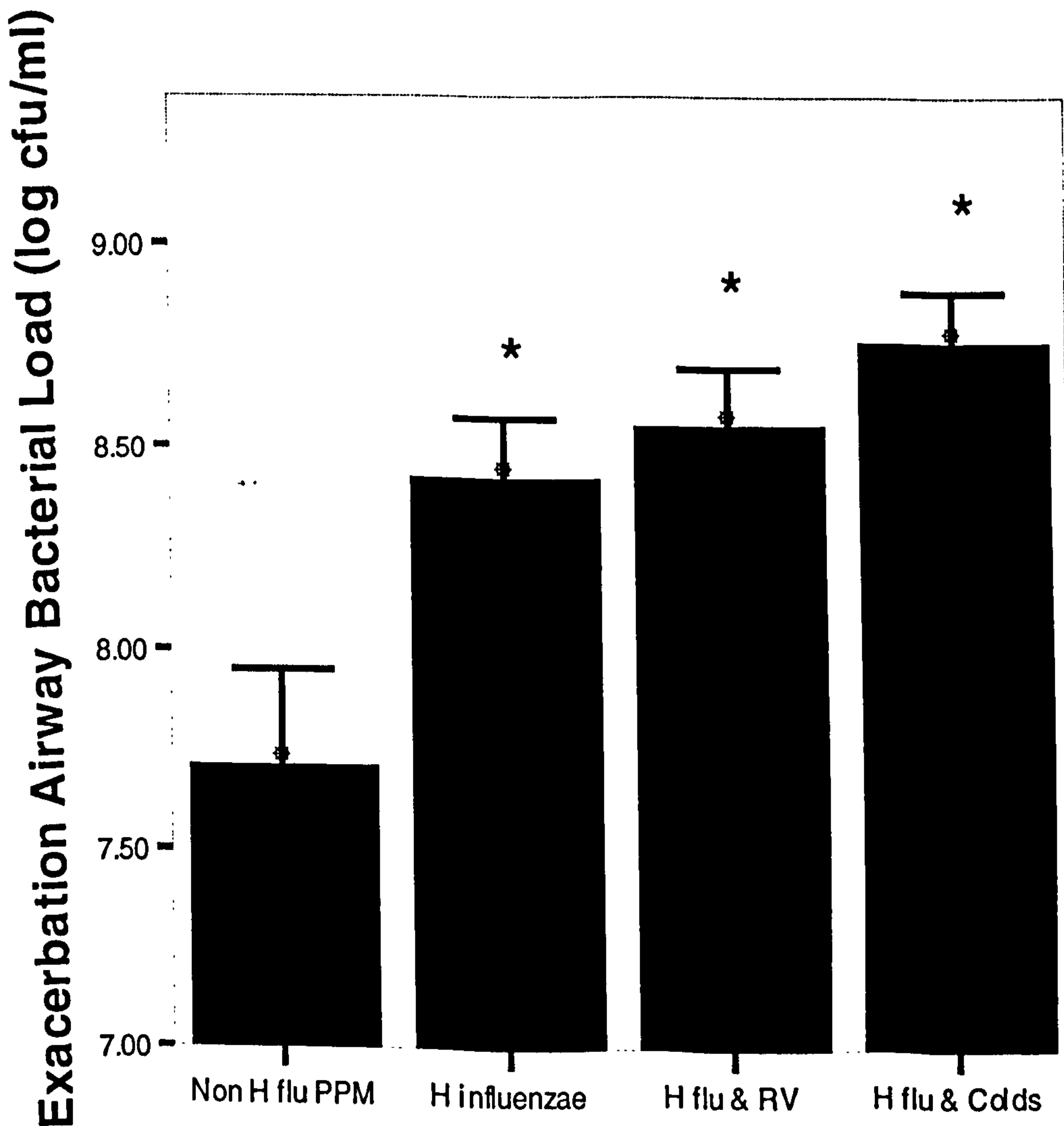


FIGURE 6.4. AIRWAY BACTERIAL LOAD AT EXACERBATION (CFU PER ML), FOR DIFFERENT CATEGORIES OF ISOLATED PATHOGEN. Columns represent mean values with error bars as standard error of mean; All non-*H influenzae* PPMs, *H influenzae*, *H influenzae* and HRV, *H influenzae* and Cold symptoms. n=56. PPM = Potentially Pathogenic Micro-organism

RV = Human rhinovirus H flu = *Haemophilus influenzae* * represent significantly different from all PPMs category.



The observed FEV₁ fall associated with colds at exacerbation was more marked in the presence of a lower airway bacterial pathogen; -20.3(14.81) % with both colds and a bacterial pathogen compared to -3.63 (5.57) % with a cold alone, p = 0.026 or -3.13 (14.88) %, with a bacterial pathogen alone, p= 0.001. (Figure 6.2). The specific effect of the interaction between colds and bacterial pathogens was assessed with a multivariate regression analysis with the % FEV₁ fall at exacerbation as the dependent variable, the effect of the interaction was additional to the independent effects of each individual factor; 95% CI -13.13 to -2.09, p= 0.009.

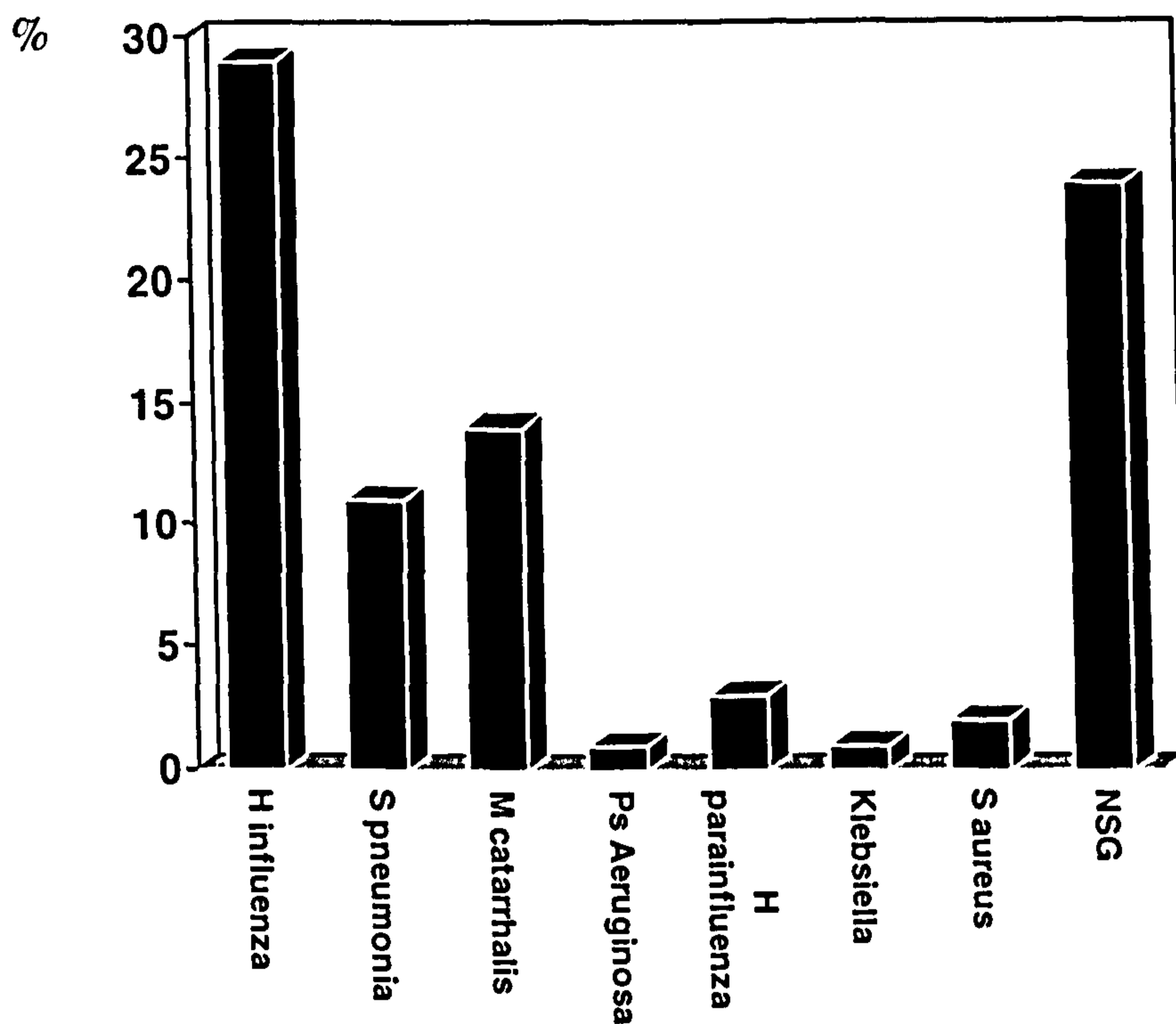
Similarly exacerbation symptoms were more severe (higher symptom count at exacerbation onset) in those exacerbations associated with a PPM in the presence of cold symptoms; 4.0 (3.0 - 4.5), compared to those exacerbations with a PPM but not associated with cold symptoms; 3.0 (2.0-3.0), p = 0.019, or with neither a PPM nor cold symptoms; 3.0 (2.0-3.0), p = 0.029. (Figure 6.3)

Exacerbations associated with both *Haemophilus influenzae* and HRV exhibited a greater bacterial load ($10^{8.56(0.31)}$ vs $10^{8.05(0.77)}$ log cfu/ml, p = 0.018) and serum IL6 (13.75 (10.53-16.91) vs 6.29 (3.31-9.75) pg/ml, p = 0.028) than those without both pathogens. The exacerbation airway bacterial load associated with *Haemophilus influenzae* and HRV compared to other PPMs is illustrated in Figure 6.4.

6.3.5 ASSOCIATIONS BETWEEN HRV AND BACTERIAL PATHOGENS

In this further analysis we sampled 68 exacerbations in 68 patients (mean (SD) without corresponding baselines. Patient characteristics: age 67.8(8.22) yr, FEV₁1.1(0.43)l, FVC 2.12(0.73)l, % predicted FEV₁ 44.07(15.77), pack yr history 55.3(36.0), at exacerbation, sputum was analysed for microbiology, HRV using PCR and cytokines using ELISA as described previously.

FIGURE 6.5 DETECTION RATES OF BACTERIAL PATHOGENS IN SPUTUM FROM 68 EXACERBATIONS.



HRV was detected in 26.5 % of patients exacerbation sputums. Detection of HRV was associated with active smoking χ^2 4.0 p=0.05 and greater pack years history of smoking, rho=0.252, p=0.045. HRV at exacerbation was associated with isolation of *S. pneumoniae*; χ^2 4.94, p=0.02, and a trend to higher airway inflammation (IL6 218.9(124.4-268.8) vs 113.9(42.2-283.6)pg/ml, p=0.06, figure 6.5. Exacerbations when *S. pneumoniae* was isolated from sputum exacerbations exhibited a greater drop

in FEV₁ rho=0.243, p=0.034 than those without this pathogen. No other significant inter-pathogen associations were present in this analysis.

FIGURE 6.6 SPUTUM INTERLEUKIN-6 LEVELS IN EXACERBATION SAMPLES FROM 68 COPD EXACERBATIONS n = 68, p = 0.06.

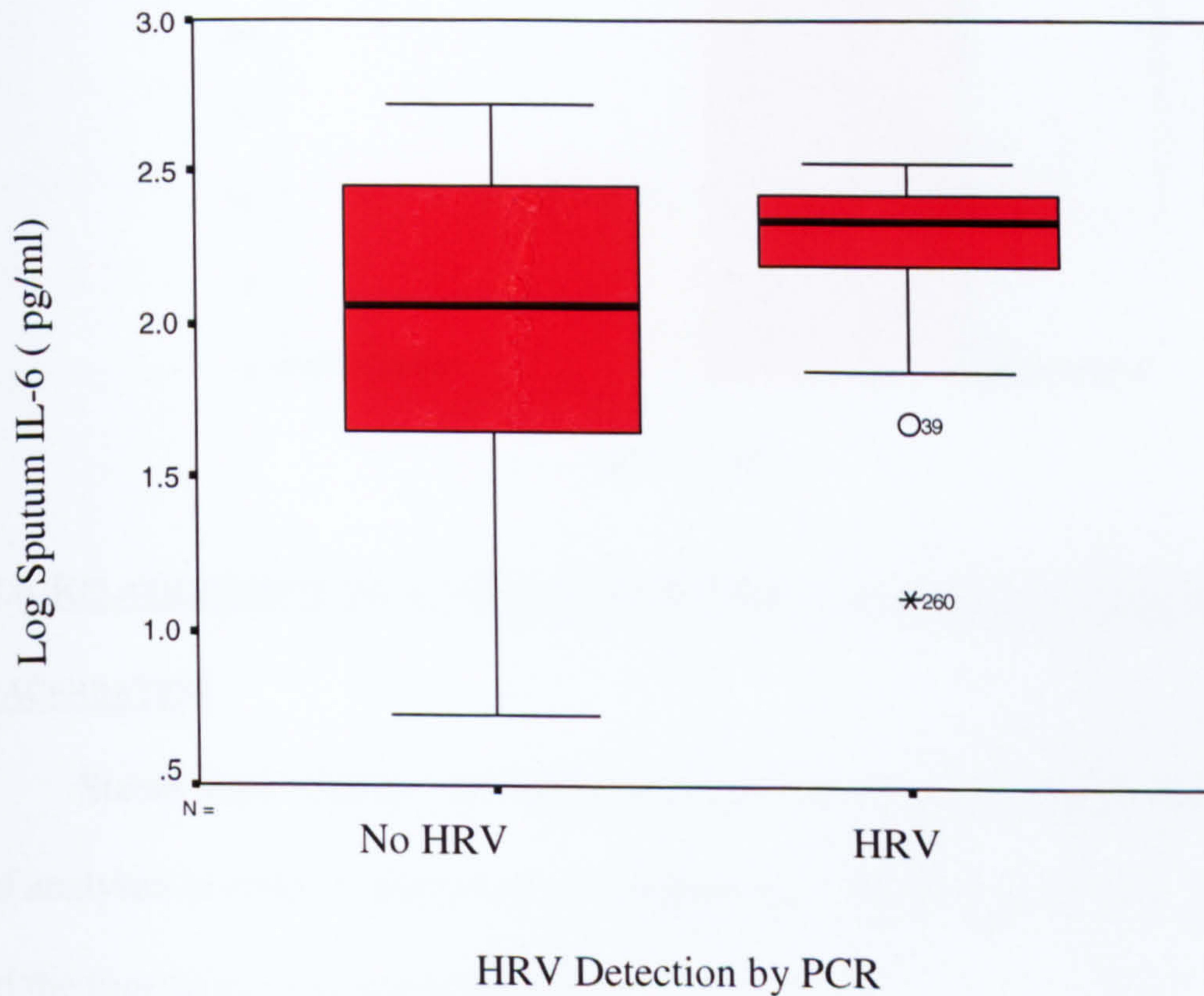
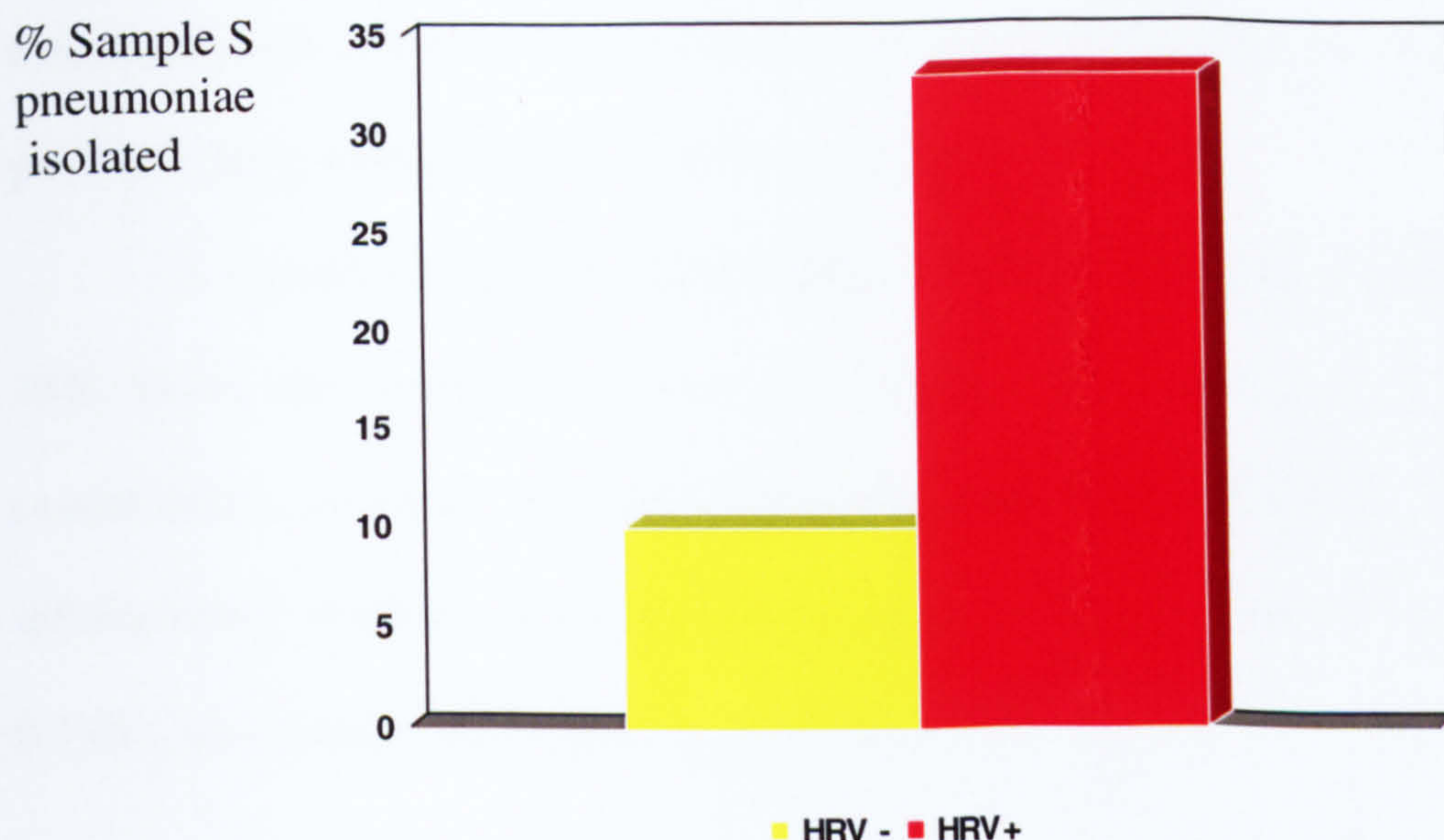


FIGURE 6.7 ISOLATION RATES FOR *STREPTOCOCCUS PNEUMONIAE* IN EXACERBATIONS WITH AND WITHOUT HRV POSITIVE PCR n=68 p=0.02.



6.3.6 RELATIONSHIP BETWEEN STABLE STATE INFLAMMATION AND TIME TO FIRST EXACERBATION

Stable state sputum and blood samples from 120 cohort patients were taken and analysed in order to determine the relationship between stable state inflammation and the time to the next exacerbation.

Diary cards were reviewed to ensure patients were in the stable state for at least six weeks prior to baseline sampling and patients were followed prospectively to first exacerbation- diagnosed using the standardised symptom based criteria.

Patient baseline characteristics were typical for the East London Study. 81 males, mean (SD) age 66 (7.64) years FEV₁ 1.08 (0.42) l, FVC 2.51(0.81) l, FEV₁ % predicted 41.1 (15.7) %. We analysed sputum (n=80) for IL-6, IL-8 and blood (n=120) for IL-6 and fibrinogen. Patients completed diary cards of symptoms and were prospectively followed to first exacerbation. Relationships between stable state inflammation and time to first exacerbation after sampling were determined.

The median (IQR) stable state levels were: Sputum IL-6 140.7(46.8-231.6) ng/ml, IL-8 3430 (2046-4679) ng/ml, Serum IL-6 4.3 (2.8-5.5) ng/ml and Plasma Fibrinogen 3.91(3.53-4.42) g/l. Higher levels of stable state airway inflammation were associated with a shorter time to first exacerbation; \log_{10} sputum IL-6; ($r = -0.37$, $p=0.001$), \log_{10} sputum IL-8 ($r = -0.56$, $p<0.001$).

A regression analysis to determine effect size revealed a sputum IL8 level 33% above the median was associated with a 26% reduction in time to next exacerbation. However no relationship was seen between stable state systemic inflammatory markers and time to exacerbation; \log_{10} serum IL-6 ($r=0.223$, $p=0.1450$), \log_{10} plasma fibrinogen; ($r=-0.12$, $p=0.9$).

6.3.7 PROTEOMIC ANALYSIS OF SPUTUM AT BASELINE AND EXACERBATION

A pilot study was recently performed to determine the suitability of sputum samples collected from patients with moderate COPD and processed using standard protocols, for analysis using MSI Protein arrays and to profile changes in 107 cytokines from clinically stable state to exacerbation in five patients.

The patients were sampled when determined to be in a clinically stable state and followed prospectively until they experienced an exacerbation of their symptoms, when confirmed clinically with review of diary cards were sampled again. All samples were processed in the departmental laboratory with aliquots frozen at -70°C for this analysis. Baseline and exacerbation sputum aliquots were transferred to MSI for protein microarray analysis as described in the methods section of this chapter.

TABLE 6.3 CHARACTERISTICS OF 5 BASELINE AND 5 EXACERBATION SPUTUM SAMPLES
USED FOR PROTEIN MICROARRAY.

Patient	Sample Type	TBC log cfu/ml	Bacteria	HRV PCR
001	Base Line	8	H parainfluenza	Negative
001	Exacerbation	8.9	H Influenza	Negative
002	Base Line	7	NSG	Negative
002	Exacerbation	8.3	B catarrhalis	Negative
003	Base Line	9.6	H influenza	Negative
003	Exacerbation	8.7	H influenza	Negative
004	Base Line	8.3	Citrobacter sp	Negative
004	Exacerbation	9	NSG	Positive
005	Base Line	8.6	Enterobacter	Negative
005	Exacerbation	6.6	Pseudomonas	Negative

Sputum as sampled and processed is a suitable substrate for protein microarray analysis, all sample replicates passed quality control. 62 of the 107 (58%) analytes were detectable in the sputum samples assayed (Mean fluorescence intensity >1000) in the baseline sputum samples. 16 analytes were found to be modulated at exacerbation compared to matched stable samples; (GRO-beta, ICAM-3, TIMP-1, ENA- 78, Flt3Lig, IL-13, IL-15, IL-3, IL-4, MIP-1delta, NT3, NT4, PARC, TARC, sgp130, IGFBP-3). These results with p values are tabulated below. Table 6.4.

Of the analytes detected a number which have been demonstrated to be modulated in sputum at exacerbation using larger sample populations and more conventional ELISA techniques did not appear to be modulated significantly in this preliminary study. For example IL-8, IL-6, Gro-alpha and markers of neutrophil activation. This discrepancy is likely to be due to the small sample size in this study.

Analyte intensities within each patient were highly correlated between baseline and exacerbation. (Corr coeff > 0.85). This suggests that variation between individuals is an important factor in the analyte levels detected and hence a

longitudinal sampling of matched baseline and exacerbation samples is a valuable sampling methodology

TABLE 6.4 PROTEOMIC ANALYTES WITH EVIDENCE OF MODULATION AT EXACERBATION. N = 5 (paired analysis).

Analyte	p-value	Direction of change in exacerbated samples	Fold change
GROb	0.0773	increased	3.1
ICAM3	0.0730	increased	1.5
TIMP1	0.0387	increased	1.4
ENA-78	0.0038	decreased	2.5
Flt3Lig	0.0955	decreased	1.7
IL-13	0.0730	decreased	1.5
IL-15	0.0848	decreased	1.3
IL-3	0.0900	decreased	1.5
IL-4	0.0744	decreased	1.6
MIP-1d	0.0874	decreased	2.3
NT3	0.0509	decreased	1.9
NT4	0.0825	decreased	1.7
PARC	0.0033	decreased	3.1
TARC	0.0101	decreased	2.1
sgp130	0.0639	decreased	2.0
IGFBP-3	0.0962	decreased	1.6

6.4 DISCUSSION

The results of this study show evidence of a synergistic effect between viral and bacterial infection in modulating the severity of symptoms, lung function changes and inflammation at exacerbations of COPD. The findings demonstrate that changes in lower airway bacterial load are associated with the variability in inflammation and lung function seen at exacerbation in patients with moderate to severe COPD, effects which were more pronounced in proven rhinoviral and putative viral infections associated with cold symptoms.

These data also suggest that pathogens associated with more severe exacerbations such as *Haemophilus influenzae*, may have greater effects on the lower airway and indeed on the patient as a whole due to a process which is mediated, at least in part via a greater stimulus to inflammation, associated with higher airway bacterial loads.

Patients with more severe disease in this study demonstrated greater rises in airway and systemic inflammation than those with milder disease. This suggests that the heterogeneous nature of exacerbation severity is dependent not only upon the nature of infective triggers but also upon the baseline severity of disease.

This study has been performed using the well validated technique of daily diary card symptom recording and analysis, to confirm both the diagnosis of exacerbations and also the stable state. (Bhowmik 2000, Wedzicha 2000, Seemungal 1998, Seemungal 2000). The study of exacerbations utilised two designs, this has allowed analysis of data from sampling the same patients in both stable and exacerbating clinical states and to describe both cross sectional analyses at exacerbation, and also changes from baseline and furthermore how these changes in exacerbation parameters were modulated by the corresponding infectious agents.

The paired baseline- exacerbation analysis has shown that the severity of the fall in lung function and the rise in inflammation seen at exacerbation is related to the extent of the rise in airway bacterial load. A relationship between airway inflammation and airway bacterial load has previously been described in the stable state, (Hill 2000, Patel 2002, Banerjee 2004) with higher loads associated with greater falls in FEV₁ over a one year study (Wilkinson 2003). A number of previous studies have identified that bacterial pathogens are commonly found in the lower airway at exacerbation (Sethi 2000, Sethi 2002, Miravittles 1999) with higher loads than in the stable state (Monso 1995). However the effect of rising numbers of bacteria on the nature of exacerbations has not been investigated. These findings suggest that changes in bacterial load may play a role in the heightened levels of airway inflammation characteristic of exacerbations. However, evidence for an association between changes in bacterial load and indices of exacerbation severity does not prove causality. It is possible that changes in airway bacterial load may simply be a secondary phenomenon to other causes of inflammation. Indeed, the findings of this study show that the key changes in symptoms and lung function at exacerbation were observed when the synergistic effects of viral and bacterial infection were found. In vitro and intervention studies are required to differentiate the exact contribution of a particular pathogen or pathogens to the inflammatory and patho-physiological changes at exacerbation. Indeed there has recently been established a human rhinoviral exposure model of COPD exacerbations (Mallia 2004). However due to the necessities of patient safety, only subjects with very mild disease would be suitable for such viral challenge experiments and the role of airway bacterial infection in such a population may differ greatly from that seen in more established disease.

Haemophilus influenzae was found in this study, as in previous studies (Monso 1995, Zalacain 1997) to be the most important bacterial pathogen identified both in terms of prevalence in the stable state and also at exacerbation, and in determining the airway bacterial load. *Haemophilus influenzae* unlike a number of other bacterial pathogens may colonise not only the airway but invade the respiratory epithelium itself. *H influenzae* colonisation has been shown to be a greater stimulus to airway inflammation than other commonly isolated pathogens (Sethi 2000, Bresser 2000). This is in agreement with the findings of our study which demonstrate that *Haemophilus influenzae* was present in greater numbers than the other PPMs identified and that its presence at exacerbation was associated with more severe drops in FEV₁. The role of less prevalent bacterial pathogens at exacerbation in particular their interactions with respiratory viruses requires further study; this will require a larger patient population or longer term follow up.

The stimulus of newly acquired *Haemophilus influenzae* at exacerbation provided a greater deleterious effect on FEV₁ than *Haemophilus influenzae* associated exacerbations in patients already colonised with this pathogen. These findings compliment previous work into the role that the presentation of new bacterial epitopes may play in stimulating the immune system. A previous study of strain changes of particular bacterial species play in the aetiology of exacerbations has identified the role that a new antigenic stimulus to the airway immune system plays in the pathogenesis of an exacerbation (Sethi 2001). It is feasible that acquisition of a new bacterial strain or type may not only provide a direct antigenic stimulus, but also overcome the established host-pathogen balance allowing bacterial proliferation, and thus a further inflammatory stimulus due to greater bacterial numbers.

Respiratory viral infection is an important trigger to the airway immune system. In our cohort of influenza vaccinated patients we have previously demonstrated that human rhinovirus is the most commonly isolated virus at exacerbation (Seemungal 2000). Rhinovirus can be isolated from lower airway samples and is associated with greater levels of inflammation than non viral infections (Seemungal 2000). Similarly we have shown that colds, a marker of putative viral infections (Seemungal 2000) are associated with more severe exacerbations. In this study systemic inflammation (serum IL6), exacerbation symptoms and lung function changes were all more severe when evidence for both bacterial and viral infection was present. It is possible that this effect may have been due to the separate additional inflammatory stimuli of two separate pathogens in the airway, however this explanation is not supported by the multivariate analysis which indicated a synergistic effect on lung function in addition to the individual effects of each pathogen type. Furthermore these exacerbations were associated with higher bacterial loads than when both pathogens were not present, which may suggest a synergistic interactive effect of viral infection which allows greater proliferation of airway bacteria. Viral infection therefore may impact on exacerbation severity indirectly by increasing bacterial load in addition to the direct effects of viral infection itself, eg heightened inflammation or airway hyper-responsiveness, independent of other pathogens.

Whilst human rhinovirus is the commonest virus identified at exacerbation (Seemungal 2001, Rohde 2003) and hence the target of investigation in this study, a number of other respiratory viruses have been identified in previous studies during these events, for example coronavirus, influenza, parainfluenza and adenovirus (Rohde 2003, Seemungal 2001). The role of these other viral pathogens and interactions with bacterial infection requires study. Similarly as discussed previously

the role that atypical bacteria such as *Mycoplasma* and *Chlamydia* play at exacerbation remains uncertain (Blasi 2002, Seemungal 2002) and requires investigation.

The mechanisms by which viral infection may facilitate airway bacterial growth are likely to be complex. However any disruption of the innate defences of the respiratory epithelium in a lower airway colonised with bacteria may unsettle a fine balance between host immunity and bacterial numbers. Rhinoviral infection is known to increase mucous production and neutrophilic inflammation (White 2003, Fraenkel 1995). Direct evidence that rhinoviral infection increases susceptibility to bacterial adherence to airway epithelial cells, a key process in bacterial infection, is available from in-vitro studies (Ishisuka 2003). Indeed the cross-sectional analysis confirmed an association between infection with HRV and with *Streptococcus Pneumoniae*. This suggests that the in vitro work demonstrating up-regulation of the S pneumoniae cellular binding site the epithelial cell surface platelet aggregation factor receptor by HRV infection, may occur in vivo. However, biopsy studies would be required to confirm the mechanisms of bacterial adherence and how these are modulated by concurrent viral infection in COPD.

The key cell surface binding site for major type HRV infection, ICAM-1, is itself up-regulated by HRV infection (Papi 1999) and by bacterial colonisation (Patel 2003). This increase may play a key role in neutrophil elastase-mediated inflammation which is key process at exacerbation (Nadel 2000). Hence, by a number of mechanisms viral infection may alter the immune environment which may allow either proliferation of colonising airway bacteria or a new pathogen to infect the lower airway.

This study was performed in patients with moderate to severe COPD. The role of bacterial infection and therefore potential bacterial-viral interaction is likely to vary with disease severity and therefore prevalence of bacterial colonisation. Indeed we have shown that the degree of airway and systemic inflammatory response at exacerbation was related to baseline disease severity. This suggests that the severity of inflammatory response may progress with disease severity which is in agreement with the findings of a recent longitudinal analysis of exacerbations (Donaldson 2003). Further studies are required to determine if these findings can be extrapolated to COPD patients with milder disease. Indeed the observed heterogeneity of exacerbations is likely to be further modulated by the relative frequency of particular pathogens and hence may show seasonality. This may explain differences in associated cytokine responses in studies of comparable sample size (Bhowmik 2000). Similarly differences in the technique of sampling such as spontaneous or induced sputum, may affect the observed results. However, we have previously demonstrated that the two techniques are comparable in assessing lower airway inflammation (Bhowmik 1998).

Therapy must also be considered important when considering factors modulating inflammatory responses. The patients sampled for this study were all receiving inhaled steroids both at baseline and when sampled at exacerbation. It is possible that the inflammatory responses observed at exacerbation were modified by effects of this treatment (Patel 2003). A statistical analysis of this effect was not feasible due to the ubiquity of inhaled steroid use in this patient group. Therefore the modulating influences on the nature of exacerbations are numerous. It is probable that any individual factor plays a contributing rather than a definitive role in determining the

nature and severity of a particular exacerbation and furthermore that potential interactions between these factors further modulate the characteristics of these events.

Indeed determining factors which may influence or may predict responses to exacerbation therapy may be of great benefit to the clinician. Therefore, analysis of biological, demographic and physiological indices of poor outcome requires further understanding and indeed is the focus for the next Chapter of this thesis.

In a similar manner, the ability to predict when a particular patient is at high risk of an imminent exacerbation may allow the clinician to intervene and prevent its occurrence. The finding that a relationship exists between baseline inflammation and time to next exacerbation seen in this study may indicate that this may become possible at some stage. However, the relationship between stable airway inflammation shown and subsequent occurrence of exacerbation in this study were not strong enough to offer adequate predictive power to guide a clinical intervention to prevent exacerbations. This is particularly the case as over use of currently available therapies such as corticosteroids and antibiotics carry serious consequences not only for the patient (Walsh 2002) but also for the larger community if induction of anti-microbial resistance is considered (Metlay 2002).

It is therefore important to identify new therapeutic targets for novel therapeutic interventions to prevent and better treat exacerbations. This preliminary study on 10 sputum samples has suggested that micro-array techniques may be valid method for the analysis of a broad range of sputum cytokines and other proteins in patients with COPD, however, further assay validation is required. Initial conclusions on modulation of individual analytes are largely speculative due to the very small sample size used and a number of changes in these modulated analytes have been

previously described in the literature eg. TIMP-1 (Mercer 2005) while others are new eg. ICAM-3, MIP-1delta, IL-4, IL-13.

However use of this technique in a larger sample of paired baseline and exacerbation sputa should produce highly interesting and potentially novel results.

CONCLUSION

The findings of these studies suggest that changes in airway bacterial load, the nature of the individual infective pathogens and interactions between multiple pathogens including bacteria and viruses and the airway modulate exacerbation severity. Further studies are required to improve understanding of the pathogen-host interactions at exacerbation and indeed also in the stable state. Manipulation of this complex relationship with appropriate anti-infective and anti-inflammatory therapies may benefit COPD patients by reducing both exacerbation severity and slowing progression of this highly prevalent disease. However whilst new avenues of therapeutic intervention are sought it is important to identify how we can improve the outcome of exacerbations using currently available treatments.

Whilst this work has demonstrated that airway infection is associated with exacerbation severity the factors which modulate recovery from exacerbation or indeed responses to therapy are poorly understood. The following chapter presents the findings of analyses which aim to determine these parameters.

CHAPTER 7

EXACERBATIONS OF COPD: PREDICTING AND IMPROVING RESPONSES TO THERAPY

This chapter represents the findings of a two part analysis aimed at determining which factors determine the time course of recovery from exacerbations and the clinical response to therapy. The initial analysis was of a limited dataset of sampled exacerbations and revealed that the timing of therapy may be a key factor in modulating recovery. This finding is the focus of the subsequent studies into how exacerbation reporting behaviour affects outcome and how this behaviour may be modulated.

7.1 INTRODUCTION

Exacerbations are a frequent cause of physician consultation in primary and secondary care and a major cause of hospital admission (Garcia-Aymerich 2000, Pearson 1994, Guest 1999). Consequently management of exacerbations places a considerable burden on the health services both in terms of physician consultation time and health care costs (Grasso 1998, McGuire 2001, Sullivan 2000). Reduction in exacerbation frequency would have a number of benefits for patients and health services alike. However, currently available preventative therapies have been found to have only a relatively small effect (Calverley 2003, Casaburi 2002, Burge 2000).

Current therapies for exacerbations include oxygen, antibiotics, oral corticosteroids and increased bronchodilator medications. Whilst there is evidence that antibiotics (Anthonisen 1987) and corticosteroids (Davies 1999, Niewoehner 1999, Aaron 2003, Thompson 1996) hasten the rate of recovery of certain exacerbations there is little data in the literature on the factors which predict response to therapy. However, patients with COPD often have poor understanding of their disease and symptoms, with the result that exacerbations are often not reported to health care professionals for treatment (Seemungal 1998). Thus, if delay in presentation can be shown to affect exacerbation recovery, this provides a potentially important issue that can be addressed in the management of COPD patients.

The initial analysis presented in this Chapter was performed to determine which patient and exacerbation factors are associated with improved outcome. The subsequent analysis of a larger dataset of exacerbations aimed to investigate the hypothesis that the early presentation of the patient to the physician with an exacerbation would allow early intervention, reduction of exacerbation severity and thus potentially improve clinical outcomes such as hospitalisation.

7.2 METHODS

In order to determine the relationship between patient characteristics, exacerbation symptomology and exacerbation recovery time we initially determined the influence of individual characteristics on recovery time, of both peak expiratory flow and symptoms using a non parametric correlation analysis in a sample of 57 exacerbations reported to the study clinic. The significant associations were subsequently entered into a multivariate regression model. The recovery variables were calculated as described in Chapter 3 Methods.

EFFECTS OF EXACERBATION REPORTING ON OUTCOMES: ANALYSIS OF COHORT DATA.

This analysis was conducted on data collected from the entire cohort over a six year period. 128 patients with COPD recruited during this period, who had recorded daily data for 1 year or more, were included in the analysis. Thus data collected on daily symptoms and exacerbation treatment on 1099 exacerbations was included. This daily monitoring enabled us to investigate how exacerbation outcomes and markers of severity such as recovery time were affected by the timing of presentation to the physician for treatment. We also studied how patient and exacerbation characteristics influenced the patterns of health care utilisation and outcomes of therapy.

Patient recruitment and follow up was performed as described previously. Indices of Health Related Quality Of Life (HRQOL) were obtained using the St. Georges Respiratory Questionnaire (SGRQ) at the beginning of the study either at recruitment or during the first annual review.

EXACERBATIONS: DEFINITION OF TERMS

The diagnosis of an exacerbation was based on symptomatic criteria previously validated by our group (Seemungal 1998, Seemungal 2000). Exacerbation onset was taken as the first day on which these symptom criteria were met. Figure 7.1 in the

FACTORS AFFECTING EXACERBATION RECOVERY

manuscript illustrates a diagrammatic timeline of an exacerbation with a definition of terms used.

TREATMENT DELAY

From the diary card data and treatment records, the time between exacerbation onset and physician consultation at which treatment was initiated, was calculated and called the treatment delay.

EXACERBATION RECOVERY TIME

A total daily count of individual symptoms recorded on diary cards was calculated as the sum of the 7 symptoms with the presence of a symptom scored 1 and its absence 0. In order to determine recovery to baseline levels and therefore exacerbation recovery time a diary card symptom baseline was determined from the diary card for each exacerbation. The baseline symptom count was taken as the mean total daily symptom count over days 14 to 8 preceding the onset of exacerbation. Exacerbation total recovery time was calculated as the time from exacerbation onset for a 3-day moving average of the total daily symptom count to return to this baseline. The use of a three-day moving average minimised the effect of day-to-day symptom variation without biasing the results. Treated recovery time was taken as the time between consultation and recovery.

SEVERITY OF EXACERBATION IN TERMS OF SYMPTOMS EXPERIENCED

Symptom severity at exacerbation was calculated as the difference between the average daily symptom count at baseline (days 14 to 8 preceding the onset of exacerbation) and the number of symptoms at onset of exacerbation. Individual symptoms were recorded as increased (=1) or not (=0).

TREATMENT

Patients were encouraged to report symptom changes to the study team. They were assessed within 24 hours in the study clinic by a respiratory physician prior to initiation of therapy for the exacerbation. Patients did not report all symptom changes to the study team, but also reported a number of these episodes to their primary care physician for assessment and treatment. The prescription of treatment for all exacerbations was at the discretion of the attending physician and included prednisolone and/or antibiotic therapy. Exacerbation treatment in the study therefore represented the usual practice of the primary care or study physician attending the patient. Records were kept of the date of initiation and type of treatment prescribed to patients for each exacerbation, both at our clinic and also therapy prescribed by the patient's primary care physician.

CLASSIFICATION OF EXACERBATIONS

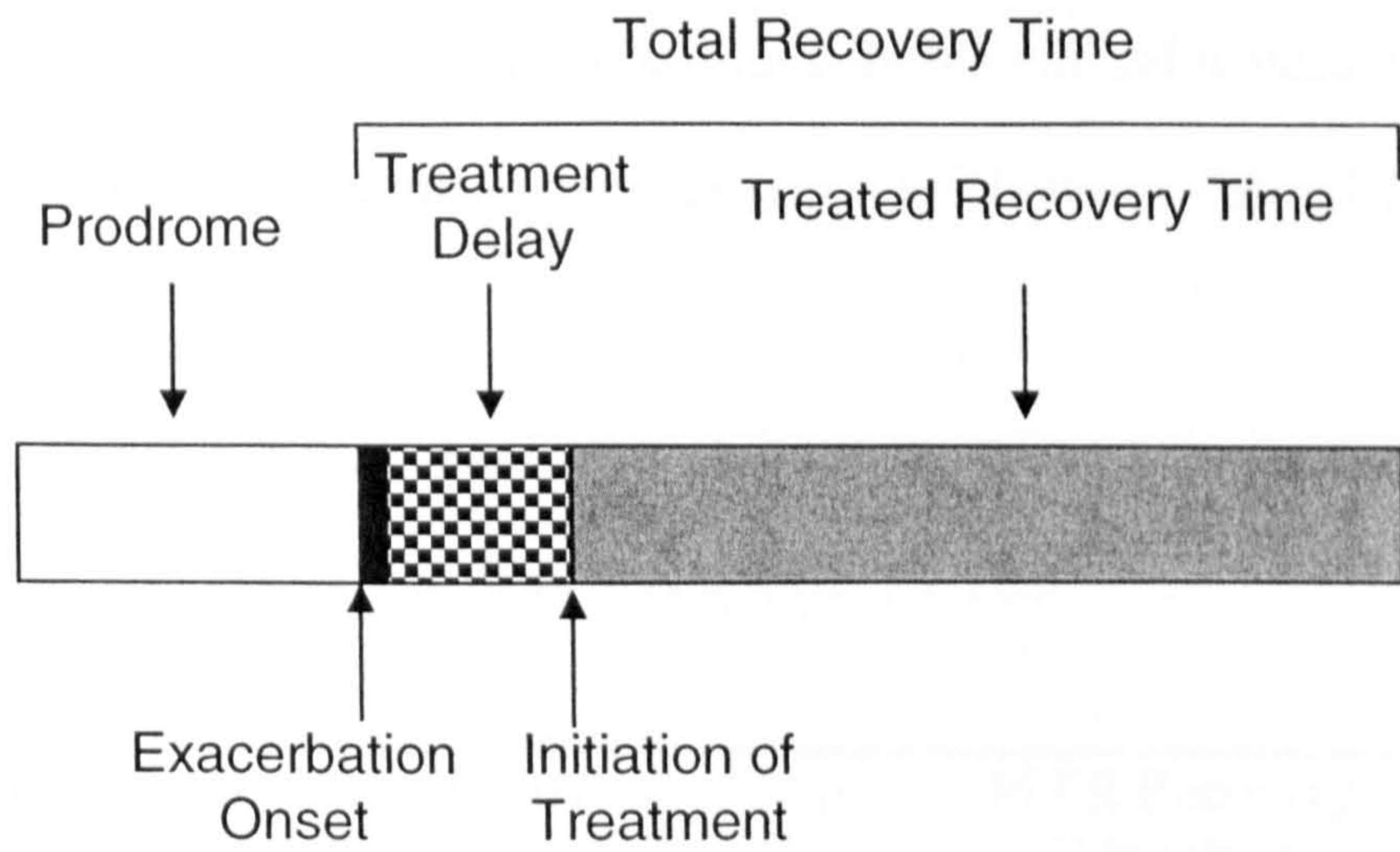
The exacerbations seen by the study clinical team or the patient's general practitioner were classified as "physician reported exacerbations" with those unseen by either GP or study clinician but recorded on diary cards termed "unreported exacerbations". Records of hospitalization were kept throughout the study. Additional detail on the diagnosis of exacerbations is available in the on-line data supplement.

STATISTICAL ANALYSIS

Exacerbation total recovery time was calculated as the time from exacerbation onset for a 3-day moving average of the total daily symptom count to return to baseline. Treated recovery time was taken as the time between consultation and recovery. Symptom severity at exacerbation was calculated as the difference in total daily symptom count at baseline and at exacerbation onset.

Assessment of the effect of delay and treatment on recovery with or without allowance for severity, type of treatment or non-recovery was made with a generalized linear model with adjustments for Poisson distribution in the dependent variable. Additional detail on the method for making these measurements is provided in an online data supplement.

FIGURE 7.1 SCHEMATIC TIMELINE OF EXACERBATIONS AND TERMS USED IN THE ANALYSIS OF TREATMENT EFFECTS ON EXACERBATION RECOVERY.



7.3 RESULTS

7.3.1 FACTORS AFFECTING EXACERBATION RECOVERY TIME

The baseline characteristics of the cohort patients were; mean (SD) age 68 (6.5), FEV₁ 1.00 (0.38)l, FEV₁ % predicted 41.8 (16.5), FVC 2.57 (0.93)l, Pa O₂ 8.8 (0.9) kpa, Pa CO₂ 5.5 (0.8) kpa. The relationships between clinical indices of exacerbation severity and exacerbation recovery time are shown in Table 7.1 below.

TABLE 7.1 RELATIONSHIPS BETWEEN CLINICAL INDICES OF EXACERBATION SEVERITY AND EXACERBATION RECOVERY TIME

Factor	Symptom Recovery Time rho=	p	PEFR Recovery Time rho=	p
Age	0.373	0.019	0.294	0.069
Smoking	0.352	0.038	0.304	0.076
PEFR Fall	0.53	0.758	0.678	0.0001
Symptom Count	0.367	0.022	0.427	0.007
Cold	0.132	0.423	0.459	0.003

There was also a significant relationship between the time between the onset of exacerbation symptoms, determined by diary card analysis, and the duration of exacerbation symptoms. This relationship is shown in figure 7.2.

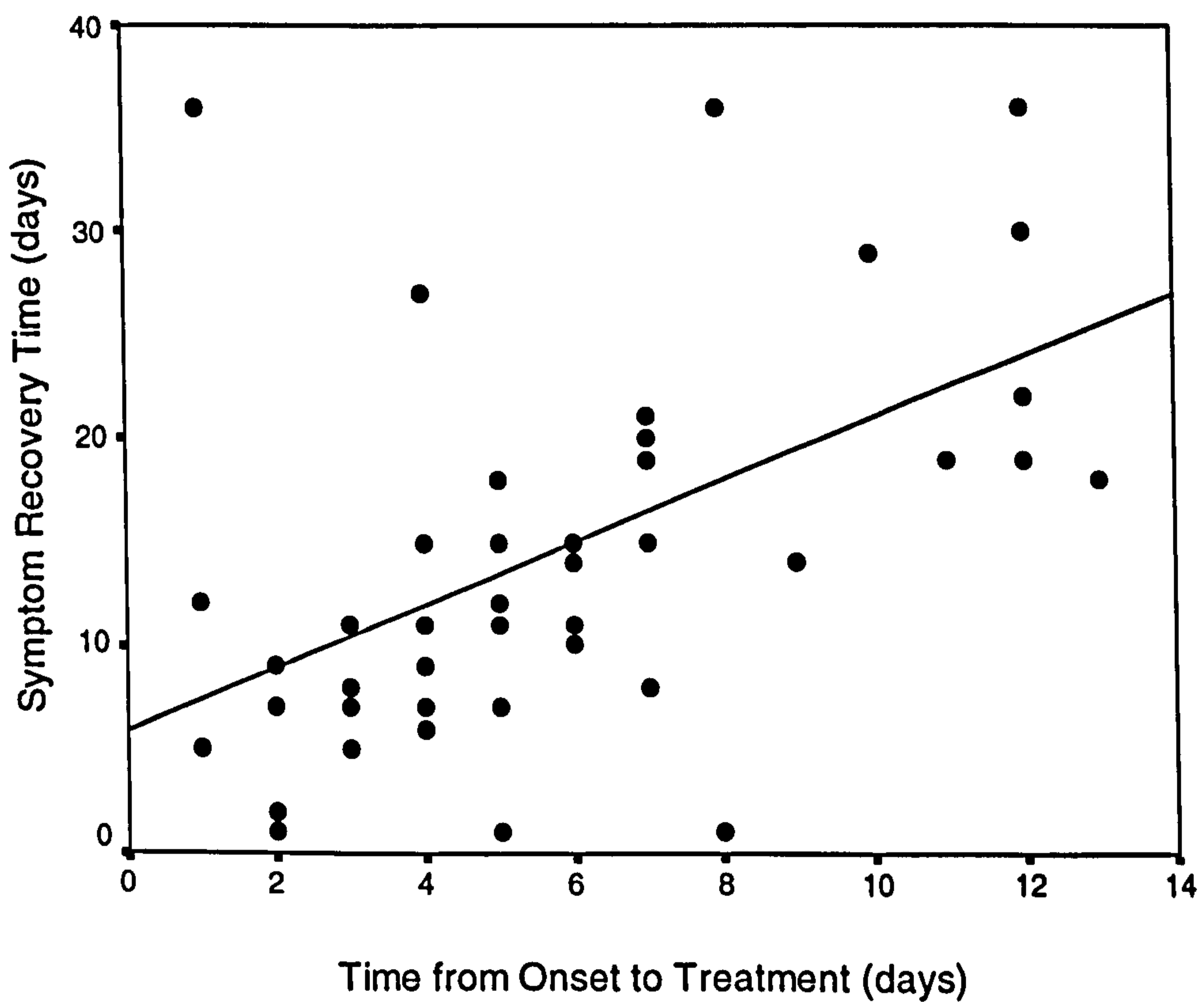
There were no significant relationships seen between baseline disease severity, airway bacterial load or pathogen type, or markers of airway inflammation (sputum IL6 or IL8) and symptom or lung function recovery time.

FACTORS AFFECTING EXACERBATION RECOVERY

A multivariate analysis of the effect of treatment delay on symptom recovery adjusted for age, active smoking and exacerbation symptom count, the effect of treatment timing remain significant ($p = 0.039$).

FIGURE 7.2 RELATIONSHIP BETWEEN REPORTING DELAY AND EXACERBATION

RECOVERY TIME. $\rho = 0.5$, $p = 0.004$, $n = 57$



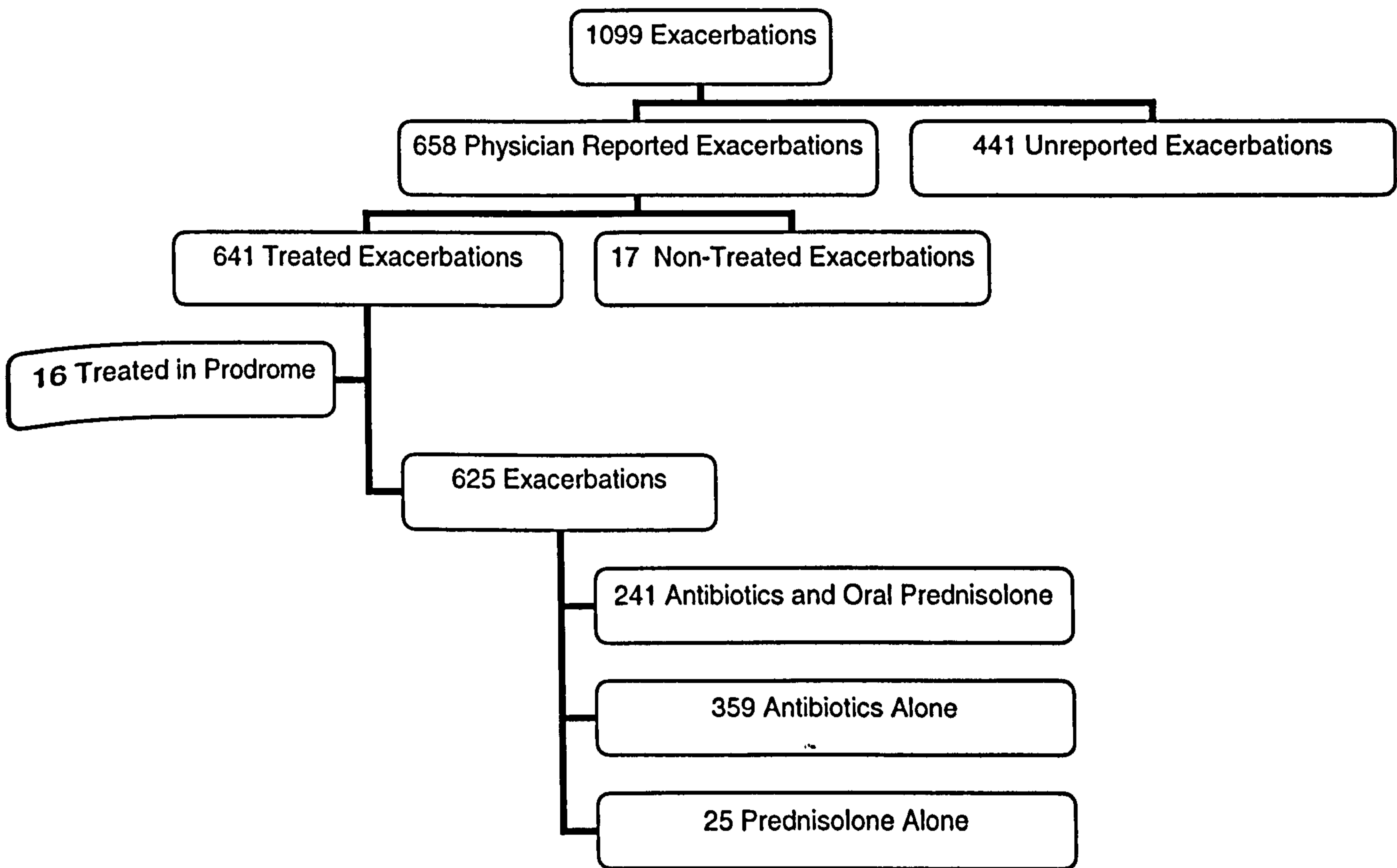
7.3.2 EFFECTS OF EXACERBATION REPORTING ON OUTCOMES: ANALYSIS OF COHORT DATA.

The physiological characteristics of the 128 (88 Male) patients at recruitment for the study are summarised in Table 1. The mean (SD) FEV₁ was 1.07 (0.43) litres and % predicted FEV₁ 40.8 (15.6) %. 115 patients took a mean (SD) daily dosage 1.2 mg (0.68) of inhaled steroids. 9 patients were on a mean of 4.9 (3.0) mg/day of oral prednisolone; 8 patients used both oral and inhaled steroids. The 128 patients in this study completed diary cards for a median of 925 (IQR 628 – 1520) days. 60 (46.9%) reported daily (chronic) dyspnoea and 65 (50.8%) daily sputum production.

TABLE 7.2 CHARACTERISTICS OF THE 128 (88 MALE) PATIENTS IN THE COHORT STUDY OF RELATIONSHIPS BETWEEN EXACERBATION THERAPY AND OUTCOMES.

	Mean (SD)
Age (years)	67.3 (7.6)
FEV ₁ (litres)	1.07 (0.43)
FEV ₁ % predicted	40.8 (15.6)
FVC (litres)	2.51 (0.81)
FEV ₁ /FVC %	43.50 (0.13)
PEF (litres/minute)	191 (88)
PaO ₂ (kPa)	8.93 (1.00)
PaCO ₂ (kPa)	5.89 (0.89)
Number of active smokers	42
Pack years of smoking	46.6 (31.4)
Inhaled steroid dosage (mg /day)	1.21 (0.68)

FIGURE 7.3 REPORTING AND TREATMENT CHARACTERISTICS OF 1099 EXACERBATIONS RECORDED IN THIS STUDY.



EXACERBATIONS.

During the study, the patients experienced a total of 1099 exacerbations. Of this total, 658 (59.9%) were reported to a physician, either a primary care physician or the study team. The median (IQR) number of exacerbations per patient per year was 2.51 (1.41 to 3.75) for all 128 patients. 8 patients had no exacerbations. Of the 1099 exacerbations, 441 (40.1%) exacerbations were diagnosed solely from review of diary card symptoms and were not seen by a physician. These unreported exacerbations were only considered in the analysis of hospitalisation and HRQOL with respect to reporting. The 658 remaining exacerbations were all seen by physicians but of these 17 (2.6%) exacerbations received no additional treatment and 16 (2.4%) involved physician consultation in the prodromal period before formal exacerbation onset as defined by the diagnostic criteria. These 33 exacerbations were excluded, leaving 625 which were analysed for the effects of physician reporting and treatment on recovery. A summary of reporting and treatment measures is illustrated in Figure 6.2.

Increased dyspnea was present in 63.7% of the 1099 exacerbations, increased sputum purulence in 26.6%, increased sputum volume in 41.3%, cold symptoms in 29.1%, increased wheeze in 31.7%, sore throat in 13.0% and increased cough in 30.7%.

PHYSICIAN REPORTED EXACERBATIONS AND TREATMENT

Figure 7.3 shows the reporting and treatment characteristics of the exacerbations recorded in the study. Of the 625 exacerbations which were treated with prescribed oral therapy, a total of 266 exacerbations were treated with oral corticosteroids. 600 of the exacerbations were treated with antibiotics, and of these 241 were treated with both oral corticosteroids and antibiotics. As 93.6% of

FACTORS AFFECTING EXACERBATION RECOVERY

exacerbations were treated with antibiotics, this precludes a meaningful statistical analysis of the effect of antibiotic therapy on exacerbation outcomes, as the non-treated group is too small. Oral prednisolone therapy hastened treated recovery by 2.63 days ($p=0.001$) compared to exacerbations not treated with prednisolone.

7.3.2.1 RELATIONSHIP BETWEEN PRESENTING SYMPTOMS AND OUTCOME

A regression analysis of the relationship between the nature of presenting symptoms and the recovery time of the exacerbation was performed. The results are summarised in Table 7.3. The regression coefficients for each individual symptom recorded on diary cards correspond to the additional days recovery time to the regression constant of 4.8 days for this analysis. The effects on exacerbation length shown in the table are summative. For example an exacerbation presenting with symptoms of dyspnoea and sputum volume calculated using this model would take $(4.80+2.84+1.61)$ 9.5 days to recover to baseline on average in this patient population.

TABLE 7.3 REGRESSION ANALYSIS OF INDIVIDUAL EXACERBATION SYMPTOMS AND RECOVERY TIME. 1099 EXACERBATIONS FROM 128 PATIENTS.

Symptom	Effect on Recovery Time if Symptom Present, days (95%CI)	p
Dyspnoea	2.84(2.17-3.51)	<0.001
Sputum Purulence	-0.37 (-1.06-0.31)	0.281
Sputum Volume	1.61 (0.93-2.29)	<0.001
Cold	1.88 (1.13-2.64)	<0.001
Wheeze	0.23 (-0.51-0.97)	0.541
Sore Throat	5.61 (4.45-6.77)	<0.001
Cough	0.211 (-0.55-0.97)	0.587

(Effect on recovery time: positive = longer, negative = shorter. Regression constant = 4.8 days, Regression coefficients are summative).

Exacerbations associated with increased dyspnoea and sputum volume and putative viral exacerbations associated with colds and sore throats took longer to recover than those without these symptoms and were thus more severe.

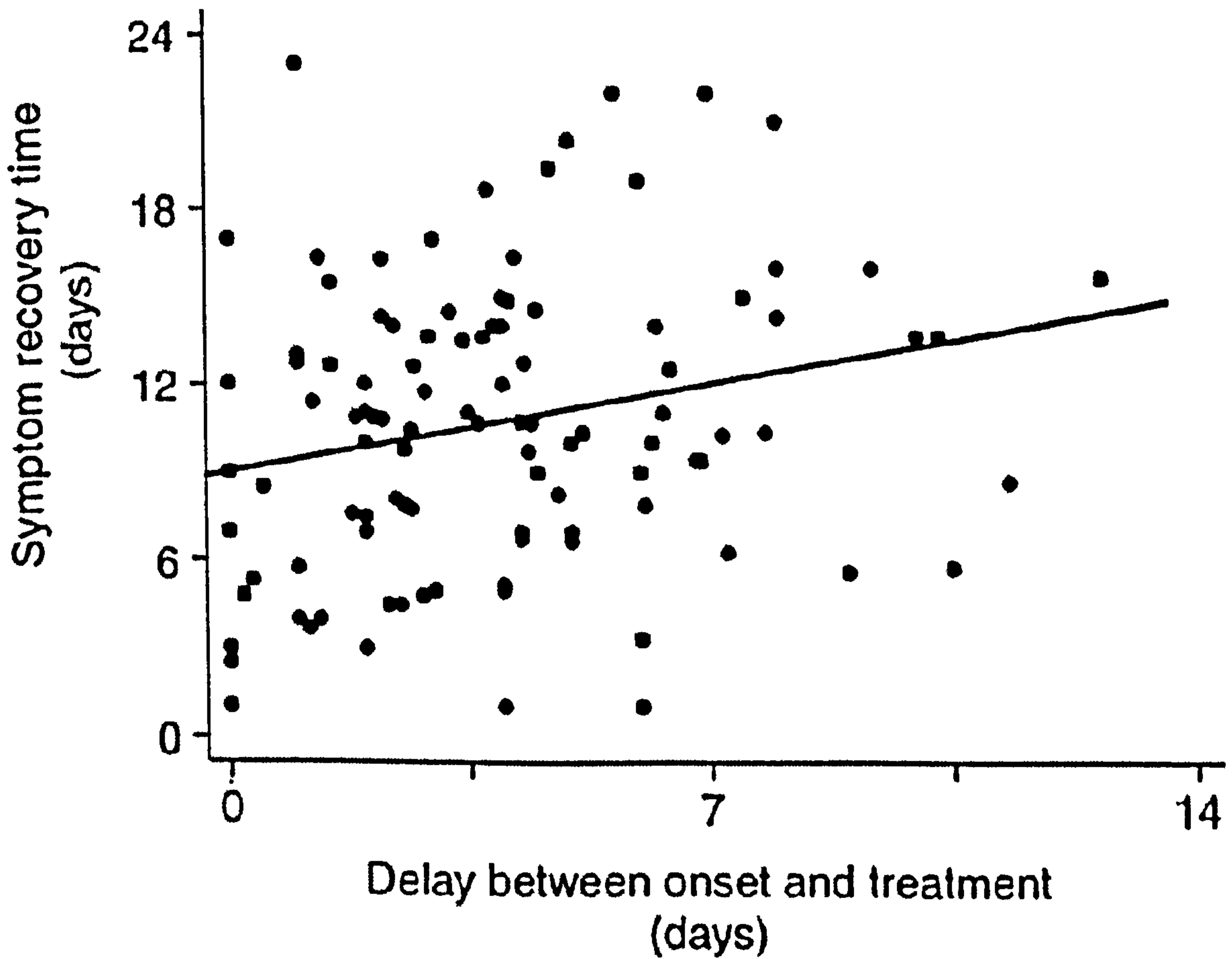
7.3.2.2 TREATMENT DELAY AND EFFECTS ON EXACERBATION RECOVERY TIME

The median (IQR) treatment delay (time from onset of exacerbation to the initiation of treatment) was 3.69 (2.00 to 5.57) days. The median exacerbation total recovery time was 10.7 (7.0 to 14.0) days. The median treated recovery time (time between the start of treatment to symptom recovery) was 6.9 (3.0 to 10.5) days. Figure 3 demonstrates the relationship between symptom recovery time and the delay between exacerbation onset and treatment. Early initiation of exacerbation therapy was associated with a faster recovery of exacerbation symptoms (regression coefficient, 95% CI, p value) (0.42 days/day delay, 0.19 to 0.65, $p < 0.001$).

The benefits of early physician consultation and treatment on exacerbation recovery time were potentially confounded by the fact that patients with more severe symptoms had longer exacerbations (2.68 days per symptom, 2.06 to 3.31, $p < 0.001$) and also tended to report earlier (-0.28 days per symptom, -0.66 to 0.10, $p = 0.15$).

Following an adjustment for symptom severity by its inclusion in the regression model, the relationship between early treatment and faster recovery became more pronounced (0.52 days/day delay, 0.31 to 0.74, $p < 0.001$). Treatment with oral corticosteroids could also confound the relationship if exacerbations reported earlier were preferentially treated with oral corticosteroids. However the relationship between recovery time and treatment delay remained significant if allowance was made for both symptom severity and the treatment with oral corticosteroids (0.57 days/day delay, 0.34 to 0.79, $p < 0.001$).

FIGURE 7.4 EFFECT OF EARLY TREATMENT ON RECOVERY OF EXACERBATION SYMPTOMS. Patient mean total recovery time (days) plotted against patient mean treatment delay ie time from onset of exacerbation symptoms to initiation of therapy) (days), in 108 patients. (Regression coefficient 0.42 day/day delay CI 0.19 to 0.65, $p < 0.001$).



FACTORS AFFECTING PHYSICIAN CONSULTATION DELAY

An analysis of patient characteristics with respect to mean treatment delay for each patient revealed that older patients received treatment earlier ($\rho = -0.19$, $p = 0.04$), however there were no observed significant relationships between treatment delay and baseline FEV₁ ($\rho=0.19$, $p=0.11$), percentage predicted FEV₁ ($\rho=0.11$, $p=0.25$), SGRQ Total score ($\rho=-0.12$, $p=0.21$), active smoking status ($\rho=0.03$, $p=0.72$) or baseline symptoms such as daily sputum production ($\rho= -0.16$, $p=0.08$) or dyspnea ($\rho=0.11$, $p=0.24$).

Table 7.4 gives the effect of a specific symptom at exacerbation onset on the treatment delay. Exacerbations (adjusted for repeated measures) involving worsening dyspnea as a presenting symptom presented earlier (regression coefficient (days), 95% CI, p value) (-0.42, -0.76 to -0.08, $p=0.016$) as did those with sputum purulence (-1.30, -1.60 to -0.92, $p=0.001$), wheeze (-0.59, -0.93 to -0.25, $p=0.001$) or sore throat (-0.78,-1.23 to -0.33, $p=0.001$).

TABLE 7.4 REGRESSION ANALYSIS OF INTERACTIONS BETWEEN EXACERBATION SYMPTOMS AND TREATMENT DELAY FOR 625 EXACERBATIONS

Symptom	Regression Coefficient (days) (95% Confidence Interval)	p value
Dyspnea	-0.42 (-0.76 to -0.08)	0.016
Sputum Purulence	-1.26 (-1.60 to -0.92)	0.001
Wheeze	-0.59 (-0.93 to -0.25)	0.001
Sore Throat	-0.78 (-1.23 to -0.33)	0.001
Cold	0.31 (-0.05 to 0.67)	0.093
Sputum Volume	0.02 (-0.31 to 0.34)	0.924
Cough	0.01 (-0.34 to 0.37)	0.945

(a negative value indicates a treatment delay shorter than those exacerbations without the symptom)

FAMILY PHYSICIAN VS. HOSPITAL OUTPATIENT PRESCRIBING.

Patients in the study could consult either the study team or their own primary care physician at exacerbation. 37.6% of the physician treated exacerbations were seen by a general practitioner and 62.4% were seen by a member of the study team. Prescribing practices at exacerbation were very similar irrespective of where the consultation took place, with primary care physician prescribing oral corticosteroids in 43.8% and antibiotics in 96.2% of treated exacerbations and the study team in 41.8% (chi-squared, $p=0.618$) and 95.9% ($p=0.868$) respectively.

The time to physician consultation was earlier in the primary care physician treated group by 0.36 days (-0.69 to -0.04, $p=0.030$) however when an adjustment for symptom severity was made no significant differences in timing of consultation was found. Despite similar prescribing practices, patients reviewed by a respiratory specialist in the study team had a shorter recovery time than those seen in general practice (0.82 days, $p=0.047$).

7.3.2.3 THE IMPORTANCE OF UNREPORTED EXACERBATIONS

SGRQ SCORES, HOSPITALISATION AND UNREPORTED EXACERBATIONS

Not all the exacerbations experienced by a patient were reported to a physician for treatment. The median (IQR) percentage of physician reported exacerbations for the 120 patients with an exacerbation was 66.7% (50 to 83.3). Stable SGRQ scores were recorded on 118 of these 120 patients. Figure 7.4 illustrates the relationship between SGRQ total score and its components, and the percentage of physician treated exacerbations. Patients who had a higher percentage of their exacerbations seen by a physician had a better health related quality of life (lower total SGRQ scores) ($\rho=-0.22$, $p=0.018$). This relationship was also present when considering the

FACTORS AFFECTING EXACERBATION RECOVERY

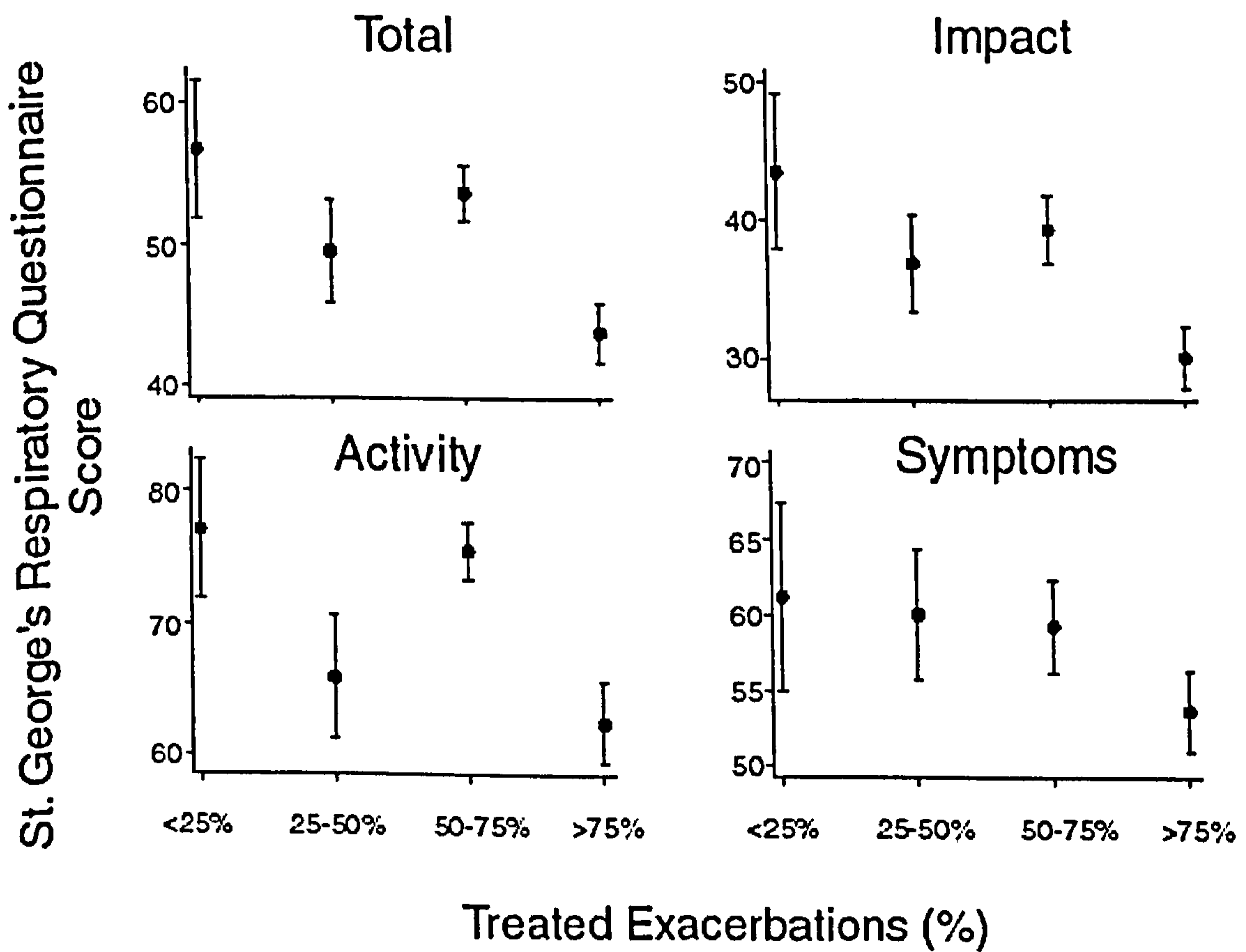
impact ($\rho=-0.19$, $p=0.04$) and activity ($\rho = -0.21$, $p = 0.02$) components of the SGRQ score separately.

UNREPORTED EXACERBATIONS AND RISK OF HOSPITALISATION

During the study 6.4% of the 1099 exacerbations required hospitalization. The median annual unreported exacerbation rate was 0.77 per year (0.33 to 1.60) for the 120 patients who had at least one. The annual rate for physician reported, but not hospitalised exacerbations, was 1.53 per year (0.86 to 2.37) and for hospitalized exacerbations the median rate was 0 per year (0 to 0.40).

Of the 120 patients, those who tended not to seek treatment from their general practitioner or the study clinicians, at exacerbation (as measured by a high annual rates of unreported exacerbations) were more likely to be admitted to hospital for treatment of an exacerbation than those who routinely reported their exacerbations for treatment ($\rho 0.21$, $p=0.04$).

FIGURE 7.5 RELATIONSHIP BETWEEN TOTAL AND COMPONENT SGRQ SCORES (HIGHER SCORES REPRESENT POORER HEALTH RELATED QUALITY OF LIFE) AND PERCENTAGE OF RECORDED EXACERBATIONS (IN QUARTILE GROUPS) THAT WERE REPORTED TO AND TREATED BY A PHYSICIAN. The Rho and p values from Spearman's Rank correlations of total and component SGRQ scores with proportion of exacerbations treated on a per patient basis are a) Total SGRQ score rho= -0.22, p=0.018), b) Impact Score rho=-0.19, p=0.04, c) Activity Score rho=-0.21, p=0.02 d) Symptoms Score rho=-0.13, p=0.16. Bars represent standard errors.



7.3.3 IMPROVING EXACERBATION REPORTING BEHAVIOUR

The impact of exacerbation reporting behaviour on exacerbation outcomes has been highlighted by the analysis presented above. It is possible therefore that interventions aimed at improving the timely delivery of exacerbation therapy may improve outcomes. However to date there have been few health care interventions which have been shown to improve patient reporting (Taylor 2005). We therefore studied a nurse lead intervention to determine if improvements in exacerbation reporting can be achieved within the cohort.

COPD patients were recruited from the outpatient department and local primary care clinics. In the first winter of the study (Period 1 November 03-February 04) patients were reviewed 3 monthly in the study clinic and at reported exacerbations, the cohort was followed through a second Winter (Period 2-November 04-February 05) when patients were telephoned at home every 2 weeks by a respiratory nurse specialist, questioned about symptoms and encouraged to attend the study clinic if an undiagnosed exacerbation was picked up.

RESULTS

We studied 116 patients with COPD, mean (SD) age 70.5(24.5) yrs, FEV₁ 1.25(0.55)l, FEV₁ % Predicted 50.4(21.8)%. 116 COPD patients had 213 exacerbations during periods 1 and 2. The nurse led intervention during period 2 was associated with an increased reporting rate of 61.7% compared to 45.8% in period 1, $p = 0.027$. Multivariate analysis showed that reporting rates were higher during exacerbations associated with increased dyspnoea (OR 3.2, $p=0.009$), sputum purulence (OR 2.24, $p=0.008$), increased cough (OR 2.6, $p=0.002$) and period 2 (OR 2.02, $p=0.024$). Over the two periods 31(26%) patients reported a mean of 0.55

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exacerbations more in period 2 ($p = 0.002$) and patients who reported less exacerbations in period 1 were more likely to have reported more exacerbations in period 2 ($p = 0.010$).

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7.4 DISCUSSION

This is the first prospective study to demonstrate the important effects of early treatment on COPD exacerbation outcomes. The findings show that patients who receive prompt therapy after the onset of their exacerbation are likely to recover more rapidly than those who delay reporting and thus initiation of treatment. Furthermore, patients who habitually fail to seek therapy for their exacerbations have poorer health related quality of life and are more likely to be hospitalised for the management of an exacerbation.

This study has been performed using well-characterised patients collecting daily data over a number of years and has used a symptom-based definition of exacerbations that is well validated (Seemungal 1998, Seemungal 2000, Seemungal 2001, Wilkinson 2003, Donaldson 2003). The collection of such prospective data allowed us to establish precisely the start of the exacerbation from the diary card entries and then the point at which therapy is commenced, in addition to in-depth analysis of the time-course and recovery time of the exacerbations. Our group and others have previously demonstrated that the recovery time of an exacerbation is an important measure of its severity and can be affected by various etiological factors such as respiratory viruses (Seemungal 2000, Seemungal 2001, Rohde 2003) and therapies (Anthonisen 1987, Davies 1999, Niewoehner 1999, Aaron 2003, Thompson 1996). The delay between symptom onset and therapy and its effect on exacerbation outcomes has not been studied previously as most studies evaluating exacerbations have focused on health care utilization and therefore have missed the period between symptom onset and the initiation of therapy. The finding that earlier treatment improves exacerbation recovery has confirmed for the first time the clinical suspicion that treating these episodes promptly offers additional benefit to the patient. It is also

the first to establish the size of this effect and the role that improving exacerbation reporting behaviour may play in reducing morbidity.

A possible alternative interpretation of this finding would be that patients with milder exacerbations seek treatment earlier and thus recover more quickly; however the data on the nature of symptoms at presentation demonstrates that this was not the case. Patients with more symptoms at exacerbation onset tended to present earlier for treatment and those exacerbations with more symptoms were indeed more severe as they took longer to recover. Therefore the milder and less symptomatic exacerbations were in fact presenting slightly later and when this effect is taken into account the benefit of early treatment became more pronounced.

In this study we have studied exacerbations across a spectrum of exacerbation severities and not only those more severe exacerbations requiring hospitalisation. The patients in this study were treated either by the dedicated study team or by their primary care physician and the exacerbation treatment reflected prescribing practices of both physician groups, which were very similar as reflected by similar exacerbation recovery times for both treatment groups. In the linear regression model of the effects of treatment delay on recovery time, allowance was made for the exacerbation treatment prescribed. A potential bias of variation in oral antibiotic therapy was not apparent as over 93% of treated exacerbations received this form of therapy. The effect of oral prednisolone prescription was to shorten exacerbations, if the prescription of this treatment was included in the regression model, the key finding that early treatment hastens recovery time became more pronounced.

These reported exacerbations which received treatment are in keeping with another definition of exacerbations recently suggested (Rodriguez Roisin 2000). Therefore, the findings of this study may be generalised to the COPD population

treated both in primary and secondary care and the validated methods used are suitable for future investigation into the relationships between reporting behaviour and exacerbation outcomes.

Exacerbations are heterogeneous in aetiology and in the nature of presenting symptoms (Seemungal 2001, Patel 2002). The symptom characteristics of an exacerbation affected presentation of the exacerbation to the physician. Symptoms of increased dyspnea, sputum purulence, wheeze and sore throat were associated with earlier presentation, while the presence of a common cold at exacerbations was associated with a trend towards later presentation. The factors affecting how COPD patients interpret changes in their symptoms are likely to be complex including their understanding of COPD which is often poor (Rennard 2002), the relationship with disease severity, and psychological overlay in a group of patients with high levels of anxiety and depression (Okubadejo 1996). Further studies into the mechanisms of symptom recognition are required to determine how to improve COPD patients' reporting behaviour and therefore exacerbation outcomes. These may include investigation of the role of patient education, self management plans and methods of assessing and improving compliance.

An additional advantage of collecting daily prospective data on symptom changes is that episodes when patients experience an exacerbation and record their symptoms, but do not consult their general practitioner or a study physician, can be analysed. In this study of 1099 exacerbations recorded on diary cards only 658 were reported to a physician for treatment and these rates are in keeping with published clinical trials (Burge 2000). The finding that on an annual basis, patients who are less likely to report their exacerbations are more likely to undergo emergency hospital admission for treatment is an important one. These patients may be less aware of the

importance of the changes in their symptoms at exacerbation or indeed they may subjectively experience less severe symptoms for a given severity of exacerbation. Thus, they are less likely to seek treatment from a physician in the early stages of their exacerbation. Furthermore, we found that patients who had a lower proportion of their exacerbations treated had worse health-related quality of life, as measured by the St George's Respiratory Questionnaire total score, impact score and activity score than those patients who sought treatment for a higher proportion of exacerbations. We can postulate that failure to report and therefore receive treatment for exacerbations contributes to additional morbidity from these events and thus adversely affects health-related quality of life. However, it may also be the case that patients with poor health related quality of life are less likely to seek physician intervention for their exacerbation, and thus a cycle of decline in quality of life and appropriate health care utilisation may become established. Identification and education of the patient group who delay or fail to seek treatment for exacerbations in particular may increase and improve the rate and timing of physician consultation, reduce patient morbidity and the considerable burden of inpatient treatment of exacerbations on health care services.

Analysis of factors that affected time to presentation of an exacerbation showed that older patients were likely to receive therapy earlier, but there was no relation between disease severity, baseline symptoms or the patient's health status. Adequate access to health care is important and the affect of age on presentation in our study suggests that the more elderly patients are receiving medical attention for exacerbations earlier than the younger patients in the group. This finding may be related to the higher degree of disability and co-morbidity in the elderly (Yohannes 2002), causing them to recognise and report symptoms at an earlier stage.

This study has been performed in a population of COPD patients who were encouraged to report exacerbations, who were likely to have heightened awareness of changes in their symptoms due to the use of daily diary cards and who had improved access to healthcare. Therefore, the findings of this study that early treatment of COPD exacerbations hastens recovery time and that patients who do not report to a physician are more likely to be hospitalised may be an underestimation of the scale of these effects in the general population of patients with COPD. Indeed COPD patients often have a poor understanding of their disease (Rennard 2002) and often delay or fail to report symptom changes to their physician. To date there has been no significant initiatives to encourage reporting and early presentation of exacerbations in this patient group.

Exacerbations of COPD are complex in their pathobiology and are markedly heterogeneous in the nature of presenting symptoms and clinical outcomes. Analysis of presenting symptoms has demonstrated that exacerbations associated with increased breathlessness, colds, sore throats and with increased sputum volume are likely to take longer to recover despite therapy than those without these symptoms. Increased dyspnoea may be a surrogate marker for disease severity and the other symptoms associated with longer recovery time are known to be markers of putative viral infections which we have previously shown to be associated with greater inflammation and lung function changes. The presence of cough, wheeze and sputum purulence did not increase recovery time in this analysis and this may be due to the fact that the aetiology of the processes that lead to these symptom changes; eg. sputum purulence due to bacterial infection responds relatively well to prescribed therapy ie. oral antibiotics. Greater understanding of the pathophysiology and

symptomatology of exacerbations may aid the development of novel therapies and assist the clinician in targeting treatment for these important clinical events.

The nurse-lead intervention study has shown that regular telephone contact with COPD patients can improve exacerbation reporting rates and could therefore potentially improve outcomes and reduce hospitalisation rates. However, it must be ensured that regular telephone contact does not result in over reporting of trivial symptoms as exacerbations. Further studies of novel healthcare interventions are required in COPD to determine whether altering exacerbation reporting behaviour may influence outcomes.

CONCLUSION

This study has demonstrated the important finding that the early recognition of exacerbation symptoms and prompt treatment by a physician is beneficial to the recovery of the exacerbation. This result suggests that improving patient and physician understanding of the nature of exacerbations and the benefits of early treatment will improve the outcomes of therapy of exacerbations of this extremely prevalent disease. There is a vital role for new, more efficacious treatments for COPD exacerbations. However by optimising the use of existing exacerbation therapies and by improving patients' awareness of exacerbations and access to health care, we can improve the current excessive burden of morbidity and mortality resulting from exacerbations.

CHAPTER 8

SUMMARY DISCUSSION AND AREAS FOR FUTURE RESEARCH

This chapter summarises the key findings of this study with particular attention to the original aims outlined in Chapter 2. Many of the findings in this thesis are novel and their implications have been discussed in the relevant chapter discussions. The key points are summarised below. This thesis concludes with some suggestions for future research in this area and a discussion of the possible implications for improving treatment for patients with COPD based upon the findings of this study.

8.1 CONCLUSIONS

The key findings of my studies are:

- Lower airway bacterial colonisation is common in this population of patients with moderate to severe COPD, with isolation rates of potentially pathogenic organisms of 57% in the stable state with the most prevalent pathogen being *Haemophilus influenzae*. These findings are in keeping with previous studies.
- Prospective patterns of bacterial colonisation were investigated. Colonisation was dynamic in nature with changes in isolation rates of particular pathogens

but no seasonal pattern was observed. Seasonal variations in airway bacterial loads were seen with higher prevalence in the summer months.

- Airway bacterial load and the degree of inflammation varied according to the nature of bacterial isolate in the stable state.
- The degree of neutrophilic airway inflammation measured as sputum IL-8 levels was related to the airway bacterial load in this population.
- Airway bacterial colonisation was associated with higher levels of systemic inflammation in terms of serum IL-6 and plasma fibrinogen. Higher levels of these markers were seen with the airway pathogens; *Haemophilus influenzae* and *Pseudomonas aeruginosa*. This finding suggests a possible mechanism for the systemic inflammation and resulting co-morbidities seen in COPD and thus a possible therapeutic opportunity to modulate systemic inflammation.
- For the first time, a relationship between airway bacterial colonisation and lung function decline was described. Patients with high and rising bacterial loads manifested a faster decline in FEV₁ in a 1-year follow-up study. This finding was confirmed with an analysis of cohort data with a longer follow-up.
- Faster decline in lung function was also associated with greater neutrophilic inflammation and changes in bacterial colonisation in terms of total bacterial load and nature of species isolated.

- Respiratory viruses can be detected in sputum samples from a significant proportion of stable COPD patients distant from any exacerbation symptoms.
- RSV was the most prevalent viral pathogen detected in this study and present in approximately 1/3 of stable state samples. Patterns of RSV detection were highly suggestive of persistent infection and no patterns of seasonality were observed. Sequencing of RSV PCR products confirmed high levels of homology with reference RSV strains and the sequence variability observed precludes contamination as a cause of the findings.
- Patients with frequent detection of RSV in stable sputum samples during a prospective study had higher levels of airway inflammation as measured by IL 6, IL 8 and MPO. These patients also had higher airway bacterial loads.
- These patients showed evidence of accelerated decline in FEV₁ over a two year follow up period. This relationship was significant independently of variations in baseline lung function, smoking, exacerbation frequency and airway bacterial colonisation but not of airway inflammation.
- PBMCs from patients in whom RSV was detected demonstrated an impaired IFN- γ (Th 1) response to whole RSV stimulation in an ex-vivo assay.
- At exacerbations, evidence human rhinovirus and bacterial infection was common (>70% of events). Higher airway bacterial loads , higher isolation

rates of potentially pathogenic micro-organisms and higher detection rates of human rhinovirus were seen compared to the stable state.

- The size of the rise in airway bacterial load seen at exacerbation was related to the severity of the episode both in terms of airway inflammation and falls in lung function.
- *Haemophilus influenzae* isolation at exacerbation was associated with higher airway bacterial loads and a trend towards more severe falls in lung function.
- Co-infection with both a bacterial pathogen and a virus was associated with higher airway bacterial loads, greater symptom severity and more severe falls in lung function suggesting a synergistic effect in modulating exacerbation severity.
- There was an association with the incidence of *Streptococcal* and human rhinoviral infection at exacerbation. This confirms findings of previous epidemiological studies.
- Sputum as collected and processed using the methods described in this thesis is a suitable substrate for proteomic analysis using microarray techniques. This should allow future studies to determine the exact nature of inflammatory responses at exacerbation and better understand the complexity of this heterogeneous response.

- The rate of recovery from exacerbation was related to a number of variables including patient age, smoking status, severity of initial exacerbation symptoms or lung function and evidence of viral infection as cold symptoms. A relationship between exacerbation recovery time and the delay between the onset of symptoms and treatment was also found.
- An analysis of a large dataset of exacerbations confirmed that early treatment offered substantial benefit to recovery independent patient or exacerbation characteristics.
- In this analysis patients who habitually fail to present for treatment at exacerbation exhibited poorer health-related quality of life and increased risk of hospitalisation.
- In a pilot intervention study, we demonstrated that exacerbation reporting behaviour can be improved by an intervention of regular patient telephone contact to increase reporting rates.

In consideration of the specific study aims outlined in chapter 2 the specific findings and their implications are:

1. To prospectively determine the prevalence and chronicity of lower airway bacterial colonisation in a population of patients with stable COPD.

The findings of this study confirm those of previous investigations into the bacteriology of the lower airway in COPD in that bacterial colonisation is a common phenomenon in patients with moderate to severe disease and that significant airway bacterial loads can be detected ($10^7 - 10^8$ colony forming units per ml) whilst the patient remains in a symptomatically stable state. The key pathogens were *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* in keeping with previous findings. The finding of higher airway bacterial loads in the summer may relate to antibiotic usage in the winter season but may infer that airway bacterial load per se is not the primary factor in driving exacerbation susceptibility.

2. To determine the prevalence and chronicity of lower airway viral colonisation in a population of patients with stable COPD.

For the first time, direct evidence for persistence of viral pathogens in the lower airway of COPD patients in the stable state is available as a result of this prospective study. The findings confirm the previous cross-sectional analysis that RSV is present in a significant proportion of COPD patients. The usually marked seasonal variation in RSV-induced acute bronchiolitis was not present in the stable state. The detection patterns for RSV RNA suggest that persistent infection rather than repeated re-infection is detected. Further studies including sequence identification of all RSV

isolates would enable viral epidemiological analysis to confirm the nature of viral persistence.

3. To determine the relationships between lower airway bacterial and viral infection on airway inflammation and disease progression in these patients.

Both airway bacterial and viral (RSV and HRV) detection in the stable state was associated with greater levels of sputum inflammatory markers. Neutrophilic inflammation, a key process in the genesis of airways obstruction, was elevated in subjects with higher airway bacterial loads and in those in whom viruses were detected in the stable state. The longitudinal study design permitted analysis of the associations between airway infection and lung function decline. High bacterial loads and RSV detection were associated with faster declines in FEV₁. Possible synergism between these effects was noted as subjects with evidence of RSV persistence also had higher sputum bacterial counts. However multivariate modelling suggested that RSV detection was an independent predictor of FEV₁ decline independent of the additional effects of bacterial colonisation.

4. To determine the relationships between lower airway infection and systemic inflammation.

Airway bacterial colonisation was associated with greater levels of peripheral inflammation as measured by IL 6 and fibrinogen levels in plasma, particularly when colonisation with *Haemophilus influenzae* and *Pseudomonas aeruginosa* was detected. This finding may have important implications for our understanding of the mechanisms underlying systemic inflammation as direct relationships between airway

and systemic inflammation were not found in this study, as in previous studies. RSV detection was associated with lower systemic levels of IL 6 but not fibrinogen. RSV infection was also associated with depression of PBMC responses to viral stimulation. It is possible that suppression of lymphocytic responses is a key feature of viral persistence whilst neutrophilic airway responses remain upregulated.

5. To determine the effects of lower airway bacterial and viral infection at exacerbations on the nature and severity of inflammation and clinical outcomes.

Both bacterial and rhinoviral infection were prevalent at exacerbations with evidence of greater infective load than in the stable state. Greater rises in airway bacterial load were associated with indices of exacerbation severity as were co-infection with viral and bacterial pathogens.

6. The relationship between RSV detection and responses of peripheral blood mononuclear cells to viral stimulation in ex-vivo assay.

RSV detection in the stable state was associated with depressed IFN- γ production by isolated and stimulated PBMCs from these patients. There were no observed differences in expression of TH2 cytokine or in IFN- γ production in response to general stimuli or UV-inactivated virus. This suggests that RSV may be able to down-regulate appropriate and specific anti-viral type 1 responses in patients with persistent infection. This phenomenon has been described previously in acute illnesses and has been associated with increased severity of these episodes.

7. The relationship between exacerbation reporting behaviour and clinical outcomes including exacerbation recovery, hospitalisation, and health related quality of life.

A number of factors were seen to relate to the rate of recovery from exacerbation which is an important clinical outcome which may influence time of hospital stay or absence from work. The novel finding that early treatment leads to early recovery could have important implications for service delivery. The patients who often failed to seek exacerbation treatment were at increased risk of adverse outcomes such as hospitalisation. These findings may influence both the way we must encourage patients to understand their disease and consideration of inclusion of unreported events in any definition of COPD exacerbations

8.2 FUTURE RESEARCH

This study has been largely based on the clinical observation and sampling of a cohort of patients with COPD. Whilst it has been carefully undertaken to address a particular hypothesis it has inevitably raised a number of findings and generated further hypotheses and research questions which warrant further consideration and investigation. The eventual aim of this type of clinical research is not only to enable a better understanding of a disease but to directly and beneficially impact on patients' lives by improving the range or efficacy of treatment and hence to improve clinical outcomes. This final section of this thesis outlines the direction future research may take to achieve these goals and in some cases where projects based upon the findings described in the previous chapters are being undertaken.

MECHANISTIC STUDIES ON SUSCEPTIBILITY TO INFECTION

A key question arises from consideration of this work: why are patients with COPD susceptible to airway infection and to the associated consequences described in this thesis? Since the normal lower airway is sterile, at what level or levels of the immune system does the susceptibility to infection lie? The immune defences of the airway are complex ranging from the mechanical effects of the muco-ciliary escalator, the integrity of the airway epithelium and the production of antimicrobial peptides to cellular innate responses such as macrophage-induced phagocytosis and the activity of toll-like receptors and other mechanisms which are poorly characterised in this disease. The array of adaptive immune responses is equally complex and whilst a number of studies has described cellular compositions in various compartments of the COPD lung, very little is known on the functional status of these cells. Why for

example are there numerous T cells present in the small airways? Are these effector cells and if so are they fulfilling an anti-infective or even auto-immune function?

Clearly, detailed studies characterising the innate and adaptive immunology of COPD patients compared to normals are required, as are studies of differences between different disease phenotypes such as frequent and infrequent exacerbators. Our group is currently undertaking a project to investigate the role that expression of the main rhinovirus cell adhesion molecule, ICAM -1, plays in driving susceptibility to this virus and hence to exacerbation. Mechanistic as well as observational studies will be required to tease out the facets of the immune response peculiar to COPD and hopefully will identify new therapeutic targets and improve our understanding of airway infection as a whole.

INTERVENTION STUDIES AIMED AT BREAKING THE 'VICIOUS CIRCLE'

The difficulty with the interpretation of observational studies is the determination of cause and effect. The proposed circular relationship between airway inflammation, compromised airway defences, airway infection and consequent up-regulation of inflammatory responses is described as the 'vicious circle hypothesis'. Clearly therefore as discussed previously the finding that airway infection is related to inflammation and disease progression may be an epiphenomenon. A simple way of breaking this cycle of inter-association would be to eradicate the infectious component using antibiotic therapy and determine the effects on airway inflammation particularly targeting *Haemophilus influenzae* colonisation as it is associated not only with greater airway bacterial loads and inflammation but also with more severe exacerbations. Our group is currently involved in such an intervention study and if positive, the results may alter our practice in a similar manner to that observed in the

care of other diseases where airway infection is treated pro-actively such as in cystic fibrosis.

FURTHER STUDIES OF COPD PATHOGENESIS: EARLY DISEASE

The studies described in this work have been performed in a population of patients with moderate to severe COPD. Clearly the role of airway infection plays in milder disease may differ greatly from that described above. However, the study of patients with mild disease or indeed those who are yet to develop COPD would answer fundamental questions about the role of airway pathogens in the initial development of airway obstruction. It is known that only a proportion of smokers will develop COPD and the factors underpinning disease susceptibility have not been identified despite a number of genetic studies. It is possible that two environmental 'hits' are required to develop the disease, airway infection and inhalation of toxic substances such as cigarette smoke. A long term, large study would be required to examine such a hypothesis and would perhaps not only identify the reasons why certain individuals are susceptible to the effects of tobacco smoke in the lung but also to enable targeted early intervention to prevent the onset of this progressive disease.

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NAME		January 2006										NEXT APPOINTMENT		
Study Number		WORSENING SYMPTOMS? CALL US										/ /06 am		
DATE		1	2	3	4	5	6	7	8	9	10	11		
Peak Flow														
CHANGE in Symptoms														
CHANGE in Treatment														
Hours Out of the Home														

DATE		12	13	14	15	16	17	18	19	20	21	22		
Peak Flow														
CHANGE in Symptoms														
CHANGE in Treatment														
Hours Out of the Home														

DATE		23	24	25	26	27	28	29	30	31		
Peak Flow												
CHANGE in Symptoms												
CHANGE in Treatment												
Hours Out of the Home												

Instructions for filling in the DIARY CARDS

EVERY DAY...

1. After taking morning medications record the best of 3 attempts at the PEAK FLOW blowing test in the box on the sheet.
2. Please record any **WORSENING** of symptoms from your usual daily level. The symptoms we are interested in are listed below, just put the appropriate letter in the box on the sheet. Continue recording until the symptom has gone away or got back to the level you consider 'normal'.

Letter	Symptom
A	increased BREATHLESSNESS.
B1	increased SPUTUM COLOUR.
B2	increased SPUTUM AMOUNT.
C	a COLD (such as a runny or blocked nose).
D	increased WHEEZE or CHEST TIGHTNESS.
E1	SORE THROAT.
E2	increased COUGH.
F	FEVER.

If you experience a worsening in any one of these symptoms please phone us to arrange an assessment visit, and do this BEFORE starting any antibiotic or steroid tablets. The phone number is

07762 038662.

Wayomi, Tom or Ramin will have the phone and we can usually arrange to see you later the same day or the following morning.

It is best to phone first-thing in the morning.

3. Please record any **CHANGE** to your usual treatment for as many days as it applies. Again, just put the appropriate letter in the box on the sheet.

Letter	Treatment
H	I am in Hospital.
I	I am taking more than usual INHALED STEROID (red / brown).
R	I needed to take extra RELIEVER (blue / green / grey / nebuliser). HOW MANY PUFFS? Write, eg 'R3' for 3 puffs, 'R2' for 2 etc
S	I am taking STEROID (Prednisolone) TABLETS. HOW MANY TABLETS? Write, eg 'S6' for 6 tablets, 'S5' for 5 etc
X	I am taking ANTIBIOTIC TABLETS. PLEASE RECORD WHICH (write the name on the diary card).

4. Finally, please estimate the time that you were out of your own home on the previous day.

LIST OF PROJECT PUBLICATIONS

PAPERS

TMA Wilkinson, JR Hurst, W Perera, M Wilks, GC Donaldson and JA Wedzicha.

Interaction of Bacterial and Rhinoviral Infection during Chronic Obstructive Pulmonary Disease Exacerbations. *Chest* 2006; 129: 317 - 324.

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ABSTRACTS

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T M A Wilkinson, I S Patel, A. C Whiley, M Wilks and J A Wedzicha The relationship between bacterial colonisation and lung function decline in COPD. *Thorax* 2001;56:iii20.

SPOKEN PRESENTATIONS OF WORK AND AWARDS ARISING FROM THE RESEARCH

SPOKEN PRESENTATIONS

British Thoracic Society Winter Meeting, London 2001. Spoken Presentation at Symposium: The relationship between bacterial colonisation and lung function decline in COPD.

European Respiratory Society Annual Meeting, Stockholm 2002. Spoken Presentation at Symposium: Relationship between bacterial load, airway inflammation and lung function decline in patients with COPD.

British Thoracic Society Winter Meeting, London 2002 Spoken Presentation at Symposium: The Effect of Airway Bacterial Load on Exacerbation Severity in Patients with COPD.

American Thoracic Society Annual Meeting, Seattle 2003 Spoken Presentation at Symposium: Relationship between Bacterial Infection and Exacerbation Severity in Patients with COPD.

American Thoracic Society Annual Meeting, Seattle 2003 Spoken Presentation at Symposium: Delay in Reporting Prolongs COPD Exacerbation Recovery.

European Respiratory Society Annual Meeting, Vienna 2003. Spoken Presentation at Symposium: Early Treatment Hastens Recovery of COPD Exacerbations.

American Thoracic Society Annual Meeting, Orlando 2004. Spoken Presentation at Symposium: Interaction of Haemophilus influenzae and Viral Infection at COPD Exacerbation.

American Thoracic Society Annual Meeting, Orlando 2004. Spoken Presentation at Symposium: Impact of Reporting and Early Treatment on Outcome of COPD Exacerbations.

European Respiratory Society Meeting, Glasgow UK, 2004. Spoken Presentation at Symposium: Differential inflammatory effects of colonising airway bacteria in COPD.

European COPD Network Meeting, Rome 2002. Lecture: 'COPD in the UK'.

European COPD Network Meeting, Copenhagen 2003. Lecture: 'Viral and Bacterial Pathogenesis of COPD Exacerbations'.

European COPD Network Meeting, Verona 2003. Lecture: 'Role of Respiratory Viruses in COPD Exacerbations'.

European COPD Network Masterclass, Paris 2004. Lecture: 'Airway Pathogens in COPD; Passengers or New Therapeutic Targets'.

London University Microbiology MSc Course. 2004 Lecture: 'Role of Infection in Airways Disease'.

British Thoracic Society Winter Meeting, London 2004: BTS/BLF Young Investigator of the Year Finalists Symposium. Spoken Presentation: Consequences of RSV Infection in Patients with Stable COPD.

Belgian and Dutch Universities Joint Respiratory Post Graduate Meeting, Antwerp 2005. Spoken Presentation: The role of Airway Infection in COPD.

NHLI/ Royal Brompton Hospital, Pharmacology of Asthma and COPD 2005. Lecture: Acute Exacerbations of COPD.

European Respiratory Society Meeting, Copenhagen, 2005. Spoken Presentation at Symposium: Associations and Impact of Human Rhinoviral Infection in Exacerbations of COPD.

Infectious Diseases Recognised Research Group Co. Antrim, NI. Spoken Presentation Role of Airway Infection in the Pathogenesis of COPD.

AWARDS FOR THE RESEARCH

Winner 20,000 Euro Award European Respiratory Society (ERS) COPD Researcher of the Year 2005.

Finalist & Runner Up British Thoracic Society Young Investigator of The Year Award 2004.

European Respiratory Society (ERS) Award for Best COPD Abstract, European Respiratory Society Meeting, Glasgow 2004

Winner; European Respiratory Society (ERS) United Kingdom COPD Research Award 2003.

British Lung Foundation; European Respiratory Society Travel Fellowship 2003

British Thoracic Society Travel Fellowship, European Respiratory Society meeting Stockholm 2002.

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Airway Bacterial Load and FEV₁ Decline in Patients with Chronic Obstructive Pulmonary Disease

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Chronic obstructive pulmonary disease (COPD) is characterized by an accelerated decline in lung function and progressive airway inflammation. Bacteria have been isolated from the lower airway of stable COPD patients, and airway inflammation has been related to bacterial load and type. The relationship between bacterial colonization, airway inflammation, and lung function decline remains uncertain. We studied 30 patients with COPD, mean (SD) FEV₁ 0.947 (0.329), 34.8% (13.6%) predicted, for 12 months. Sputum collected at recruitment and the end of the study was analyzed for cytokines and for quantitative bacteriology. The decline in FEV₁ was 57.6 (137.6) ml year⁻¹. Bacterial growth was identified in all subjects, with an initial count of 10^{7.47(0.91)} cfu ml⁻¹ rising to 10^{7.93(0.81)} cfu ml⁻¹ at the end of the study ($p = 0.019$). FEV₁ decline was related to this increase in airway bacterial load ($r = 0.59$, $p = 0.001$). FEV₁ decline was greater in subjects who exhibited a change in the colonizing bacterial type compared with those with persistence of a single bacterial species over the study period ($p = 0.017$). Higher sputum interleukin (IL-8) was associated with greater declines in FEV₁ ($p = 0.03$). Rising airway bacterial load and species changes are associated with greater airway inflammation and accelerated decline in FEV₁. Bacterial colonization in COPD is an important factor in disease progression.

Keywords: chronic obstructive pulmonary disease; bacterial colonization; FEV₁ decline; airway inflammation

Chronic obstructive pulmonary disease (COPD) is characterized by an accelerated and progressive decline in lung function, which is not fully reversible (1–3). Smoking is the most important etiologic factor for COPD and is known to cause inflammation in the lung (4). However, smokers exhibit a variable rate of decline in lung function, suggesting that factors such as variability in smoking behavior, susceptibility to cigarette smoke, and other factors such as airway inflammation caused by bacterial colonization may contribute to the progression of COPD.

Patients with stable COPD exhibit increased airway inflammation (5, 6). The degree of airway inflammation is positively related to the severity of airway obstruction with more bronchial inflammation in patients with lower FEV₁ (4). Furthermore, higher levels of airway inflammation, as evidenced by high sputum neutrophil counts, were associated with a greater rate of decline in FEV₁ (7). The stimulus for increasing airway inflammation as lung function declines has not yet been determined.

The lower airways of healthy individuals are sterile, but bacteria have been isolated in significant numbers in patients with clinically stable COPD, indicating the presence of lower airway bacterial colonization (LABC) (8–10). The presence of bacteria in the lower airway can result in a range of important effects on the lung, including activation of host defenses with release of inflammatory cytokines and subsequent neutrophil recruitment, mucus hypersecretion, impaired mucociliary clearance, and respiratory epithelial cell damage (11). Animal models of chronic bacterial infection in the lung have shown changes characteristic of those seen in COPD in terms of inflammatory cells, cytokine expression, and pathologic changes to both airways and alveoli (12). There is evidence that airway inflammation increases with higher airway bacterial loads determined from quantitative sputum cultures in patients with COPD (13). Thus, it has been suggested that chronic LABC contributes to progression of airways obstruction (14, 15).

Previous studies performed to evaluate the relationship between airway bacterial colonization, inflammation, and lung function have been cross-sectional in design and have not addressed the important relationship between these parameters and effects on disease progression. This study addresses the hypothesis that bacterial colonization leads to increased airway inflammation and thus contributes to the accelerated progression of airway obstruction. We have performed a prospective observational study in well-characterized patients with moderate to severe COPD to elicit the relationship between LABC using both quantitative and qualitative microbiologic techniques and the progression of airway obstruction.

METHODS

Patient Selection

Thirty patients with COPD were recruited from volunteers in the East London COPD cohort and gave informed consent. Ethics approval was obtained from the East London and City Health Authority Research Ethics committee. The inclusion criteria for this prospective cohort study have previously been published and include FEV₁ of less than 70% predicted and β_2 agonist reversibility of less than 15% of baseline and/or 200 ml. Patients were assessed clinically and with a chest radiograph at recruitment to ensure the absence of other significant respiratory disease (16). Patients completed daily diary cards, for symptoms and recorded peak expiratory flow. Exacerbations were diagnosed from the diary card data as previously described (16–18). We ensured that each patient had been clinically stable (exacerbation free) for at least 6 weeks before both recruitment and sampling at the end of the study by patient interview and review of diary cards.

Patients were followed prospectively for 1 year. Patients who suffered an exacerbation around the end of the study period were only sampled when they had been clear of exacerbation symptoms and had completed any exacerbation treatment for at least 6 weeks. The mean sampling interval (after allowance for ensuring patients were fully stable before the second sample point), therefore, was 1.11 years.

Measurement of Lung Function

Lung function was measured with a rolling seal spirometer (Sensor Medic Corp., Yorba Linda, California). Lung function measurements

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TABLE 1. CHARACTERISTICS OF PATIENTS IN THE STUDY

Characteristic	Mean (SD)
Number, female/male	8/22
Age, years	66.43 (10.25)
FEV ₁ , L	0.95 (0.33)
FEV ₁ percentage predicted	34.81 (13.61)
FVC, L	2.51 (0.70)
FEV ₁ /FVC percentage	38.40 (10.70)
PEF, L/min	218.07 (76.14)
P _{aO₂} , kPa	8.55 (1.11)
P _{aCO₂} , kPa	6.20 (1.00)
Pack-years of smoking	74.3 (66.5)
Inhaled steroid dosage Beclomethasone equivalent, mg/day	1.55 (0.92)

were taken between 9:30 A.M. and 11:30 A.M., 1 hour after the patient's usual bronchodilator medication inclusive of 200 µg of salbutamol via metered dose inhaler. At least three spirometry readings were taken at each visit, and the best performance was recorded.

Sputum Sampling

Sputum was sampled at the beginning and the end of the study. Immediately after lung function measurement, patients were asked to expectorate spontaneously sputum into a sterile pot. Patients unable to produce a sample of sputum spontaneously underwent sputum induction (17). Sputum samples containing less than 25 squamous epithelial cells per low-powered field and more than 25 leukocytes per high-powered field were accepted for processing. The sample was separated from saliva, and a portion was taken and analyzed for bacteriology (19); the remainder was processed using previously published methods (17, 20, 21) and analyzed for inflammatory cytokines (17, 21). Sputum interleukin (IL)-6 and IL-8 levels were measured using ELISA (R&D Systems, Abingdon, UK) (17). Twenty of the baseline samples have been used for an analysis of the relationship between LABC and exacerbation frequency (22).

Quantitative Bacterial Analysis

Samples were processed by using sputolysin. Serial dilutions were made and cultured on appropriate media. These were incubated for 18 hours at 37°C in an atmosphere of air +5% CO₂. After incubation, bacterial colonies were enumerated and subcultured for identification by standard methods (19, 22). The number of colony forming units per gram of sputum was calculated from the total number of colonies obtained and the dilution to give the total bacterial count for each sample expressed in cfu ml⁻¹.

Statistical Analysis

Normally distributed data are reported by means (SDs) and skewed data by medians (interquartile range [IQR]). Correlations were assessed using the Pearson or Spearman correlation coefficient (two tailed). Continuous variables with normal distributions were compared by *t*-test, whereas those with non-normal distributions were compared by the Mann-Whitney U or Wilcoxon signed ranks test.

During the analysis, patients were divided into groups dependent on exacerbation frequency during the study. Patients with an exacerbation frequency that was higher or lower than the median were termed "frequent" or "infrequent" exacerbators, respectively (16); *p* values of 0.05 or less were regarded as significant. The SPSS version 10.0 (SPSS Chicago, IL) statistical package was used for data analysis. An extended version of the methods is available in an online supplement.

RESULTS

Patient Characteristics

The baseline physiologic characteristics of the 30 patients who were recruited for the study are summarized in Table 1. The 30 patients were followed for a median (IQR) period of 1.05 (1–1.22) years to ensure all sampling data were collected in the stable state. The mean (SD) FEV₁ was 0.947 (0.329) L, and the predicted FEV₁ was 34.8% (13.6%), with a range from 13.80 to

69.95% predicted. Therefore, all patients can be classified as suffering from moderate to severe COPD according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (3). Eleven of the patients were current smokers at the time of recruitment and did not alter their smoking habits during the study. Twenty eight of the patients were receiving inhaled steroids mean (SD) dosage 1.55 mg (0.92) beclomethasone equivalents; no changes to the dose of inhaled steroid occurred during the study. At the first sample point, 24 patients produced sputum spontaneously, the remainder being induced, compared with 22 patients expectorating spontaneously at the second sample point.

FEV₁ Decline

The mean (SD) FEV₁ at recruitment was 0.947 (0.329) L and declined to 0.883 (0.367) L at the end of the 1.05 (1–1.22)-year sample interval. The mean annual rate of decline was 57.6 (137.6) ml per year; expressed as percentage of initial FEV₁, this equates to 6.08% of baseline FEV₁ decline per year.

The 30 patients had a total of 86 exacerbations during the study period, 40 (46.5%) of which were reported to the study team; the remainder of the exacerbations were diagnosed from diary card review, a proportion of which (17.4%) had been independently reported to a general practitioner. Fifty-two exacerbations received antibiotic treatment during the study. The median (IQR) exacerbation frequency in this study was 2.39 (1.95) exacerbations per year. Patients with an exacerbation frequency higher than this median (frequent exacerbators) had more severe airways obstruction with a mean FEV₁ of 0.86 L compared with infrequent exacerbators with a mean FEV₁ of 1.07 (*p* = 0.05).

Quantitative Bacteriology

All cultures of sputum samples grew significant numbers of bacteria ranging from 10^{5.4} to 10^{9.6} cfu ml⁻¹. The mean (SD) total bacterial count at sample 1 was 10^{7.47(0.91)} cfu ml⁻¹ and rising to 10^{7.93(0.81)} in sample 2 (*p* = 0.019) or a rise from 29,512,092 cfu ml⁻¹ to 85,113,804 cfu ml⁻¹ when expressed without log transformation.

Bacterial Load and FEV₁ Decline

Patients with an increasing airway bacterial load demonstrated a more severe decline in FEV₁ over the study period compared with patients with stable or decreasing airway bacterial load who exhibited less marked declines or slight improvements in FEV₁. This relationship between FEV₁ decline and changes in bacterial load (Figure 1) was statistically significant in terms of absolute FEV₁ decline (*r* = 0.593, *p* = 0.001) and decline expressed as a percentage of baseline FEV₁ (*r* = 0.633, *p* < 0.001). The total bacterial count of the second sample was itself related to the absolute rate of decline over the study (*r* = 0.560, *p* = 0.001) (Figure 2). The total bacterial count of the first sample was not predictive of the subsequent decline in FEV₁ over the study (*r* = -0.125, *p* = 0.369).

A linear regression analysis of the relationship between bacterial load and FEV₁ decline revealed that a 10-fold increase (10¹ cfu ml⁻¹) in bacterial load is associated with an 82.4-ml decline in FEV₁ over the study period; the regression coefficient was 0.095 (95% confidence interval, 0.032–0.132) (*p* = 0.002). As the mean increase in bacterial load was from 10^{7.47} to 10^{7.93} cfu ml⁻¹, this represents a decline in FEV₁ attributable to the airway bacterial load of 33.3 ml/year for this patient group. A multivariate regression analysis of potential factors in the observed decline in lung function (L/year) was performed: regression coefficient (95% confidence interval), change in bacterial load (log cfu ml⁻¹) 0.0631 (0.034–0.133, *p* = 0.003), number of cigarettes

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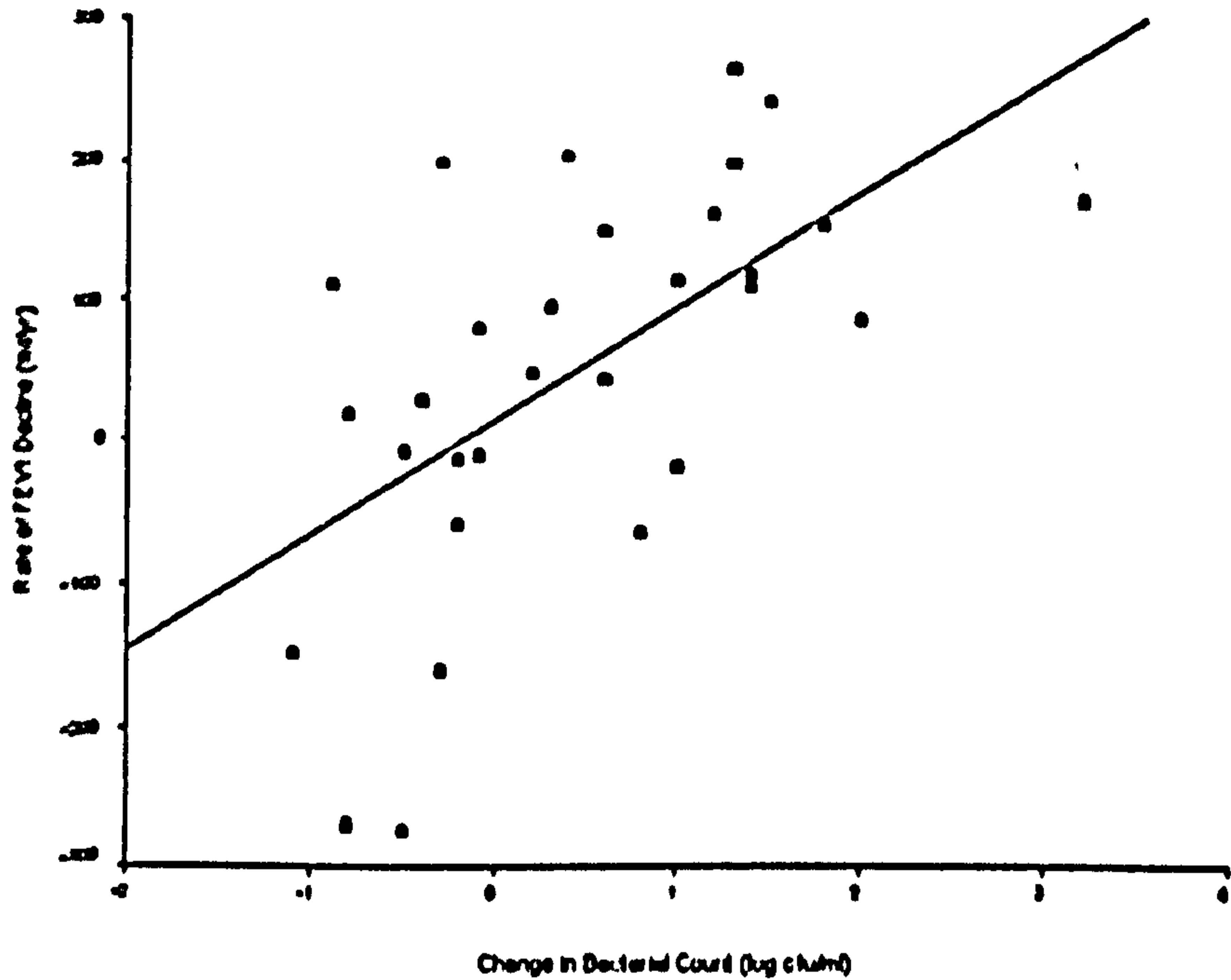


Figure 1. Correlation between change in FEV₁ and change in total bacterial count over study period. Figure shows decline in FEV₁, expressed as milliliters of loss (negative values indicate improving lung function) against log change in total bacterial count (positive values indicate increasing numbers of bacteria over study) ($r = 0.59, p = 0.001$).

smoked per day -0.011 ($-0.11-0.89, p = 0.818$), exacerbation frequency -0.002 ($-0.27-0.022, p = 0.819$), and baseline FEV₁ 0.001 ($-0.002-0.005, p = 0.368$). The change in bacterial load and the decline in FEV₁ were the strongest and the only significant relationship in this analysis.

Bacterial Isolates

The results of the qualitative bacteriology from sputum samples taken at the beginning (sample 1) and the end (sample 2) of the study are shown in Figure 3. The graph illustrates the relative frequency of each bacterial isolate expressed as a percentage of the 30 samples at each time point. Sixteen (53.0%) and 17 (56.6%) patients were colonized with a potentially pathogenic organism

at recruitment and completion of the study, respectively. The remainder of patients' sputum produced nonspecific growth of bacteria, defined as growth of bacterial species not usually associated as respiratory pathogens in immunocompetent individuals such as *Streptococcus viridans* group, *Neisseria* spp, *Corynebacterium* spp, and coagulase negative staphylococci. The relative frequencies of individual bacterial species are as shown. Five and six subjects respectively displayed colonization with more than one potentially pathogenic microorganisms at recruitment and completion. At each time point, *Haemophilus influenzae* was the most prevalent individual bacterial type present in 9 (30%) and 7 (23.3%) at samples 1 and 2, respectively.

The nature of bacterial colonization was dynamic with changes

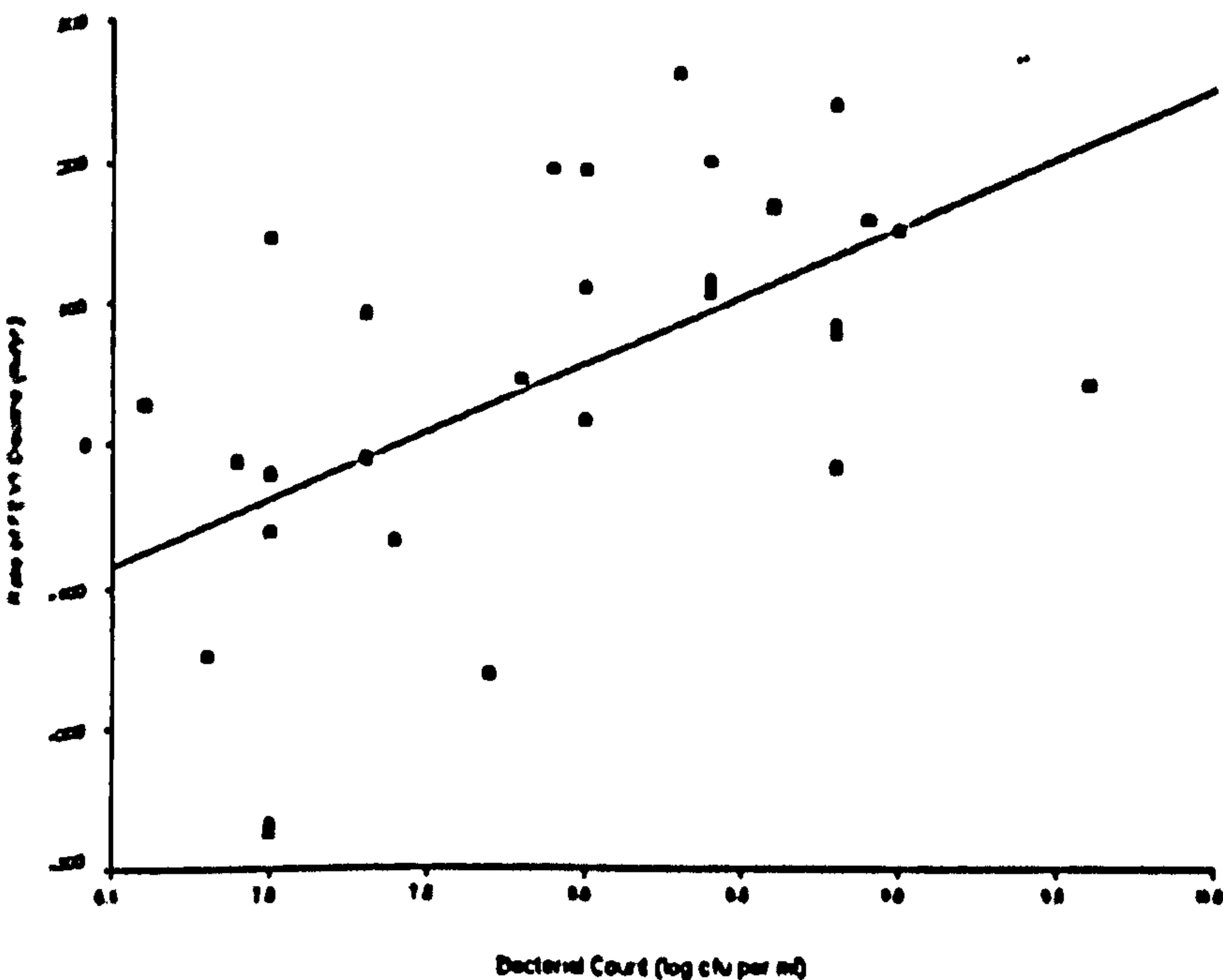


Figure 2. Correlation between rate of change in FEV₁ over study period (adjusted to ml/year) and total bacterial count at the end of the study. Figure shows decline in FEV₁, expressed as milliliters of loss (negative values indicate improving lung function) against log change in total bacterial count from sputum taken at the end of the study ($r = 0.56, p = 0.001$).

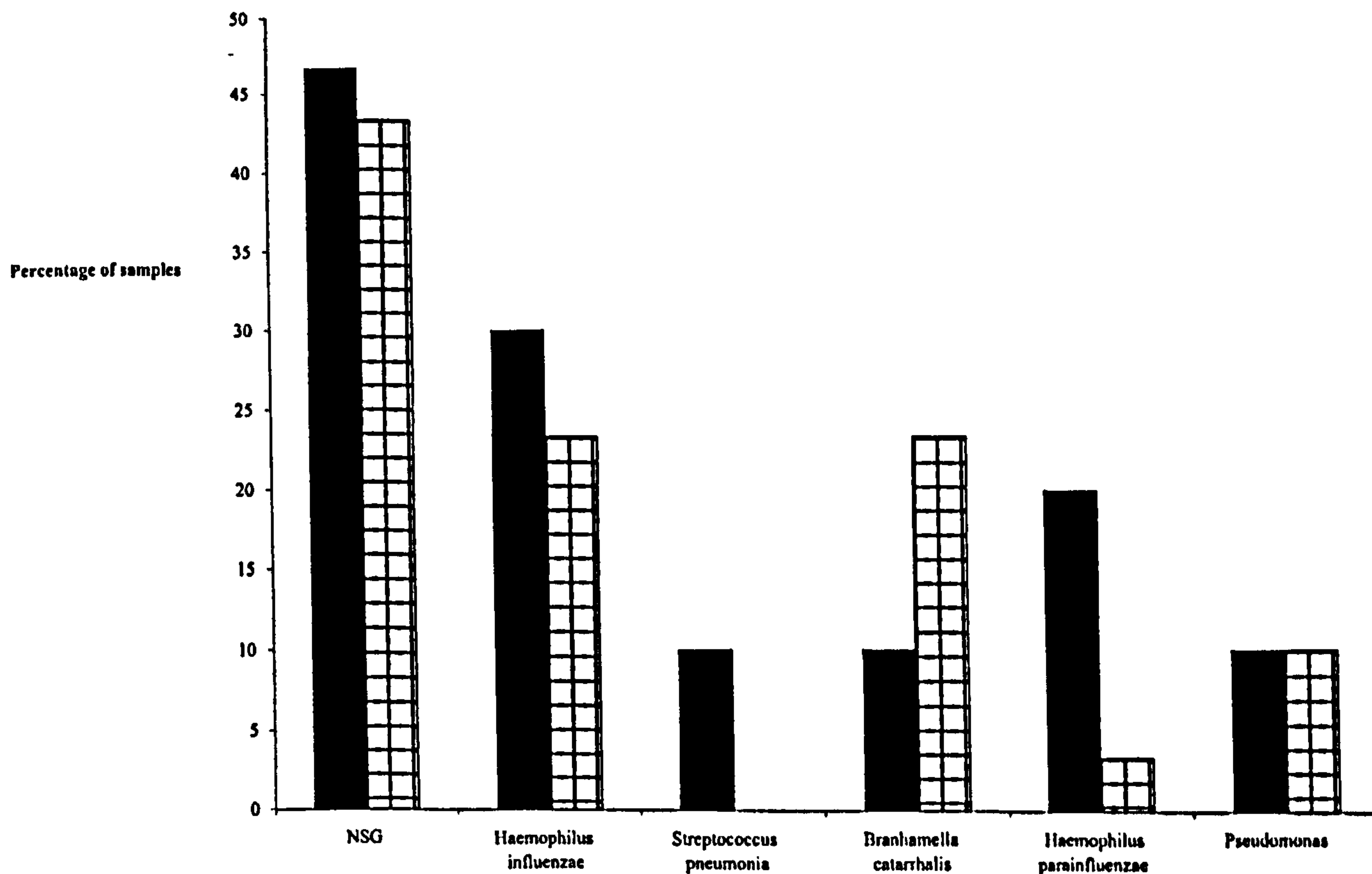


Figure 3. Relative frequency of bacterial isolates from first (black bars; n = 30) and second (hatched bars; n = 30) sputum sample expressed as a percentage of samples. NSG = nonspecific growth.

in the species of bacterial isolate over the sample interval. Fifty percent of the subjects grew entirely different bacterial species at each sample point, whereas the other 50% demonstrated persistence of a specific bacterial species or nonspecific growth across the sampling interval. Patients who demonstrated changes in colonizing bacterial species during the study exhibited higher mean bacterial loads ($10^{8.18}$ log cfu ml⁻¹) than those who maintained the same species in both samples ($10^{7.55}$ log cfu ml⁻¹, p = 0.03).

The decline in FEV₁ seen in the study group was significantly greater in those subjects with unstable bacterial type at a 102-ml (IQR 19–196, n = 18) decline in FEV₁ per year compared with a 3.6-ml (IQR -158–112, n = 12) decline per year in the group with persistence of one bacterial type at both time points (p = 0.017); this relationship with FEV₁ decline expressed as a percentage of baseline is illustrated in Figure 4. Changes in bacterial

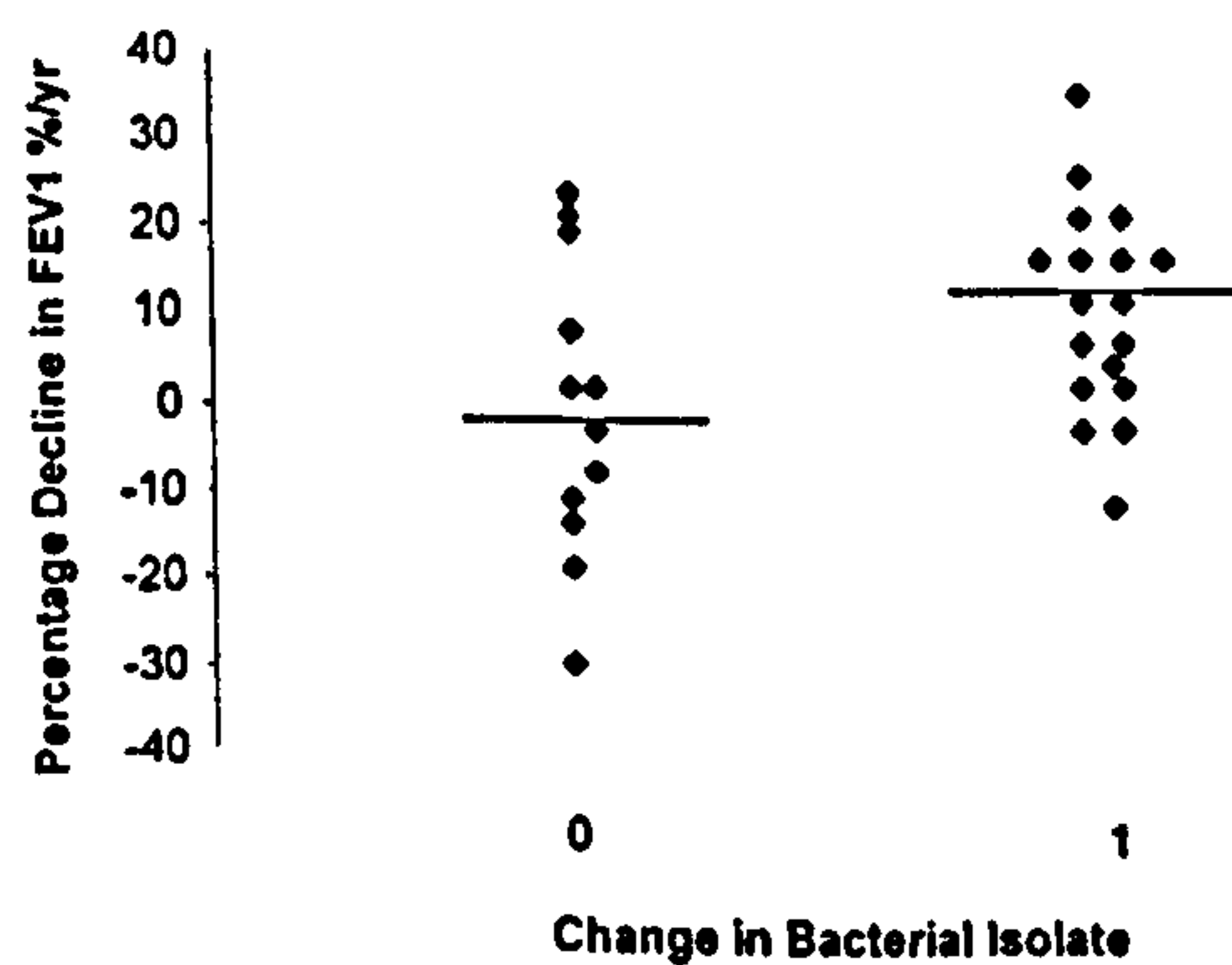


Figure 4. Differences in FEV₁ decline between subjects with persistent (0) and changing (1) bacterial isolate. 0 = Same bacterial species identified in sputum at beginning and end of study; 1 = Change in species of bacterial isolate; p = 0.017. Decline expressed as percentage of baseline FEV₁ (negative values indicate improving lung function).

species were not related to the number of cigarettes smoked per day (rho = 0.056, p = 0.767), the sputum collection method (induced or spontaneous) (rho = 0.012, p = 0.84), the recorded exacerbation frequency (rho = 0.149, p = 0.44), or the antibiotic usage during the study (rho = 0.048, p = 0.808). An analysis of each of the individual bacterial species and the associated lung function changes did not reveal any attributable significant differences in FEV₁ decline between subjects colonized with different bacterial species, although numbers in each subgroup were too small to draw any valid conclusions from this analysis.

Sputum Cytokines

Sputum IL-6 and IL-8 levels were measured on all samples. The median (IQR) IL-6 levels were similar 114 (283) pg ml⁻¹ and 51 (297) pg ml⁻¹ in samples 1 and 2, respectively, and IL-8 levels were 3,183 (1,688) pg ml⁻¹ and 3,012 (1,684) pg ml⁻¹. Levels of sputum IL-6 and IL-8 were related to one another in each patient (rho = 0.378, p = 0.007). The absolute changes in IL-6 between samples 1 and 2 correlated with the changes in IL-8 seen between the two samples (rho = 0.542, p = 0.011). The sputum IL-8 levels were related to pack-years of smoking; those patients with IL-8 higher than the median having smoked for a mean of 100 pack-years and those with lower IL-8 for 42 pack years (p = 0.018). Patients exhibiting a decline in lung function exhibited higher overall sputum median (IQR) IL-8 levels 3,343 (1,592) pg ml⁻¹ compared with those with stable or improving FEV₁ 2,160 (2,050) pg ml⁻¹ (p = 0.032). Similarly, patients with a higher bacterial load (greater than the overall mean, sample 1 and 2 combined) had higher overall IL-8 levels (2,938 pg ml⁻¹) compared with those with bacterial counts lower than the mean (2,329 pg ml⁻¹) (p = 0.05). There were no significant relationships between sputum IL-6 levels and bacterial counts or lung function decline.

DISCUSSION

This is the first longitudinal prospective study to assess directly the relationship between lower airway bacterial load and decline in lung function in patients with moderate to severe COPD. We have demonstrated a significant relationship between the sputum bacterial load and disease progression in COPD, showing that the rate of decline of FEV₁ was proportional to the rise in colonizing bacterial load over the 1-year study. Individuals who exhibited changes in the nature of bacterial colonization suffered from faster declines in lung function than those with persistence of one or more bacterial species. Quantitative estimations showed that subjects with higher or rising bacterial loads similarly demonstrated greater declines in FEV₁ compared with those with lower or decreasing airway bacterial loads. These falls in FEV₁ were also associated with elevated levels of the potent neutrophil chemo attractant IL-8.

The lower airways of healthy nonsmoking individuals are sterile, although a number of studies have identified bacteria in the lower airways of patients with stable COPD (8–10). The prevalence of LABC is increased by active smoking and with progressive airways obstruction (9, 10), and as our patient group had more severe COPD, this explains the high prevalence of LABC found. Bacterial colonization is proinflammatory and can result in a range of pathologic effects that are deleterious to lung function, including mucus hypersecretion and embarrassment of mucociliary clearance (23, 24). Bacteria can affect the airway epithelium directly and via the recruitment of neutrophils (25, 26) with release of excessive amounts of neutrophil derived proteases resulting in damage to airway epithelial cells (14). Airway inflammation has been shown to increase as airway obstruction worsens, but bacterial colonization was not originally considered as an explanation for this finding (4). Bacterial colonization has been shown to be detrimental to lung function in a number of pathological conditions, including cystic fibrosis and bronchiectasis (27, 28). Evidence that LABC contributes to worsening lung function comes from a study that found that *H. influenzae* colonization was associated with increased airway inflammation in patients with chronic bronchitis and airflow obstruction compared with patients with chronic bronchitis but without airflow obstruction, where airway inflammation was reduced (29). We have again confirmed that higher airway bacterial load is associated with greater airway inflammation in terms of sputum IL-8 levels. Furthermore, patients exhibiting a decline in FEV₁ during the study had higher levels of IL-8 than those with a stable or improving FEV₁. This finding suggests a mechanistic link between airway bacterial load, airway inflammation, and the associated deleterious effects on FEV₁.

We have previously reported that some patients with COPD develop frequent exacerbations, and this patient group has increased stable airway inflammatory cytokines (IL-6 and IL-8) compared with those with a history of infrequent exacerbations (30). Patients with frequent exacerbations also demonstrate a faster decline in FEV₁ than infrequent exacerbators, with exacerbations contributing to approximately 25% of the observed lung function decline in COPD (31). Analysis of data from the Lung Health Study (32) revealed that lower respiratory illnesses in smokers are deleterious to FEV₁ and lends further support to the hypothesis that lower airway infection and associated inflammation contribute to lung function decline.

Previous longitudinal studies of the mechanisms of lung function decline have used possible surrogate markers of airway infection such as mucus hypersecretion. The Copenhagen City Heart Study found that chronic mucus hypersecretion was associated with an excess FEV₁ decline of 22.8 ml/year in men and 2.6 ml/year in women together with increased risk of hospital-

ization (33). As bacterial colonization is associated with mucous secretion (34), and patients with mucous secretion have more airway inflammation (7); this again provides support for the role of LABC in the accelerated decline of FEV₁. We have found that the presence of bacterial colonization is directly related to exacerbation frequency, and patients with colonization have longer and thus more severe exacerbations (22). As patients with a past history of frequent exacerbations have increased airway inflammation (30), the nature of stable bacterial colonization may be an important factor in disease progression due to the effect of exacerbations.

In this study, the relationships between FEV₁ decline and features of LABC were strongest in the analyses, which included measures of "instability" of LABC such as changes in bacterial load and type. It is possible that such changes generate a renewed stimulus to inflammation in the airway, in turn causing a more rapid decline in lung function. Indeed, there is increasing evidence that bacterial colonization is a highly dynamic process and that changes in bacterial type are associated with the etiology of exacerbations (35). Bacterial colonization itself is likely to be affected by exacerbations and their treatment, but to what degree this remains uncertain. The interrelationships between host defenses and bacterial infection in the stable state and at exacerbation are highly complex. We postulate that bacterial colonization may accelerate FEV₁ decline by both increasing airway inflammation in the clinically stable state and by affecting the FEV₁ decline due to more frequent and severe exacerbations. This study suggests a significant effect of bacterial colonization on disease progression in COPD.

The multivariate regression analysis of the data from this study did not find a significant influence of active cigarette smoking on FEV₁ decline over 1 year. Indeed, although it is established that cigarette smoking is a risk factor for bacterial colonization and itself leads to increased airway inflammation, the effects of smoking cessation on airway inflammation in severe COPD may not be as clearly defined as in a milder patient group. In this colonized COPD population with severe airways disease, lower airway inflammation may persist despite smoking cessation (36). Thus, the most significant influence on airway inflammation in this study group and consequent FEV₁ decline may be the airway bacterial load.

Many studies of lung function decline have largely been performed using patients diagnosed with bronchitis or airflow obstruction (1, 33). Therefore, understanding of the natural history of lung function decline in more severe COPD is based largely on an extrapolation of observations from patients with milder COPD. Quantitative assessment of airway bacterial load and the related sputum markers of inflammation suggests a threshold level of colonisation in the order of 10⁵–10⁶ cfu ml⁻¹ above which LABC is a persisting drive to airway inflammation (13). However, the degree of airways obstruction at which the clinically significant effects of LABC occur and how bacterial colonization affects the natural history of COPD in the longer term remains uncertain and requires further study.

It is possible that the findings of changes in total bacterial count and FEV₁ may represent changes associated with unrecorded exacerbations. However, diary cards were used to record all changes in symptoms on a daily basis and could therefore be used to detect all exacerbations both reported and unreported as previously described (16). Furthermore, direct questioning of patients at each study visit was used to clarify symptomology and use of rescue medication during the previous 3 months. Therefore, exacerbations both reported to the study team or primary care requiring extra medication and those unreported but recorded on diary cards were included in assessment of baseline status. There was no identifiable relationship between

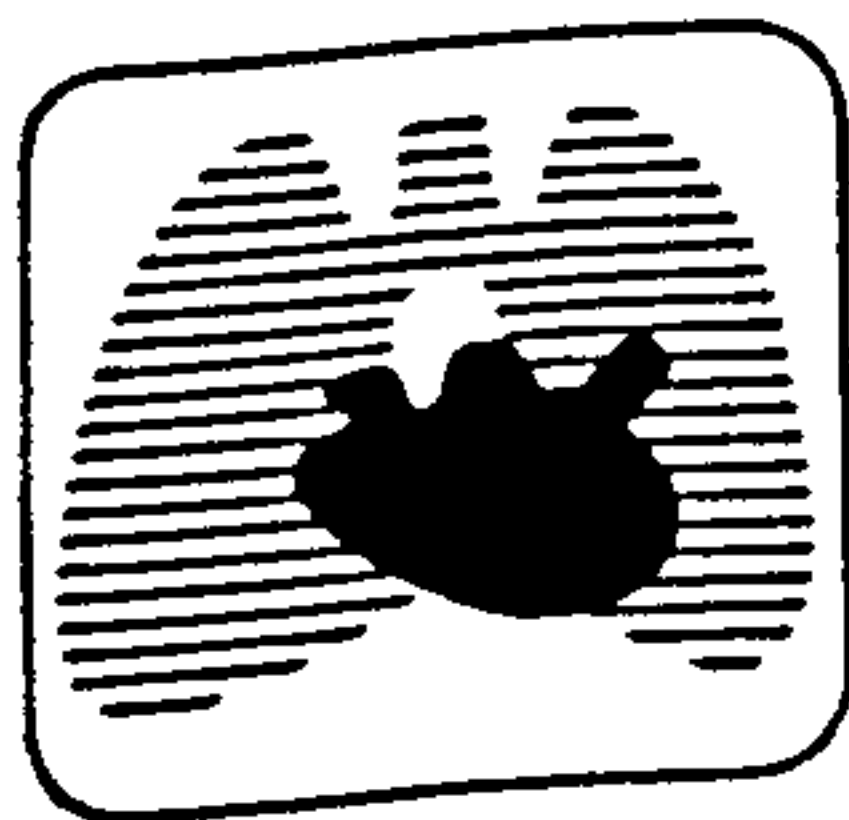
the changes in FEV₁ observed and the frequency or timing of exacerbations in this study. However, this study was not powered to investigate the relationship between exacerbation numbers and lung function decline demonstrated previously by our group (16). Assessment of diary cards for the 6-week symptom-free period effectively assured that patients had returned to baseline before sampling.

This study has shown that LABC is an important determinant of decline of lung function in this group of COPD patients with moderate to severe disease. These findings suggest that appropriate antimicrobial therapy in colonized patients may have an important therapeutic effect, offering an opportunity to alter the natural history of this highly prevalent disease. Studies performed over a longer period are required to investigate further the interactions between LABC, smoking, and exacerbations and their effect on the accelerated decline in lung function, which is characteristic of COPD.

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Effect of Interactions Between Lower Airway Bacterial and Rhinoviral Infection in Exacerbations of COPD*

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Study objectives: The inflammatory responses and associated clinical severity of COPD exacerbations are greatly variable, and the determinants of these factors are poorly understood. We examined the hypothesis that bacteria and viruses may modulate this heterogeneity and that interactions between bacterial and viral infection may affect changes in airway bacterial load and the clinical features and inflammatory responses of exacerbations in patients with COPD.

Design: Prospective cohort study.

Setting: Outpatient Department, London Chest Hospital, London, UK.

Patients: Thirty-nine patients with COPD.

Measurements: We prospectively studied 56 COPD exacerbations, obtaining clinical data and paired sputum and serum samples at baseline and exacerbation. Qualitative and quantitative microbiology, polymerase chain reaction detection for rhinovirus, and estimation of cytokine levels by enzyme-linked immunosorbent assay were performed.

Results: A total of 69.6% of exacerbations were associated with a bacterial pathogen, most commonly *Haemophilus influenzae*. Rhinovirus was identified in 19.6% of exacerbations. The rise in bacterial load at exacerbation correlated with the rise in sputum interleukin (IL)-8 ($r = 0.37$, $p = 0.022$) and fall in FEV₁ ($r = 0.35$, $p = 0.048$). Exacerbations with both rhinovirus and *H influenzae* had higher bacterial loads ($10^{8.56}$ cfu/mL vs $10^{8.05}$ cfu/mL, $p = 0.018$) and serum IL-6 (13.75 pg/mL vs 6.29 pg/mL, $p = 0.028$) than exacerbations without both pathogens. In exacerbations with both cold symptoms (a marker of putative viral infection) and a bacterial pathogen, the FEV₁ fall was greater (20.3% vs 3.6%, $p = 0.026$) and symptom count was higher ($p = 0.019$) than those with a bacterial pathogen alone.

Conclusions: The clinical severity and inflammatory responses in COPD exacerbations are modulated by the nature of the infecting organism: bacterial and viral pathogens interact to cause additional rises in inflammatory markers and greater exacerbation severity.

(CHEST 2006; 129:317-324)

Key words: bacteria; COPD; exacerbations; viruses

Abbreviations: CI = confidence interval; HRV = human rhinovirus; IL = interleukin; IQR = interquartile range; PCR = polymerase chain reaction; PPM = potentially pathogenic microorganism

Exacerbations of COPD are characterized by increased airway^{1,2} and systemic inflammation.³ However, there is marked variability in the nature of the inflammatory response at exacerbation and thus

the symptoms, clinical severity, and time course of these events.⁴ Individual factors such as respiratory viruses⁵⁻⁷ and particular bacterial pathogens⁸ are associated with indexes of more severe exacerbations. However, interactions between individual

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pathogens and the mechanisms that underlie the heterogeneity of exacerbations are poorly understood.

Bacterial pathogens are commonly identified in the lower airway of COPD patients in the stable state.^{1,9,10} Airway inflammation is directly related to the number of bacteria in the lower airway in stable COPD, and greater airway bacterial load is itself a stimulus to faster disease progression.¹¹ While it is known that airway bacterial load rises at exacerbation,⁹ it is not known to what extent these rises modulate changes in airway inflammation and exacerbation severity, or what factors determine changes in bacterial load.

Respiratory viruses have been implicated as important infective triggers of exacerbations,^{5-7,12} with human rhinovirus (HRV) being the most commonly

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identified viral pathogen.⁵ Virus-associated exacerbations are longer and thus more severe than nonviral exacerbations,⁵ but whether this is due to the direct effects of viral infection on the airway or a mechanism involving changes in lower airway bacteria is not known. We examined the hypothesis that the heterogeneity of inflammatory, symptomatic, and physiologic responses at COPD exacerbation is modulated by airway bacterial and viral infection and that a combination of these pathogens would result in greater airway and systemic inflammation and hence clinical and physiologic indexes of exacerbation severity.

MATERIALS AND METHODS

Patient Selection

Patients with COPD were recruited from the outpatient department of the London Chest Hospital into the East London COPD cohort. The inclusion criteria for this study have previously been published^{2-6,11,13-14} and include a postbronchodilator $FEV_1 < 70\%$ of predicted for age and height, β_2 -agonist reversibility $< 15\%$ of baseline and/or < 200 mL, and a FEV_1/FVC ratio $< 70\%$. Patients were assessed clinically and with chest radiography at recruitment to ensure the absence of other significant respiratory disease. Patients were observed for this study from April 2001 to July 2002. Ethics approval for the study was obtained from the East London and City Health Authority Research Ethics committee; all patients gave written informed consent. This patient cohort has been the subject of previous articles^{2-6,11} on various aspects of COPD exacerbation.

Diary Card Monitoring and Follow-up

At recruitment, patients were taught how to record on diary cards each morning postbronchodilator peak expiratory flow (Mini-Wright; Clement Clark International; Harlow, UK). Pa-

tients recorded a change in their symptoms using a letter-annotated system. When well or stable, the patients were instructed not to record any of the symptom letters in the diary. However, when they perceived an increase over their normal, stable condition in symptoms (major and minor, see below), they noted the corresponding symptom letter on their diary card. Therefore, the patients recorded symptom letters if a symptom was perceived as worse, eg, dyspnea, or of new onset, eg, a sore throat (as the latter is not usually present).

Stable State

Patients were reviewed at recruitment and with their diary cards every 3 months in the study clinic to monitor compliance with data collection and to record changes in medication and baseline lung function. A review of diary cards was utilized to ensure that stable sampling was performed when subjects had been clear of exacerbation symptoms and had completed any exacerbation treatment for at least 6 weeks.

Exacerbations

Patients were encouraged to report symptom changes to the study team; they were assessed within 24 to 48 h in the study clinic by a respiratory physician prior to initiation of therapy for the exacerbation. The diagnosis of an exacerbation was based on symptomatic criteria previously validated by our group.²⁻⁶ An exacerbation was defined as the presence for at least 2 consecutive days of increase in any two major symptoms (dyspnea, sputum purulence, sputum amount) or increase in one major and one minor symptom (wheeze, sore throat, cough, symptoms of a common cold). Exacerbation symptoms were binary coded as present or absent, and the sum of these at exacerbation onset was termed the *symptom count*, which has been validated as a marker of clinical severity.⁴ Lung function measurement and sputum and blood sampling were performed on patients prior to the initiation of exacerbation treatment.

Measurement of Lung function

Lung function was measured with a rolling seal spirometer (SensorMedics; Yorba Linda, CA). Lung function measurements were obtained between 9:30 AM and 11:30 AM, 1 h after the patient's usual bronchodilator medication. At least three spirometry readings were obtained at each visit, and the best performance was recorded.

Sputum and Blood Sampling

Sputum was sampled if the subject met criteria for the stable state at the 3-month review and also at presentation of exacerbation. Immediately following lung function measurement, the patients were asked to spontaneously expectorate sputum into a sterile container. Patients unable to produce a sample of sputum spontaneously underwent sputum induction.¹³

Once a sample was obtained, sputum plugs were separated from saliva using sterile forceps, and one third of the sputum was taken and analyzed for quantitative bacterial culture.¹⁵ The remainder was homogenized and centrifuged, and aliquots of supernatant were stored at -70°C for later cytokine analysis.^{2,11,13} Sputum samples containing < 25 squamous epithelial cells per low-power field and > 25 leukocytes per high-power field were accepted for analysis. An aliquot of phosphate-buffered saline solution-processed sputum was frozen at -80°C for subsequent RNA extraction and polymerase chain reaction (PCR). The remainder was analyzed for inflammatory cyto-

kines.^{2,11,13} Sputum interleukin (IL)-6 and IL-8 levels were measured using an enzyme-linked immunosorbent assay (R&D Systems; Abingdon, UK).² Contemporaneous blood samples were obtained, centrifuged at 4°C, and serum decanted and stored at -80°C for subsequent analysis of IL-6 levels using an enzyme-linked immunosorbent assay (R&D Systems).

Quantitative Bacterial Analysis

Samples were processed homogenized. Tenfold serial dilutions of the homogenized sample were made in brain heart infusion broth, and 100- μ L aliquots were plated out onto the surface of a range of different media, including blood agar, chocolate agar, MacConkey agar, and cysteine lactose electrolyte-deficient agar. These were incubated for 18 to 36 h at 37°C in an atmosphere of air + 5% carbon dioxide. After incubation, bacterial colonies were counted and subcultured for identification by standard methods.¹⁵ *Haemophilus influenzae* and *Haemophilus parainfluenzae* were identified and differentiated by their growth patterns on peptone agar on which discs containing nicotinamide adenosine dinucleotide or haemin were placed (Oxoid Unipath; Basingstoke, UK). The number of colony forming units per milliliter of sputum was calculated from the number of colonies obtained and the dilution of the sputum. Bacteriology data are expressed as the total bacterial count in log base 10 U. Potentially pathogenic microorganisms (PPMs) are bacteria known to be common pathogens of the respiratory tract in subjects with COPD (*Streptococcus pneumoniae*, *H influenzae*, *H Moraxella catarrhalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and other Gram-negative enteric bacteria).

RNA Extraction, Reverse Transcription, and Picornavirus PCR

RNA extraction from the sputum was performed using a standard extraction kit (Qiagen; Southampton, UK). Reverse transcription was performed using random hexamers, and picornavirus PCR was performed as previously described.⁶ This PCR technique has been validated in the detection of rhinovirus in these samples using confirmatory nucleic acid sequencing.⁶

Statistical Analysis

Normally distributed data are reported by means and SDs and skewed data are presented as medians and interquartile range (IQR). Correlations were assessed using the Pearson or Spearman correlation coefficient (two tailed), as appropriate. Continuous variables with normal distributions were compared by *t* test, whereas those with nonnormal distributions were compared by the Mann-Whitney *U* or Wilcoxon signed-ranks test. Changes in parameters from the stable state to exacerbation were assessed using a paired analysis of the stable sample data taken preceding the exacerbation studied; *p* values ≤ 0.05 were regarded as significant. In the analysis of changes in parameters between stable state and exacerbation, the prior stable sampling point closest to the subjects corresponding exacerbation was used creating a data set of paired baseline and exacerbation samples for each exacerbation. The analysis of group data was initially adjusted for repeated measures by selecting the first exacerbation sampled per patient (*n* = 39) to assess the changes in measured indexes from baseline to exacerbation. These observed changes were comparable to the larger data set of 56 exacerbations (in 39 patients), which was therefore used to compare individual exacerbation characteristics and etiologies, as in previous studies.⁵ Multivariate analysis was performed using a multiple linear regression analysis. Data analysis was performed using statistical software (SPSS version 10.0; SPSS; Chicago, IL).

RESULTS

Patient Characteristics

Table 1 shows the baseline characteristics of the 39 patients in the East London COPD Cohort sampled during the study. Fifty-six paired stable and exacerbation samples were obtained from 39 patients for this analysis. Of these 39 patients, 15 were receiving long-term oxygen therapy, all patients were receiving long-term inhaled corticosteroids (median, 500 μ g/d; IQR, 400 to 1,500 μ g/d of beclomethasone equivalents), no patients were receiving long-term oral corticosteroids, and all patients received regular inhaled bronchodilators. The remainder of the patients did not have an exacerbation during the sampling period (*n* = 26), did not report an exacerbation to the study team, received antibiotic treatment before sampling, or were unable to provide an adequate sputum sample (*n* = 14). The sampled patients did not differ significantly in terms of baseline characteristics from those who were not sampled (Table 1).

Changes in Lung Function and Inflammatory Markers at Exacerbation

Table 2 shows the stable and exacerbation FEV₁, airway bacterial load, sputum IL-6 and IL-8, and blood IL-6 levels for all the 56 sampled exacerbations and on a per-patient basis (*n* = 39). In both analyses, the mean FEV₁ fell at exacerbation and the mean airway bacterial load rose significantly. Exacerbations were associated with increased airway inflammation in terms of sputum IL-8. The rises in levels of sputum and serum IL-6 did not reach statistical significance.

Airway Bacteriology

Airway bacterial load rose in all samples (*n* = 56) from 10^{7.50(0.74)} log cfu/mL in the stable state to 10^{8.09(0.76)} log cfu/mL at exacerbation, and also rose significantly in data adjusted for repeated measures

Table 1—Patient Baseline Characteristics (*n* = 39)*

Characteristics	Data
Age, yr	68.8 (6.9)
FEV ₁ % predicted	40.6 (15.6)
PaO ₂ , kPa	8.86 (0.89)
PaCO ₂ , kPa	5.67 (0.74)
Smoking history, pack-yr	41.0 (30–52)
Male gender, %	60
Active smokers, %	23
Chronic sputum producers, %	43

*Data are presented as mean (SD) or median (IQR) unless otherwise indicated.

Table 2—FEV₁, Infective and Inflammatory Changes for Baseline (Stable State) and Exacerbation Sample Points and on a Per-Patient Basis (n = 39) and for All 56 Sampled Exacerbations (n = 56)*

Variables	Baseline	Exacerbation	p Value†
Per patient (n = 39)			
FEV ₁ , L	0.95 (0.36)	0.87 (0.30)	0.012
Bacterial load, log cfu/mL	7.47 (0.73)	8.16 (0.76)	0.001
Sputum IL-8, pg/mL	3,647 (2,930–4,466)	4,409 (3,983–4,787)	0.002
Sputum IL-6, pg/mL	146.0 (20.4–246.9)	187.6 (49.9–269.1)	0.322
Serum IL-6, pg/mL	4.73 (3.34–7.07)	6.0 (4.25–13.18)	0.228
All patients (n = 56)			
FEV ₁ , L	0.96 (0.37)	0.89 (0.32)	0.015
Bacterial load, log cfu/mL	7.50 (0.74)	8.09 (0.76)	0.001
Sputum IL-8, pg/mL	3,604 (2,913–4,390)	4,288 (3,991–4,765)	0.005
Sputum IL-6, pg/mL	146.7 (29.4–233.0)	185.0 (50.0–280.0)	0.477
Serum IL-6, pg/mL	4.6 (3.1–7.1)	6.6 (4.0–11.7)	0.136

*Data are presented as mean (SD) or median (IQR).

†Statistical comparison of baseline and exacerbation.

(n = 39) [$10^{7.47 (0.73)}$ to $10^{8.16 (0.76)}$; p = 0.001]. The prevalence of PPMs rose from 48.2% at baseline to 69.6% at exacerbation (n = 56), with the remainder of samples demonstrating nonspecific bacterial growth. The most frequently isolated organism was *H influenzae* in 14.3% of stable and 37.5% of exacerbation samples, with *S pneumoniae* in 8.9% and 14.3%, *M catarrhalis* in 7.1% and 14.3%, *H parainfluenzae* in 10.7% and 0%, *S aureus* in 3.6% and 0%, *P aeruginosa* in 1.8% and 1.8%, and Gram-negative enteric bacteria in 1.8% and 1.8%, respectively; the remainder demonstrated nonspecific bacterial growth.

Relationships Between Bacterial Load, Airway Inflammation, and Lung Function Changes at Exacerbation

Changes in airway bacterial load (n = 39) were related to exacerbation severity in terms of changes in lung function and airway inflammation. The rise in airway bacterial load from baseline to exacerbation was related to the percentage fall in FEV₁ (r = 0.35, p = 0.048). The magnitude of the rise in airway IL-8 at exacerbation was related to the rise in airway bacterial load (ρ = 0.37, p = 0.022).

The changes in airway and serum IL-6 observed from stable state to exacerbation were not related to changes in bacterial load (ρ = -0.76, p = 0.649 and ρ = 0.144, p = 0.482, respectively). However, the rise in systemic inflammation was related to that of airway inflammation; the change in sputum IL-6 correlated with the change in serum IL-6 (ρ = 0.435, p = 0.023, n = 39).

Changes in airway and systemic markers of inflammation at exacerbation were modulated by existing disease severity. The observed change in sputum IL-8 at exacerbation per patient was inversely related

to the baseline FEV₁ (percentage of predicted) [ρ = -0.298, p = 0.05], as was the change in sputum IL-6 (ρ = -0.358, p = 0.02) and the change in serum IL-6 (ρ = -0.392, p = 0.03). Thus, patients with more severe COPD exhibited greater rises in inflammation at exacerbation compared with those with more mild disease.

Effects of Individual Pathogens and Their Interactions

H influenzae: *H influenzae*-related exacerbations were associated with higher airway bacterial load (n = 56; $10^{8.52 (0.39)}$ log cfu/mL) compared to cases in which it was not isolated ($10^{7.85 (0.81)}$ log cfu/mL; p = 0.001). There was a trend toward more severe drops in FEV₁ (expressed as a percentage of baseline): -11.91% (SD, 15.32%) with *H influenzae* present, vs -1.20% without *H influenzae* (SD, 15.09%) [p = 0.057]. Where *de novo H influenzae* infection occurred (ie, *H influenzae* present at exacerbation but not present in stable sample), there was again a greater exacerbation bacterial load ($10^{8.56 (0.40)}$ log cfu/mL) compared to when *H influenzae* was not isolated ($10^{7.81 (0.84)}$ log cfu/mL) [p = 0.001], and the percentage fall in FEV₁ was significantly worse in this group (-14.60% [SD, 12.39%]) compared to the non-*H influenzae* exacerbations (-1.17% [SD, 15.99%]) [p = 0.027].

HRV and Colds: HRV PCR findings were positive in 11 of 56 exacerbations (19.6%); cold symptoms, a measure of putative viral infections, were present in 6 of 18 cases (32.1%). The presence of cold symptoms and HRV-positive PCR sputum were related (continuity adjusted χ^2 , 4.11; p = 0.04). Exacerbations associated with colds were associated with a greater percentage fall in FEV₁ (-14.03%; SD,

13.91%) than those without colds (-3.01%; SD, 16.38%) [p = 0.043].

Effect of Viral and Bacterial Infections on All Sampled Exacerbations: The observed FEV₁ fall associated with colds at exacerbation was more marked in the presence of a lower airway bacterial pathogen: -20.3% (SD, 14.81%) with both colds and a bacterial pathogen, compared to -3.63% (SD, 5.57%) with a cold alone (p = 0.026) or -3.13% (SD, 14.88%) with a bacterial pathogen alone (p = 0.001) [Fig 1]. The specific effect of the interaction between colds and bacterial pathogens was assessed with a multivariate regression analysis with the percentage of FEV₁ fall at exacerbation as the dependent variable; the effect of the interaction was additional to the independent effects of each individual factor (95% confidence interval [CI], -13.13 to -2.09; p = 0.009).

Similarly, exacerbation symptoms were more severe (higher symptom count at exacerbation onset) in those exacerbations associated with a PPM in the presence of cold symptoms (4.0; IQR, 3.0 to 4.5) compared to those with a PPM alone (3.0; IQR, 2.0 to 3.0) [p = 0.019] or with neither a PPM nor cold symptoms (3.0; IQR, 2.0 to 3.0) [p = 0.029; Fig 2].

Exacerbations associated with both *H influenzae* and HRV exhibited a greater bacterial load (10^{8.56} [0.31] log cfu/mL vs 10^{8.05} [0.77] log cfu/mL, p = 0.018) and serum IL-6 (13.75 pg/mL; IQR, 10.53 to 16.91 pg/mL; vs 6.29 pg/mL; IQR, 3.31 to 9.75 pg/mL, p = 0.028) than those without both

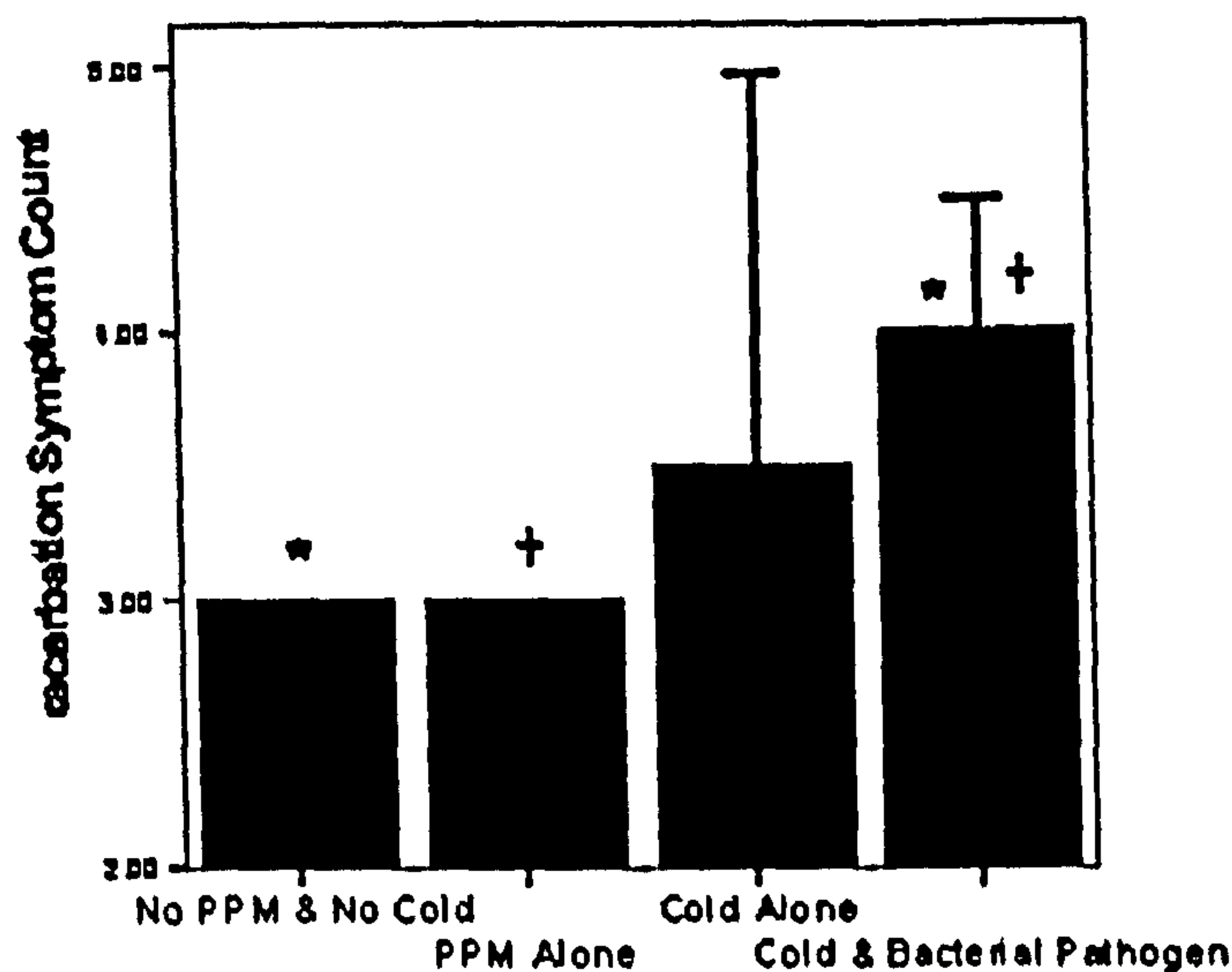


FIGURE 2. Effect of airway pathogens and pathogen combinations on symptom severity (median symptom count at exacerbation onset). Columns represent median values, bars indicate IQR, and * and + denote statistically significant (p < 0.05) differences between corresponding labeled categories (n = 56; *p = 0.029; + p = 0.019).

pathogens. The exacerbation of airway bacterial load associated with *H influenzae* and HRV compared to other PPMs is illustrated in Figure 3.

DISCUSSION

The results of this study show for the first time a synergistic effect of viral and bacterial infections in

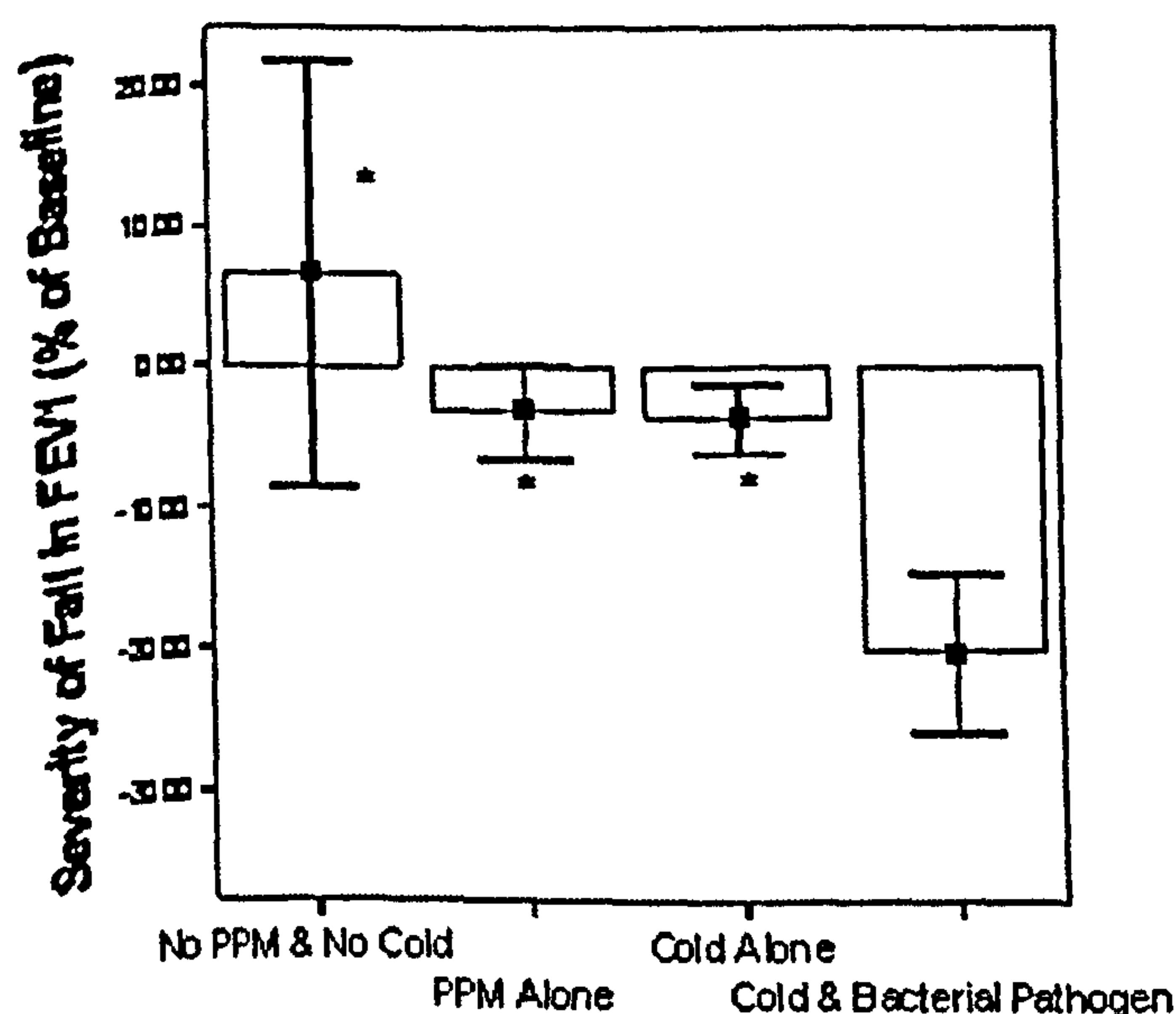


FIGURE 1. Effect of airway pathogens and pathogen combinations on percentage fall in FEV₁ at exacerbation. Columns represent mean values with error bars as SEM *Significant (p < 0.05) difference between this category and cold and bacterial pathogen category (n = 56).

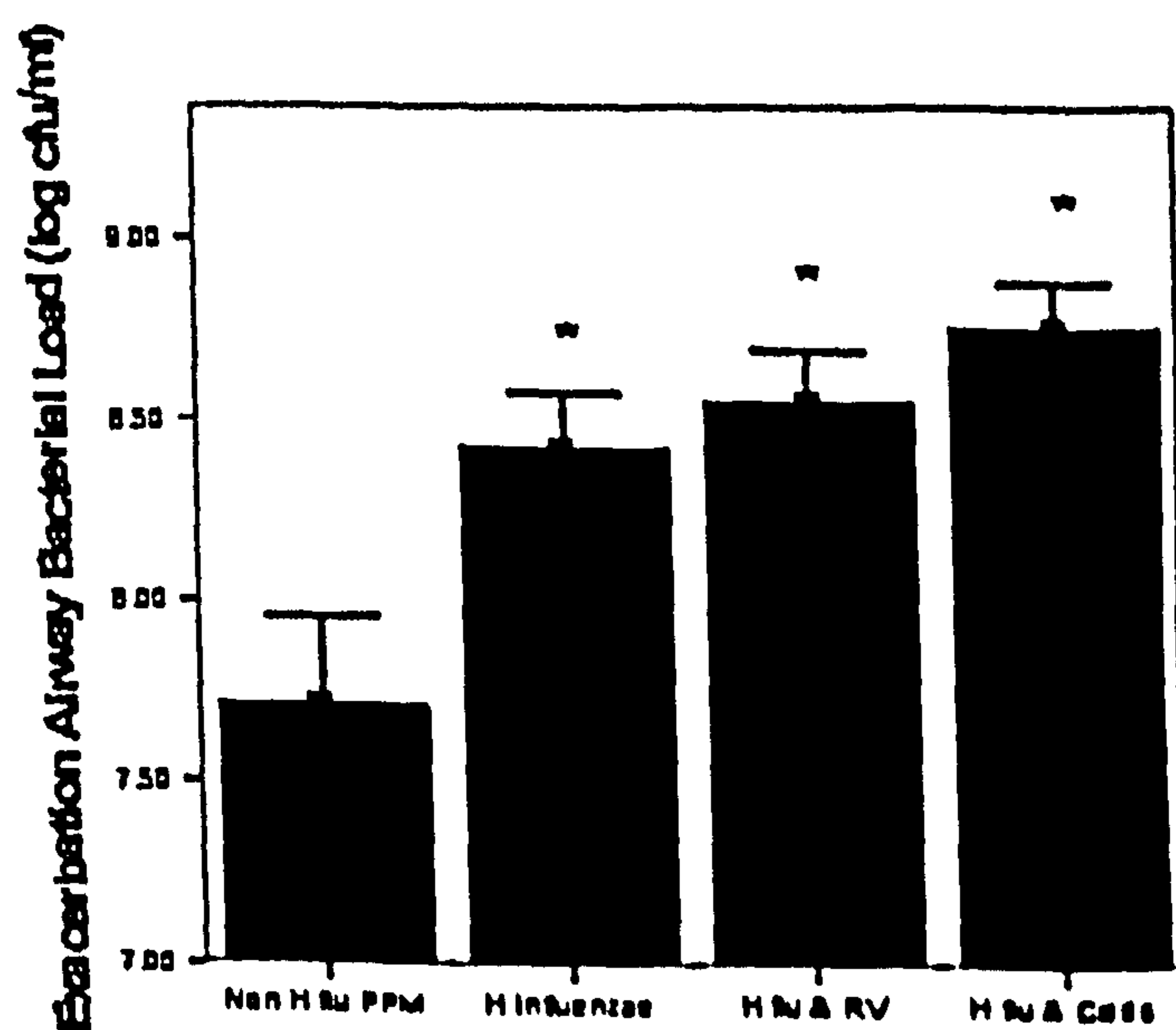


FIGURE 3. Airway bacterial load at exacerbation for different categories of isolated pathogen (n = 56). Columns represent mean values, with error bars as SEM. RV = rhinovirus; H flu = *H influenzae*. *Significantly different from all PPM categories.

modulating the severity of symptoms, lung function changes, and inflammation at exacerbations of COPD. The findings demonstrate that changes in lower airway bacterial load are associated with the variability in inflammation and lung function seen at exacerbation in patients with moderate-to-severe COPD, effects that were more pronounced in proven rhinoviral and putative viral infections. These data also suggest that pathogens associated with more severe exacerbations, such as *H influenzae*, may act at least in part via a greater stimulus to inflammation, associated with higher airway bacterial loads. Patients with more severe disease in this study demonstrated greater rises in airway and systemic inflammation than those with milder disease. This suggests that the heterogeneous nature of exacerbation severity is dependent not only on the nature of infective triggers but also on the baseline severity of disease.

This study has been performed using the well-validated technique of daily diary card symptom recording and analysis to confirm both the diagnosis of exacerbations and also the stable state.^{2-6,11,13,14} The study design has allowed us to sample the same patients in both clinical states and to describe not only cross-sectional analyses at exacerbation but also changes from baseline, and furthermore how these changes in exacerbation parameters were modulated by the corresponding infectious agents.

We have found that the severity of the fall in lung function and the rise in inflammation seen at exacerbation are related to the extent of the rise in airway bacterial load. A relationship between airway inflammation and airway bacterial load has previously been described in the stable state,^{1,16-22} with higher loads associated with greater falls in FEV₁ over a 1-year study.¹¹ A number of previous studies^{8,18,19} have identified that bacterial pathogens are commonly found in the lower airway at exacerbation with higher loads than in the stable state.⁹ However, the effect of rising numbers of bacteria on the nature of exacerbations has not been investigated. These findings suggest that changes in bacterial load may play a role in the heightened levels of airway inflammation characteristic of exacerbations. However, evidence for an association between changes in bacterial load and indexes of exacerbation severity does not prove causality; it is possible that changes in airway bacterial load may simply be a secondary phenomenon to other causes of inflammation. Indeed, the findings of this study show that the key changes in symptoms and lung function at exacerbation were observed when the synergistic effects of viral and bacterial infection were found. *In vitro* and intervention studies are required to differentiate the

exact contribution of a particular pathogen or pathogens to the inflammatory and pathophysiological changes at exacerbation.

H influenzae was found in this study, as in previous studies,^{8,9,10} to be the most important bacterial pathogen identified both in terms of prevalence in the stable state and at exacerbation, and in determining the airway bacterial load. *H influenzae*, unlike a number of other bacterial pathogens, may colonize not only the airway but the respiratory epithelium itself. *H influenzae* colonization has been shown to be a greater stimulus to airway inflammation than other commonly isolated pathogens.^{8,23} This is in agreement with the findings of our study that demonstrate that *H influenzae* was present in greater numbers than the other PPMs identified, and that its presence at exacerbation was associated with more severe drops in FEV₁. The role of less prevalent bacterial pathogens at exacerbation, in particular, their interactions with respiratory viruses requires further study.

The stimulus of newly acquired *H influenzae* at exacerbation provided a greater deleterious effect on FEV₁ than *H influenzae*-associated exacerbations in patients already having colonization with this pathogen. These findings compliment previous work¹⁰ of the role that strain changes of particular bacterial species play in the etiology of exacerbations. This has identified the role that a new antigenic stimulus to the airway immune system plays in the pathogenesis of an exacerbation. It is feasible that acquisition of a new bacterial strain or type may not only provide a direct antigenic stimulus but also overcome the established host/pathogen balance allowing bacterial proliferation, and thus a further inflammatory stimulus due to greater bacterial numbers. To date, studies to determine the possible interactions between viral infection and bacterial strain changes have not been performed; these may provide information on the complex mechanisms that result in triggering exacerbations.

Respiratory viral infection is an important trigger to the airway immune system. In our cohort of influenza-vaccinated patients, we have previously demonstrated that HRV is the most commonly isolated virus at exacerbation.⁵ Rhinovirus can be isolated from lower airway samples⁶ and is associated with greater levels of inflammation than nonviral infections.⁶ Similarly, we have shown that colds, a marker of putative viral infections,⁶ are associated with more severe exacerbations. In this study, systemic inflammation (serum IL-6), exacerbation symptoms, and lung function changes were all more severe when evidence for both bacterial and viral infection was present. It is possible that this effect may have been due to the separate additional inflam-

matory stimuli of two separate pathogens in the airway; however, this explanation is not supported by the multivariate analysis that indicated a synergistic effect on lung function in addition to the individual effects of each pathogen type. Furthermore, these exacerbations were associated with higher bacterial loads than when both pathogens were not present, which may suggest a synergistic interactive effect of viral infection which allows greater proliferation of airway bacteria. Viral infection therefore may impact on exacerbation severity indirectly by increasing bacterial load in addition to the direct effects of viral infection itself, eg, heightened inflammation or airway hyperresponsiveness, independent of other pathogens. While HRV is the most common virus identified at exacerbation⁵ and hence the target of investigation in this study, a number of other respiratory viruses have been identified in the airway during these events, for example coronavirus.^{5,7} The role of these other viral pathogens and atypical bacteria at exacerbation remains uncertain^{24,25} and requires investigation.

The mechanisms by which viral infection may facilitate airway bacterial growth are likely to be complex. However, any disruption of the innate defenses of the respiratory epithelium in a lower airway colonized with bacteria may unsettle a fine balance between host immunity and bacterial numbers. Rhinoviral infection is known to increase mucous production and neutrophilic inflammation.²⁶ Direct evidence that rhinoviral infection increases susceptibility to bacterial adherence to airway epithelial cells, a key process in bacterial infection, is available from *in vitro* studies.²⁷ Indeed, the key cell surface binding site for HRV infection, intracellular adhesion molecule-1, is itself up-regulated by HRV infection²⁸ and by bacterial colonization²⁹; this increase may play a key role in neutrophil elastase-mediated inflammation.³⁰ Hence, by a number of mechanisms, viral infection may alter the immune environment that may allow either proliferation of colonizing airway bacteria or a new pathogen to infect the lower airway.

This study was performed in patients with moderate-to-severe COPD. The role of bacterial infection and therefore potential bacterial-viral interaction is likely to vary with disease severity and therefore prevalence of bacterial colonization.^{10,18} Indeed, we have shown that the degree of airway and systemic inflammatory response at exacerbation was related to baseline disease severity. This suggests that the severity of inflammatory response may progress with disease severity, which is in agreement with the findings of a longitudinal analysis³¹ of exacerbations. Further studies are required to determine if these findings can be extrapolated to COPD patients with

milder disease. Indeed, the observed heterogeneity of exacerbations is likely to be further modulated by the relative frequency of particular pathogens and hence may show seasonality; this may explain differences in associated cytokine responses found in studies² of comparable sample size. Similarly, differences in the technique of sampling, spontaneous or induced sputum, may affect the observed results. However, we have previously demonstrated the two techniques are comparable in assessing lower airway inflammation.¹³ Therapy must also be considered important when considering factors modulating inflammatory responses. The patients sampled for this study were all receiving inhaled steroids both at baseline and when sampled at exacerbation. It is possible that the inflammatory responses observed at exacerbation were modified by effects of this treatment.³² A statistical analysis of this effect was not feasible due to the ubiquity of inhaled steroid use in this patient group. Therefore, the modulating influences on the nature of exacerbations are numerous. It is probable that any individual factor plays a contributing rather than a definitive role in determining the nature and severity of a particular exacerbation, and furthermore that potential interactions between these factors further modulate the characteristics of these events.

The findings of this study suggest that changes in airway bacterial load, the nature of the individual infective pathogens, and interactions between multiple pathogens and the airway modulate exacerbation severity. Further studies are required to improve understanding of the pathogen/host interactions at exacerbation and indeed also in the stable state. Manipulation of this complex relationship with appropriate anti-infective and anti-inflammatory therapies may benefit COPD patients by reducing both exacerbation severity and slowing progression of this highly prevalent disease.

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Early Therapy Improves Outcomes of Exacerbations of Chronic Obstructive Pulmonary Disease

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Treatment of chronic obstructive pulmonary disease (COPD) exacerbations improves outcomes; however, responses to treatment are variable, and patients with COPD often delay presentation or fail to seek therapy. The impact on exacerbation outcomes, hospitalization, and health status of delaying or failing to seek treatment is poorly understood. We studied between 1996 and 2002 a cohort of 128 patients with COPD, mean (SD) FEV₁ of 1.07 (0.43) L. Patients recorded respiratory symptoms daily and reported exacerbations to the outpatient-based study team or to their primary care physician; 1,099 exacerbations were recorded by the patients, of which 658 were reported to a physician. The time between exacerbation onset and treatment was a median (interquartile range) of 3.69 (2.0–5.57) days, and the exacerbation recovery time was 10.7 (7.0–14.0) days. Earlier treatment was associated with a faster recovery (regression coefficient 0.42 days/day delay) (confidence interval, 0.19–0.65; $p < 0.001$). Patients who reported a higher proportion of exacerbations for treatment had better health-related quality of life than those patients with more untreated exacerbations ($\rho = -0.22$, $p = 0.018$). Failure to report exacerbations was associated with an increased risk of emergency hospitalization ($\rho = 0.21$, $p = 0.04$). Patient recognition of exacerbation symptoms and prompt treatment improves exacerbation recovery, reduces risks of hospitalization, and is associated with a better health-related quality of life.

Keywords: chronic obstructive pulmonary disease; exacerbations; therapy

Chronic obstructive pulmonary disease (COPD) is characterized by acceleration in the normal decline in lung function with age and by exacerbations. These exacerbations are associated with both worsening symptoms and lung function (1). The frequency of exacerbations has been shown to be an important determinant of the impaired health-related quality of life seen in COPD (2) and to affect decline in lung function (3, 4). Exacerbations are a frequent cause of physician consultation in primary and secondary care and a major cause of hospital admission (5–7). Consequently, the management of exacerbations places a considerable burden on the health services both in terms of physician consultation time and healthcare costs (8–10). A reduction in exacerbation frequency would have a number of benefits for patients and health services alike; however, currently available preventative

therapies have been found to have only a relatively small effect (11–13).

Current therapies for exacerbations include antibiotics, oral corticosteroids, and increased bronchodilator medications. Although there is evidence that antibiotics (14) and corticosteroids (15–18) hasten the rate of recovery of certain exacerbations, there are few data in the literature on the effects or importance of the timing of initiation of exacerbation treatment on outcome measures such as recovery time, which relates to exacerbation severity (1, 19). We hypothesize that early presentation of the patient to the physician with an exacerbation would allow early intervention and a reduction of exacerbation severity and would potentially reduce disease progression; however, patients with COPD often have poor understanding of their disease and symptoms, with the result that exacerbations are often not reported to healthcare professionals for treatment (2). Thus, if delay in presentation can be shown to affect exacerbation recovery, this provides a potentially important issue that can be addressed in the management of patients with COPD.

In this study, we have followed prospectively 128 well-characterized patients with moderate to severe COPD over 6 years from November 1996 and collected daily symptoms and exacerbation treatment data on 1,099 exacerbations. This daily monitoring enabled us to investigate how exacerbation outcomes and markers of severity such as recovery time were affected by the timing of presentation to the physician for treatment. We also studied how patient and exacerbation characteristics influenced the patterns of healthcare use and outcomes of therapy. Some of the results of these studies have been previously reported in the form of abstracts (20, 21).

METHODS

Patients

This study was conducted between November 1996 and October 2002. One hundred twenty-eight patients with COPD recruited during this period, who had recorded daily data for 1 year or more, were included in the analysis. Ethics approval was obtained from the East London and City Health Authority Research Ethics committee, and all patients provided written informed consent before recruitment. The inclusion criteria for this study have previously been published (1–3, 22–24) and include FEV₁ of less than 70% predicted for age and height, β_2 agonist reversibility of less than 15% of baseline and/or less than 200 ml, and an FEV₁/FVC of less than 70%. This patient cohort has been the subject of previous articles on various aspects of COPD exacerbations (1–3, 22–24).

At recruitment, measurements were made of FEV₁, FVC, and PEF by rolling seal spirometer (Sensor Medic Corp., Yorba Linda, CA), reversibility to 400 μ g of inhaled salbutamol, and arterialized ear lobe blood gases (model 278 Blood Gas Analyzer; Ciba-Corning, Medfield, MA). A history was taken of smoking habits (years of smoking, cigarettes smoked per day, current smoking status). Patients gave a history of smoking (mean [SD] duration of 46.6 [31.4] pack-years, with 42 active smokers). Patients were asked about their stable respiratory symptoms and also about their long-term inhaled and oral corticosteroid use.

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Patient monitoring. Diary card records. At recruitment, patients were taught how to record on diary cards each morning, postbronchodilator PEF (Mini-Wright Clement Clark International Ltd., Harlow, UK). Patients recorded a change in their symptoms using a letter-annotated system. When well or stable, the patients were instructed not to record any of the symptom letters on the diary. When they perceived an increase over their normal, stable condition in symptoms dyspnea, sputum purulence or sputum volume (major symptoms), and colds (nasal discharge/congestion), wheeze, sore throat, cough (minor symptoms), they noted the corresponding symptom letter on the diary. Therefore, patients record symptom letters if a symptom was perceived as worse, for example, dyspnea or of new onset (e.g., a sore throat) (as the latter is not usually present).

The validated diagnostic criteria of the presence of at least two major or one major and one minor symptom for at least 2 days were then used in the analysis of diary card records. This method is explained more fully in the METHODS section of the online supplement.

Patients were reviewed every 3 months in the study clinic when stable to monitor compliance with data collection, record changes in medication, and baseline lung function.

Quality of life measures. Indices of Health-Related Quality of Life were obtained using the St. George's Respiratory Questionnaire (SGRQ) at the beginning of the study either at recruitment or during the first annual review (25).

Exacerbations

Diagnosis. The diagnosis of an exacerbation was based on symptomatic criteria previously validated by our group (1, 2). An exacerbation was defined as the presence for at least 2 consecutive days of increase in any two "major" symptoms (dyspnea, sputum purulence, sputum amount) or increase in one "major" and one "minor" symptom (wheeze, sore throat, cough, symptoms of a common cold). Exacerbation onset was taken as the first day on which these symptom criteria were met (1). Figure 1 illustrates a diagrammatic timeline of an exacerbation with a definition of terms used.

Treatment delay. From the diary card data and treatment records, the time between exacerbation onset and physician consultation at which treatment was initiated was calculated and called the treatment delay.

Exacerbation recovery time. A total daily count of individual symptoms recorded on diary cards was calculated as the sum of the seven symptoms with the presence of a symptom scored 1 and its absence 0.

To determine recovery to baseline levels and therefore exacerbation recovery time, a diary card symptom baseline was determined from the diary card for each exacerbation. The baseline symptom count was taken as the mean total daily symptom count over days 14 to 8 preceding exacerbation onset. Exacerbation total recovery time was calculated as the time from exacerbation onset for a 3-day moving average of the total daily symptom count to return to this baseline. The use of a 3-day moving average minimized the effect of day-to-day symptom variation without biasing the results. Treated recovery time was taken as the time between consultation and recovery.

Severity of exacerbation in terms of symptoms experienced. Symptom severity at exacerbation was calculated as the difference between the average daily symptom count at baseline (Days 14 to 8 preceding the exacerbation onset) and the number of symptoms at exacerbation onset. Individual symptoms themselves were recorded as increased (1) or not (0) (see the online supplement for further explanation).

Treatment. Patients were encouraged to report symptom changes to the study team. They were assessed within 24 hours in the study clinic by a respiratory physician before initiation of therapy for the exacerbation. Patients did not report all symptom changes to the study team but also reported a number of these episodes to their primary care physician for assessment and treatment. The prescription of treatment for all exacerbations was at the discretion of the attending physician and included prednisolone and/or antibiotic therapy. Exacerbation treatment in the study therefore represented the usual practice of the primary care or study physician attending the patient. Records were kept of the date of initiation and type of treatment prescribed to patients for each exacerbation, both at our clinic and also for therapy prescribed by the patient's primary care physician.

Classification of exacerbations. The exacerbations seen by the study clinical team or the patient's general practitioner were classified as "physician reported exacerbations" with those unseen by either but recorded on diary cards termed "unreported exacerbations." Records of hospitalization were kept throughout the study. Additional detail on the diagnosis of exacerbations is available in the online supplement.

Statistical Analysis

Normally distributed data are presented as mean (SD) and skewed data as median (interquartile range) values and associations tested by Spearman's correlation or chi-squared test. A p value of less than 0.05 was considered statistically significant.

Exacerbation total recovery time was calculated as the time from exacerbation onset for a 3-day moving average of the total daily symptom count to return to baseline (1). Treated recovery time was taken as the time between consultation and recovery. Symptom severity at exacerbation was calculated as the difference in total daily symptom count at baseline and at exacerbation onset. Figure 1 illustrates a diagrammatic timeline of an exacerbation with a definition of terms used.

Assessment of the effect of delay and treatment on recovery with or without allowance for severity, type of treatment, or nonrecovery was made with a generalized linear model with adjustments for Poisson distribution in the dependent variable. Additional detail on the method for making these measurements is provided in the online supplement.

RESULTS

Patient Characteristics

The physiologic characteristics of the 128 (88 male) patients at recruitment for the study are summarized in Table 1. The mean (SD) FEV₁ was 1.07 (0.43) L, and the percentage predicted FEV₁ was 40.8 (15.6). One hundred fifteen patients took a mean (SD) daily dosage 1.2 mg (0.68) of inhaled steroids. Nine patients were on a mean of 4.9 (3.0) mg/day of oral prednisolone; eight patients used both oral and inhaled steroids. The 128 patients in this study completed diary cards for a median of 925 (interquartile range, 628–1,520) days. Sixty (46.9%) reported daily (chronic) dyspnea and 65 (50.8%) daily sputum production.

TABLE 1. Characteristics of the 128 (88 male) patients in this study

Age, yr	67.3 (7.6)
FEV ₁ , L	1.07 (0.43)
FEV ₁ % predicted	40.8 (15.6)
FVC, L	2.51 (0.81)
FEV ₁ /FVC %	43.50 (0.13)
PEF, L/min	191 (88)
PaO ₂ , kPa	8.93 (1.00)
PaCO ₂ , kPa	5.89 (0.89)
Number of active smokers	42
Pack years of smoking	46.6 (31.4)
Inhaled steroid dosage, mg/day	1.21 (0.68)

Data are expressed as mean (SD).

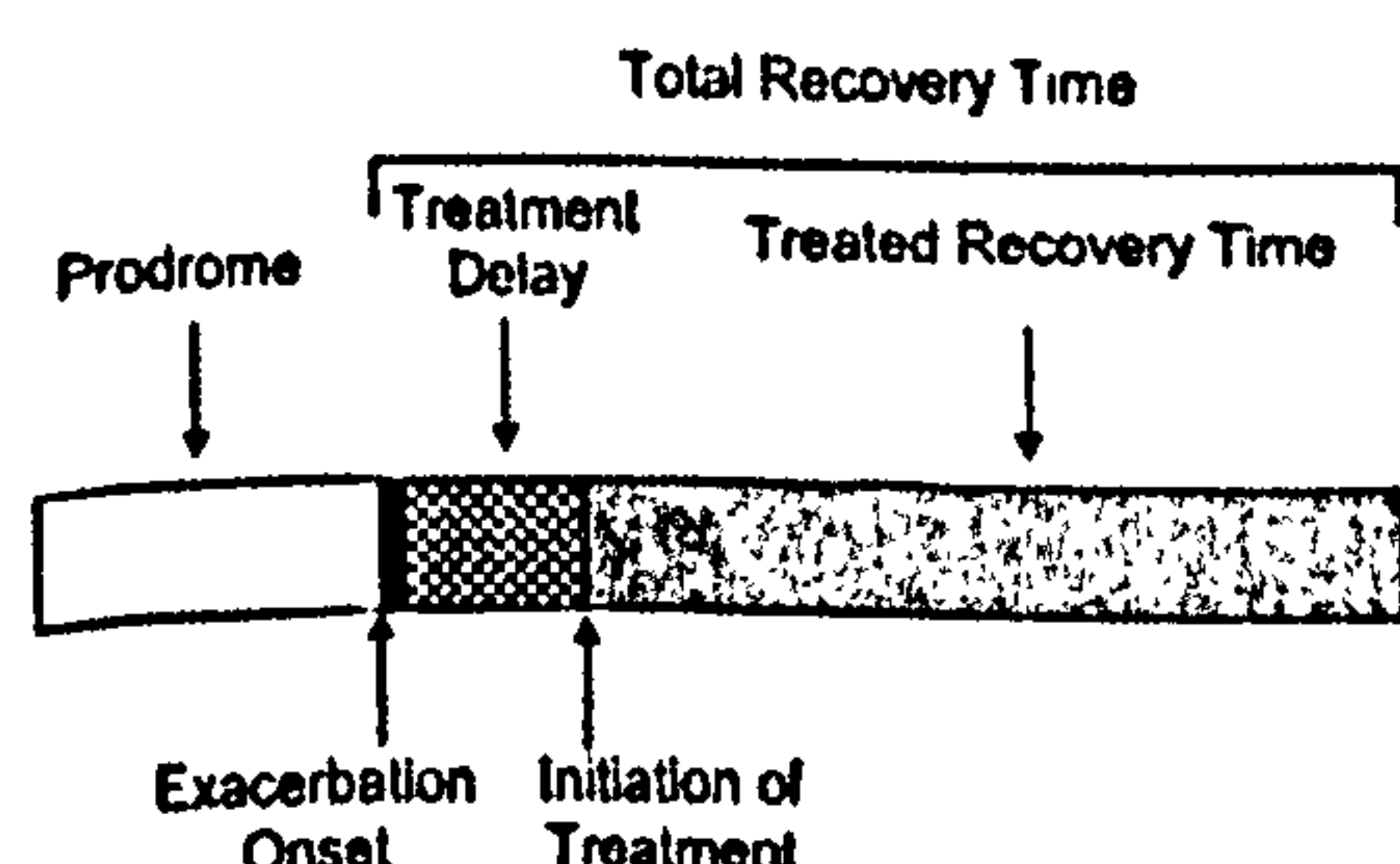


Figure 1. Schematic timeline of exacerbations and terms used in the analysis.

Exacerbations

During the study, the patients experienced a total of 1,099 exacerbations. Of this total, 658 (59.9%) were reported to a physician, either a primary care physician or the study team. The median (interquartile range) number of exacerbations per patient per year was 2.51 (1.41 to 3.75) for all 128 patients. Eight patients had no exacerbations. Of the 1,099 exacerbations, 441 (40.1%) were diagnosed solely from review of diary card symptoms and were not seen by a physician. These unreported exacerbations were only considered in the analysis of hospitalization and Indices of Health-Related Quality of Life with respect to reporting. The 658 remaining exacerbations were all seen by physicians; however, of these, 17 (2.6%) exacerbations received no additional treatment, and 16 (2.4%) involved physician consultation in the prodromal period before formal exacerbation onset, as defined by the diagnostic criteria. These 33 exacerbations were excluded, leaving 625 that were analyzed for the effects of physician reporting and treatment on recovery. A summary of reporting and treatment measures is illustrated in Figure 2.

Increased dyspnea was present in 63.7% of the 1,099 exacerbations, increased sputum purulence in 26.6%, increased sputum volume in 41.3%, cold symptoms in 29.1%, increased wheeze in 31.7%, sore throat in 13.0%, and increased cough in 30.7%.

Physician-Reported Exacerbations and Treatment

Exacerbation therapy. Figure 2 shows the reporting and treatment characteristics of the exacerbations recorded in the study. Of the 625 exacerbations that were treated with prescribed oral therapy, a total of 266 exacerbations were treated with oral corticosteroids. Six hundred of the exacerbations were treated with antibiotics, and of these, 241 were treated with both oral corticosteroids and antibiotics. As 93.6% of exacerbations were treated with antibiotics, this precludes a meaningful statistical analysis of the effect of antibiotic therapy on exacerbation outcomes, as the nontreated group is too small. Oral prednisolone therapy hastened treated recovery by 2.63 days ($p = 0.001$) compared with exacerbations not treated with prednisolone.

Effect of consultation delay and treatment on recovery time. The median (interquartile range) treatment delay (time from the onset of exacerbation to the initiation of treatment) was 3.69 (2.00 to 5.57) days. The median exacerbation total recovery time was 10.7 (7.0 to 14.0) days. The median treated recovery time

(time between the start of treatment to symptom recovery) was 6.9 (3.0 to 10.5) days. Figure 3 demonstrates the relationship between symptom recovery time and the delay between exacerbation onset and treatment. Early initiation of exacerbation therapy was associated with a faster recovery of exacerbation symptoms (regression coefficient, 95% confidence interval, p value) (0.42 days/day delay, 0.19 to 0.65, $p < 0.001$).

The benefits of early physician consultation and treatment on exacerbation recovery time were potentially confounded by the fact that patients with more severe symptoms had longer exacerbations (2.68 days per symptom, 2.06 to 3.31, $p < 0.001$) and also tended to report earlier (-0.28 days per symptom, -0.66 to 0.10 , $p = 0.15$). After an adjustment for symptom severity by its inclusion in the regression model, the relationship between early treatment and faster recovery became more pronounced (0.52 days/day delay, 0.31 to 0.74, $p < 0.001$). Treatment with oral corticosteroids could also confound the relationship if exacerbations reported earlier were preferentially treated with oral corticosteroids; however, the relationship between recovery time and treatment delay remained significant if allowance was made for both symptom severity and the treatment with oral corticosteroids (0.57 days/day delay, 0.34 to 0.79, $p < 0.001$).

Factors affecting physician consultation delay. An analysis of patient characteristics with respect to mean treatment delay for each patient revealed that older patients received treatment earlier ($\rho = -0.19$, $p = 0.04$); however, there were no observed significant relationships between treatment delay and baseline FEV₁ ($\rho = 0.19$, $p = 0.11$), percentage predicted FEV₁ ($\rho = 0.11$, $p = 0.25$), SGRQ total score ($\rho = -0.12$, $p = 0.21$), active smoking status ($\rho = 0.03$, $p = 0.72$), or baseline symptoms such as daily sputum production ($\rho = -0.16$, $p = 0.08$) or dyspnea ($\rho = 0.11$, $p = 0.24$).

Table 2 gives the effect of a specific symptom at exacerbation onset on the treatment delay. Exacerbations (adjusted for repeated measures) involving worsening dyspnea as a presenting symptom presented earlier (regression coefficient [days], 95% confidence interval, p value) (-0.42 , -0.76 to -0.08 , $p = 0.016$) as did those with sputum purulence (-1.30 , -1.60 to -0.92 , $p = 0.001$), wheeze (-0.59 , -0.93 to -0.25 , $p = 0.001$), or sore throat (-0.78 , -1.23 to -0.33 , $p = 0.001$).

Family physician versus hospital outpatient prescribing. Patients in the study could consult either the study team or their own primary care physician at exacerbation; 37.6% of the

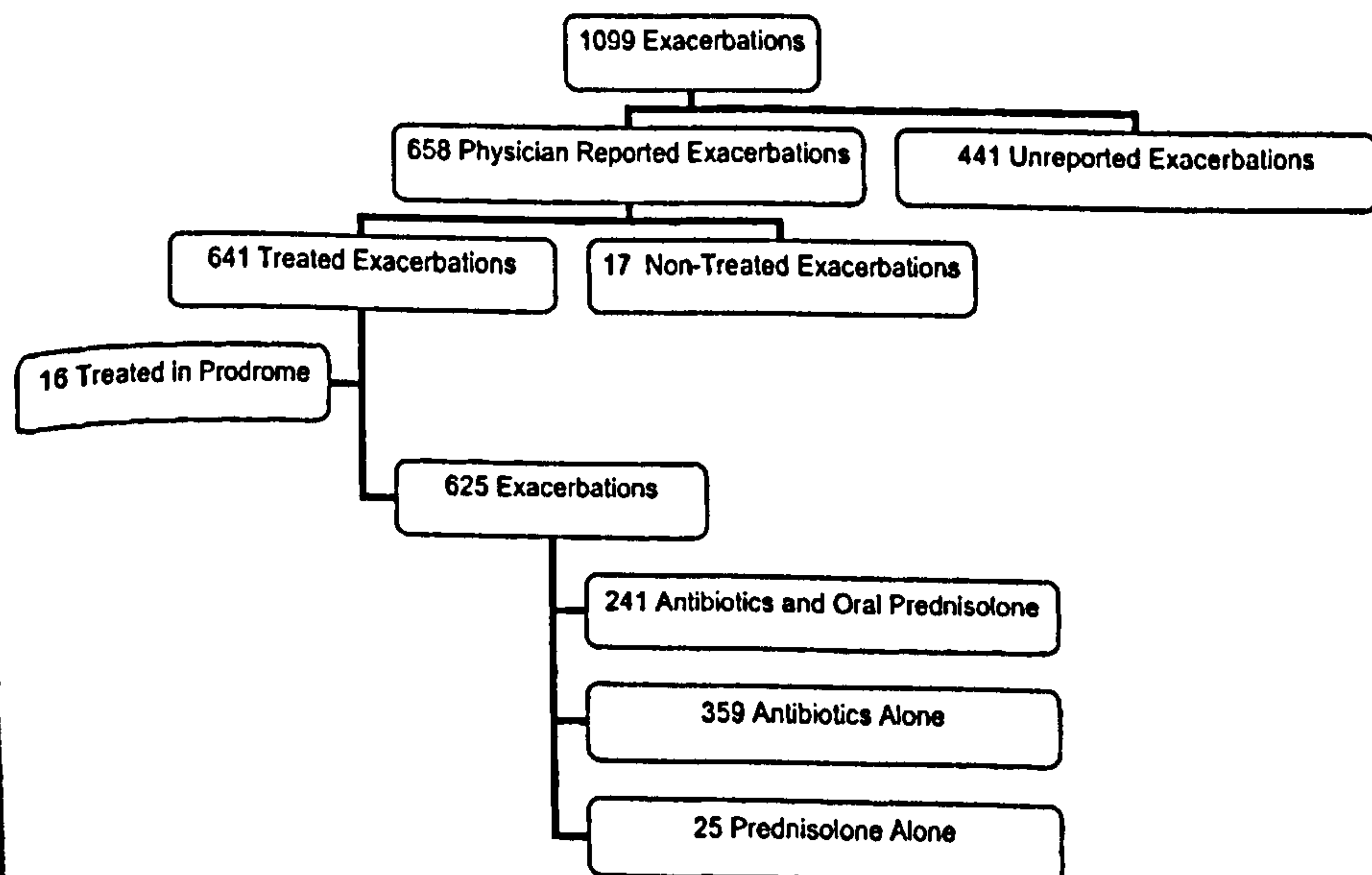


Figure 2. Reporting and treatment characteristics of 1,099 exacerbations recorded in this study.

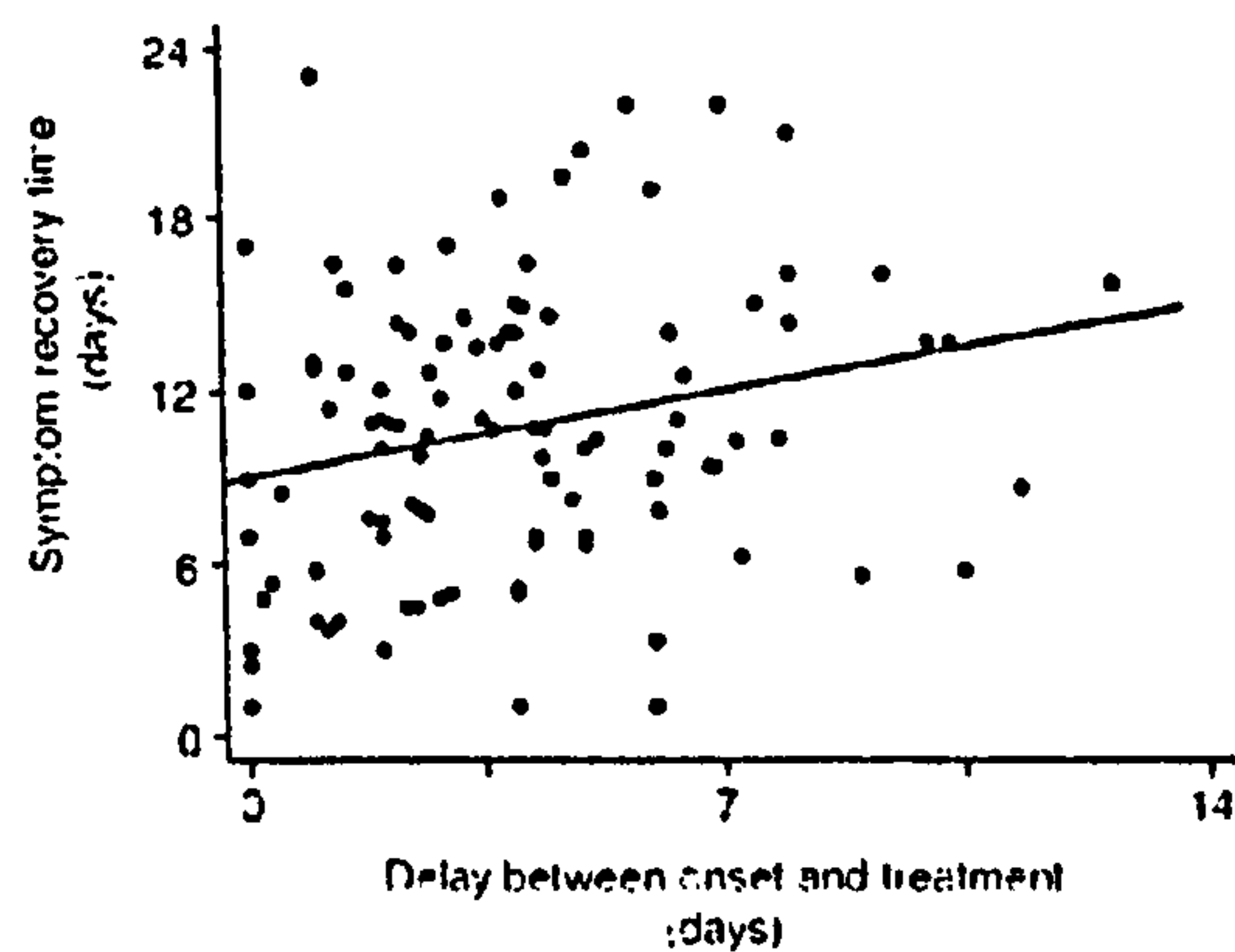


Figure 3. Effect of early treatment on recovery of exacerbation symptoms. Patient mean total recovery time (days) plotted against the patient mean treatment delay (i.e., time from onset of exacerbation symptoms to initiation of therapy) (days) in 108 patients (regression coefficient 0.42 day/day delay; confidence interval, 0.19 to 0.65; $p < 0.001$).

physician-treated exacerbations were seen by a general practitioner, and 62.4% were seen by a member of the study team. Prescribing practices at exacerbation were very similar irrespective of where the consultation took place, with the primary care physician prescribing oral corticosteroids in 43.8% and antibiotics in 96.2% of treated exacerbations and the study team in 41.8% (chi-squared, $p = 0.618$) and 95.9% ($p = 0.868$), respectively.

The time to physician consultation was earlier in the primary care physician-treated group by 0.36 days (-0.69 to -0.04 , $p = 0.030$); however, when an adjustment for symptom severity was made, no significant differences in timing of consultation were found. Despite similar prescribing practices, patients reviewed by a respiratory specialist in the study team had a shorter recovery time than those seen in general practice (0.82 days, $p = 0.047$).

SGRQ Scores, Hospitalization, and Unreported Exacerbations

Untreated exacerbations and SGRQ scores. Not all of the exacerbations experienced by a patient were reported to a physician for treatment. The median (interquartile range) percentage of physician-reported exacerbations for the 120 patients with an exacerbation was 66.7% (50 to 83.3%). Stable SGRQ scores were recorded on 118 of these 120 patients. Figure 4 illustrates the relationship between SGRQ total score and its components, and the percentage of physician-treated exacerbations. Patients who had a higher percentage of their exacerbations seen by a physician had a better health-related quality of life (lower total

SGRQ scores) ($\rho = -0.22$, $p = 0.018$). This relationship was also present when considering the impact ($\rho = -0.19$, $p = 0.04$) and activity ($\rho = -0.21$, $p = 0.02$) components of the SGRQ score separately.

Unreported exacerbations and risk of hospitalization. During the study, 6.4% of the 1,099 exacerbations required hospitalization. The median annual unreported exacerbation rate was 0.77 per year (0.33 to 1.60) for the 120 patients who had at least one. The annual rate for physician-reported but not hospitalized exacerbations was 1.53 per year (0.86 to 2.37), and for hospitalized exacerbations the median rate was 0 per year (0 to 0.40).

Of the 120 patients, those who tended not to seek treatment from their general practitioner or the study clinicians at exacerbation (as measured by high annual rates of unreported exacerbations) were more likely to be admitted to the hospital for treatment of an exacerbation than those who routinely reported their exacerbations for treatment ($\rho = 0.21$, $p = 0.04$).

DISCUSSION

This is the first prospective study to demonstrate the important effects of early treatment on COPD exacerbation outcomes. The findings show that patients who receive prompt therapy after the onset of their exacerbation are likely to recover more rapidly than those who delay reporting and thus initiation of treatment. Furthermore, patients who habitually fail to seek therapy for their exacerbations have poorer health-related quality of life and are more likely to be hospitalized for the management of an exacerbation.

This study has been performed using well characterized patients collecting daily data over a number of years and has used a symptom-based definition of exacerbations that is well validated (1-3, 22-24). The collection of such prospective data allowed us to establish precisely the start of the exacerbation from the diary card entries and then the point at which therapy is commenced, in addition to in-depth analysis of the time course and recovery time of the exacerbations. Our group and others have previously demonstrated that the recovery time of an exacerbation is an important measure of its severity and can be affected by various etiologic factors such as respiratory viruses (22, 26, 27) and therapies (14-18). The delay between symptom onset and therapy and its effect on exacerbation outcomes has not been studied previously, as most studies evaluating exacerbations have focused on healthcare use and therefore have missed the period between symptom onset and the initiation of therapy. The finding that earlier treatment improves exacerbation recovery has confirmed for the first time the clinical suspicion that treating these episodes promptly offers additional benefit to the patient. It is also the first to establish the size of this effect and the role that improving exacerbation reporting behavior may play in reducing morbidity.

A possible alternative interpretation of this finding would be that patients with milder exacerbations seek treatment earlier and thus recover more quickly; however, the data on the nature of symptoms at presentation demonstrate that this was not the case. Patients with more symptoms at exacerbation onset tended to present earlier for treatment, and those exacerbations with more symptoms were indeed more severe, as they took longer to recover. Therefore, the milder and less symptomatic exacerbations were in fact presenting slightly later, and when this effect is taken into account, the benefit of early treatment became more pronounced.

In this study, we have studied exacerbations across a spectrum of exacerbation severities and not only those more severe exacerbations requiring hospitalization. The patients in this study were treated either by the dedicated study team or by their primary care

TABLE 2. REGRESSION ANALYSIS OF INTERACTIONS BETWEEN EXACERBATION SYMPTOMS AND TREATMENT DELAY FOR 625 EXACERBATIONS

Symptom	Regression Coefficient (days) (95% CI)	p Value
Dyspnea	-0.42 (-0.76 to -0.08)	0.016
Sputum purulence	-1.26 (-1.60 to -0.92)	0.001
Wheeze	-0.59 (-0.93 to -0.25)	0.001
Sore throat	-0.78 (-1.23 to -0.33)	0.001
Cold	0.31 (-0.05 to 0.67)	0.093
Sputum volume	0.02 (-0.31 to 0.34)	0.924
Cough	0.01 (-0.34 to 0.37)	0.945

Definition of abbreviation: CI = confidence interval.

A negative value indicates a treatment delay shorter than those exacerbations without the symptom.

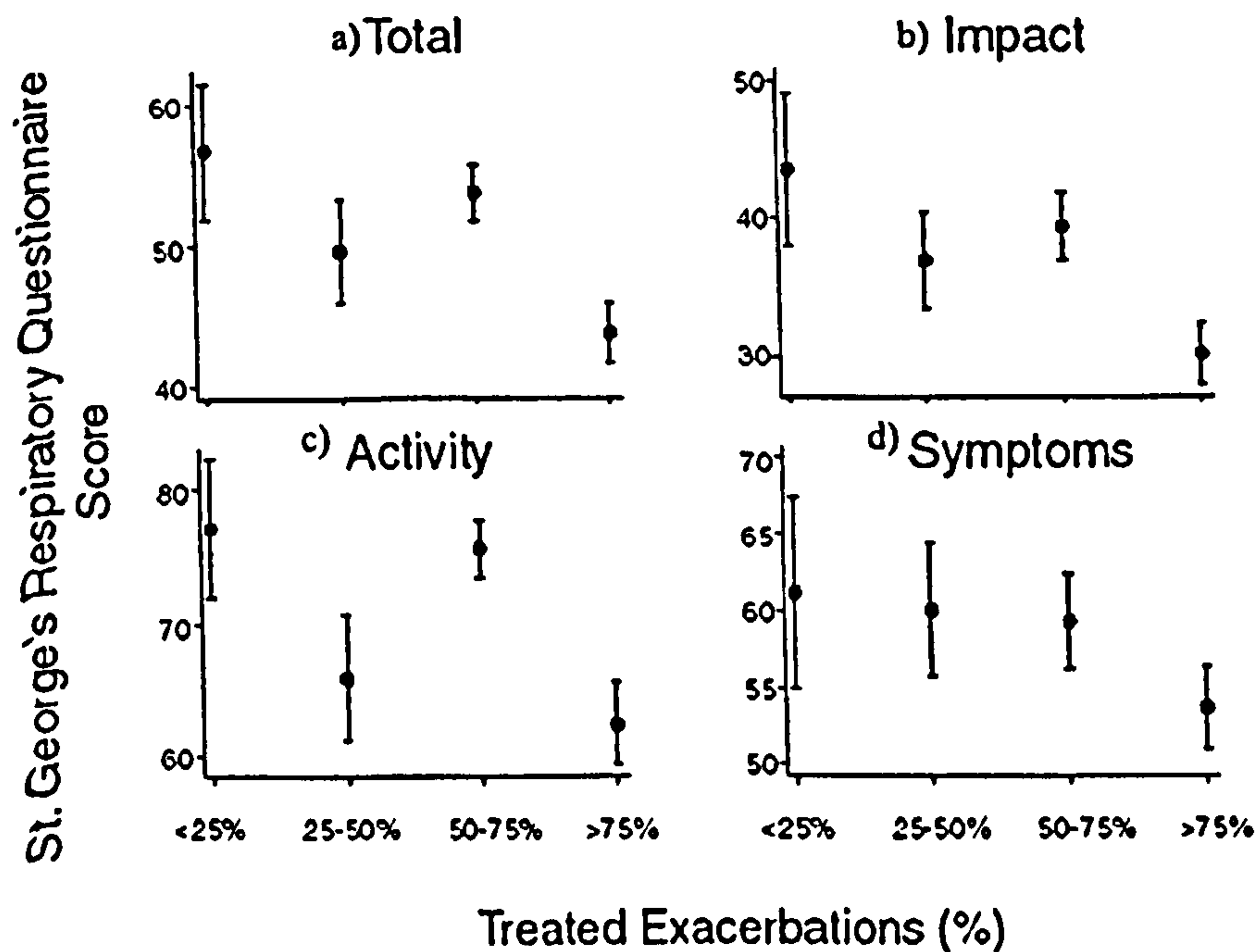


Figure 4. Relationship between total and component St. George's Respiratory Questionnaire (SGRQ) scores (higher scores represent poorer health-related quality of life) and percentage of recorded exacerbations (in quartile groups) that were reported to and treated by a physician. The rho and p values from Spearman's Rank correlations of total and component SGRQ scores with proportion of exacerbations treated on a per patient basis are (a) total SGRQ score, rho = -0.22, p = 0.018; (b) impact score, rho = -0.19, p = 0.04; (c) activity score, rho = -0.21, p = 0.02; (d) symptoms score, rho = -0.13, p = 0.16. The bars represent SEs.

physician, and the exacerbation treatment reflected prescribing practices of both physician groups, which were very similar, as reflected by similar exacerbation recovery times for both treatment groups. In the linear regression model of the effects of treatment delay on recovery time, allowance was made for the exacerbation treatment prescribed. A potential bias of variation in oral antibiotic therapy was not apparent, as over 93% of treated exacerbations received this form of therapy. The effect of oral prednisolone prescription was to shorten exacerbations; if the prescription of this treatment was included in the regression model, the key finding that early treatment hastens recovery time became more pronounced.

These reported exacerbations that received treatment are in keeping with another definition of exacerbations recently suggested (28). Therefore, the findings of this study may be generalized to the COPD population treated both in primary and secondary care, and the validated methods used are suitable for future investigation into the relationships between reporting behavior and exacerbation outcomes.

Exacerbations are heterogeneous in etiology and in the nature of presenting symptoms (22, 29). The symptom characteristics of an exacerbation affected presentation of the exacerbation to the physician. Symptoms of increased dyspnea, sputum purulence, wheeze, and sore throat were associated with earlier presentation, whereas the presence of a common cold at exacerbations was associated with a trend toward later presentation. The factors affecting how patients with COPD interpret changes in their symptoms are likely to be complex, including their understanding of COPD, which is often poor (30), the relationship with disease severity, and psychologic overlay in a group of patients with high levels of anxiety and depression (31). Further studies into the mechanisms of symptom recognition are required to determine how to improve the reporting behavior of patients with COPD and therefore exacerbation outcomes. These may include investigation of the role of patient education, self-management plans, and methods of assessing and improving compliance.

An additional advantage of collecting daily prospective data on symptom changes is that episodes when patients experience an exacerbation and record their symptoms but do not consult their general practitioner or a study physician can be analyzed.

In this study of 1,099 exacerbations recorded on diary cards, only 658 were reported to a physician for treatment, and these rates are in keeping with published clinical trials (13). The finding that on an annual basis patients who are less likely to report their exacerbations are more likely to undergo emergency hospital admission for treatment is an important one. These patients may be less aware of the importance of the changes in their symptoms at exacerbation, or indeed, they may subjectively experience less severe symptoms for a given severity of exacerbation. Thus, they are less likely to seek treatment from a physician in the early stages of their exacerbation, which may prevent the need for hospitalization. Furthermore, we found that patients who had a lower proportion of their exacerbations treated had worse health-related quality of life, as measured by the SGRQ total score, impact score, and activity score, than those patients who sought treatment for a higher proportion of exacerbations. We can postulate that failure to report and therefore receive treatment for exacerbations contributes to additional morbidity from these events and thus adversely affects health-related quality of life. However, it may also be the case that patients with poor health-related quality of life are less likely to seek physician intervention for their exacerbation, and thus, a cycle of decline in quality of life and appropriate healthcare use may become established. Identification and education of the patient group who delay or fail to seek treatment for exacerbations in particular may increase and improve the rate and timing of physician consultation reduce patient morbidity and the considerable burden of inpatient treatment of exacerbations on healthcare services.

Analysis of factors that affected time to presentation of an exacerbation showed that older patients were likely to receive therapy earlier, but there was no relationship between disease severity, baseline symptoms, or the patient's health status. Adequate access to healthcare is important, and the affect of age on presentation in our study suggests that the older patients are receiving medical attention for exacerbations earlier than the younger patients in the group. This finding may be related to the higher degree of disability and comorbidity in the older population (32), causing them to recognize and report symptoms at an earlier stage.

This study has been performed in a population of patients with COPD who were encouraged to report exacerbations, who

were likely to have heightened awareness of changes in their symptoms because of the use of daily diary cards, and who had improved access to healthcare. Therefore, the findings of this study that early treatment of COPD exacerbations hasten recovery time and that patients who do not report to a physician are more likely to be hospitalized may be an underestimation of the scale of these effects in the general population of patients with COPD. Indeed, patients with COPD often have a poor understanding of their disease (30) and often delay or fail to report symptom changes to their physician. To date, there have been no significant initiatives to encourage reporting and early presentation of exacerbations in this patient group.

This study has demonstrated the important finding that the early recognition of exacerbation symptoms and prompt treatment by a physician is beneficial to the recovery of the exacerbation. This result suggests that improving patient and physician understanding of the nature of exacerbations and the benefits of early treatment will improve the outcomes of therapy of exacerbations of this extremely prevalent disease. There is a vital role for new, more efficacious treatments for COPD exacerbations. However, by optimizing the use of existing exacerbation therapies and by improving patients' awareness of exacerbations and access to healthcare, we can improve the current excessive burden of exacerbation-related morbidity and mortality.

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Respiratory Syncytial Virus, Airway Inflammation, and FEV₁ Decline in Patients with Chronic Obstructive Pulmonary Disease

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Background: Respiratory syncytial virus (RSV) is increasingly recognized as an important pathogen in adults with cardiopulmonary disease. It has been associated with acute exacerbations of chronic obstructive pulmonary disease (COPD); however, it has also been detected in the lower airway in the stable state, but the consequences of RSV in stable disease have not previously been determined. We therefore studied the consequences of RSV persistence in adults with COPD and its effect on airway inflammation and lung function decline.

Methods: A total of 241 sputum samples from 74 patients with COPD (FEV₁% predicted, 39.2%; interquartile range, 29.6–57.8%) were collected quarterly in the stable state over 2 yr. RSV was detected by polymerase chain reaction (PCR), quantitative microbiology was performed, and inflammatory cytokines were quantified by ELISA. **Results:** RSV RNA was detected in 32.8% of sputum samples. Patients in whom RSV was more frequently detected (> 50% of samples RSV PCR-positive, n = 18) had higher airway inflammation and faster FEV₁ decline over the study (101.4 ml/yr [95% confidence interval, 57.1–145.8]) compared with those with less frequent detection of RSV (n = 56; 51.2 ml/yr [31.7–70.8]; p = 0.01). The observed relationship between RSV detection and accelerated lung function decline was independent of smoking status, exacerbation frequency, and lower airway bacterial load.

Conclusions: Persistent RSV detection in patients with COPD is associated with airway inflammation and accelerated decline in FEV₁. Chronic RSV infection may be a novel therapeutic target to alter the natural history of COPD.

Keywords: chronic obstructive pulmonary disease; respiratory syncytial virus; FEV₁ decline

Chronic obstructive pulmonary disease (COPD) is a major and growing cause of ill health and death worldwide. The Global Burden of Disease Study predicts that it will rise to being the third commonest cause of death by 2020 (1). Although cigarette smoking is the major cause, evidence exists that chronic lung infection may sustain inflammation in the small airways and lung parenchyma, leading to accelerated and progressive loss of lung function characteristic of COPD (2–4). There is some evidence that

chronic airway infection with adenovirus may play a role in the development of airway obstruction, but studies have been cross-sectional in nature and relationships between viral infection and disease progression have not been demonstrated (5). Furthermore, a role for RNA viruses in the pathogenesis of COPD has been little explored.

Respiratory syncytial virus (RSV) is a negative-strand RNA virus of the Paramyxoviridae family, genus *Pneumovirus*, and is the major cause of acute lower respiratory tract infections in young children, where it occurs in winter epidemics but is rarely identified in the summer (6, 7). Human RSV has no animal reservoir and the source of these winter epidemics remains unknown. Recent studies have also identified RSV as an important pathogen in the elderly (8–10) and in adults with cardiopulmonary disease (8, 11).

We have previously detected RSV in nasopharyngeal samples from patients with COPD in a cross-sectional study (12). Although the other commonly detected respiratory viral pathogens, such as human rhinovirus, were much more prevalent at exacerbation than in the stable state, RSV was detected at similar rates regardless of whether the patient was stable or having an exacerbation (12). Using quantitative polymerase chain reaction (PCR), these findings have been confirmed in patients with stable COPD and at exacerbation, with low viral loads identified in comparison to those seen in children with seasonal bronchiolitis (13). However, longitudinal studies to determine whether RSV is able to persist in the lower airways of patients with stable COPD are lacking and the clinical consequences of RSV persistence have not been determined.

We therefore prospectively studied a cohort of patients with well-characterized COPD with repetitive sampling in the stable state to determine the nature of RSV persistence in the lower airway. We investigated the effects of RSV persistence on lung function decline and airway inflammation and investigated interactions with lower airway bacterial colonization. Some results of this study have previously been reported in the form of abstracts (14, 15).

METHODS

Patient Selection

Patients with COPD were recruited from outpatient clinics into our COPD cohort. Ethics approval for the study was obtained from the East London and City Health Authority Research Ethics committee; all patients gave written, informed consent. The inclusion criteria for this study have previously been published (2, 12, 16–20) and include FEV₁ of less than 70% predicted for age and height, β_2 -agonist reversibility of less than 15% of baseline and/or less than 200 ml, and an FEV₁/FVC of less than 70%. Work from this cohort has been published in a number of previous studies (2, 4, 12, 16–20). Additional information is included in the online supplement.

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Diary Card Monitoring and Follow-up

At recruitment, patients were taught how to record post-bronchodilator PEF on diary cards each morning (Mini-Wright, Clement Clark International Ltd, Harlow, UK).

Patients were reviewed at recruitment and with their diary cards every 3 mo in the study clinic to monitor compliance with data collection, record changes in medication, and baseline lung function. Review of diary cards was used to ensure that stable sampling was performed, when subjects had been clear of exacerbation symptoms and had completed any exacerbation treatment for at least 6 wk.

Measurement of Lung Function

Lung function was measured with a rolling seal spirometer (Sensor Medic Corp., Yorba Linda, CA). Lung function measurements were taken between 9:30 and 11:30 A.M., 1 h after the patient's usual bronchodilator medication. At least three spirometry readings were taken at each visit and the best performance recorded.

Sputum Sampling

Sputum was sampled if the subject met criteria for the stable state at three monthly reviews. Additional detail on the sampling and processing methods is provided in the online supplement. An aliquot of phosphate-buffered saline-processed sputum was frozen at -80°C for subsequent RNA extraction. The remainder was analyzed for inflammatory cytokines using ELISA (2, 16, 19), sputum interleukin 6 (IL-6) and IL-8 (R&D Systems, Abingdon, UK) (2), and myeloperoxidase (MPO; EMD Biosciences, San Diego, CA).

Quantitative Bacterial Analysis

Samples were processed by using sputolysin; serial dilutions were made and cultured on appropriate media. These were incubated for 18 h at 37°C in an atmosphere of air plus 5% CO_2 . After incubation, bacterial colonies were enumerated and subcultured for identification by standard methods (21). The number of colony forming units per gram of sputum was calculated from the total number of colonies obtained and the dilution to give the total bacterial count for each sample expressed in colony forming units per milliliter (cfu ml^{-1}).

RNA Extraction, Reverse Transcription, RSV PCR, and Product Sequencing

RNA extraction from the sputum was performed using a standard extraction kit (Qiagen, Crawley, UK). Reverse transcription was performed using random hexamers, and nested RSV virus PCR was performed as previously described (12) using taq polymerase. Positive and negative controls were run with all samples analyzed. A random sample of the PCR product from RSV-positive sputa was taken and both strands sequenced using a standard dye terminator method. These were compared with a reference RSV strain using Clone Manager (Scientific and Educational Software, Cary, NC) to align the sequences. Additional detail on the methods is provided in the online supplement.

Statistical Analysis

Baseline recruitment data are presented as medians (interquartile ranges [IQRs]). The annual exacerbation frequency was calculated by dividing the total number of exacerbations per patient by the number of days the patient recorded data and multiplying by 365. Normally distributed data are reported by means (SD) and skewed data by medians (IQR). Appropriate comparative statistical tests were performed dependent on the distribution of the data.

We hypothesized that a greater frequency of detection of RSV in the stable airway is associated with greater inflammation and thus faster decline in lung function. Studies to date had been cross-sectional in nature and hence pilot data on the periodicity of RSV infection were not available; we therefore predetermined a cut-off limit of 50% detection rate to divide patients into high and low RSV groups. To analyze the effects of RSV colonization independently of patient characteristics (smoking status, exacerbation frequency, bacterial load, and starting FEV_1) on decline in lung function, we used the general linear mixed model (xtreg) procedure in STATA-5 software (Stata Corporation, College Station, TX). These procedures are designed for panel (cohort) data and are particularly useful where data are correlated, as in

repeated-measures designs and where there are complex error structures (4). We included as independent variables (a) time and (b) FEV_1 measurement during the stable state, as two of four independent variables. The third variable was whether or not the patient was persistently colonized with RSV, which required 50% of their samples to be RSV positive. The fourth independent variable (d) was an interaction term obtained as the product of a and c. Allowance for confounders was made by including whether the patient had a bacterial load or inflammatory marker less or greater than the cohort median, exacerbation frequency greater or less than the cohort median, and active smoking status together with terms to adjust for the effect of these on FEV_1 decline. Also included in the regression model was the starting FEV_1 to adjust for differences in the rate of FEV_1 decline between patients with high or low starting FEV_1 . The magnitude and significance of the effect of RSV colonization on decline were unchanged with inclusion of one or all of these confounders.

As multiple sputum samples were obtained during the study and the unit of analysis was the patient, the first sample obtained from each patient suitable for estimation of inflammatory cytokines was used in the analysis. p Values of 0.05 or less were regarded as significant.

RESULTS

Patient Characteristics

Table 1 shows the baseline spirometric and other characteristics of 74 patients (45 male) who were sampled during the study. There were no differences between the baseline characteristics in terms of sex distribution, FEV_1 , $\text{FEV}_1\%$ predicted, FVC, PEF, reversibility, years of smoking, current smoking status, exacerbation frequency of these patients and the 31 patients in the cohort who were not sequentially sampled (due to recruitment in the latter part of the study, use of long-term oral steroids, or intolerance of sputum induction). There were no significant differences in these variables between sputum producers and those requiring sputum induction. The 74 patients provided 241 stable sputum samples suitable for processing for analysis. Of these patients, 16 (of whom 8 died) of the 74 patients withdrew before the end of the study period. Diary card data were collected on a mean of 656 d/person (maximum possible in study, 762 d); compliance with data collection was thus 86%.

Patterns of RSV Detection

Fifty-nine of the 74 patients sampled had RSV detected in at least one stable sputum sample during the study. Overall RSV was detected in the stable state in 32.8% of the 241 stable sputum samples collected. Sequencing of both strands of 10 randomly

TABLE 1. CHARACTERISTICS OF THE 74 PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE, MEASURED AT RECRUITMENT

	Sampled Patients (n = 74)	
	Median	IQR
Age, yr	67.4	62.2–71.4
FEV_1 , L	0.98	0.77–1.37
FEV_1 , % predicted	39.2	29.6–57.8
FEV_1 , % reversibility	7.94	0.8–13.1
FVC, L	2.41	1.86–2.90
FEV_1/FVC , %	43.9	35.5–53.1
PEF, L/min	158	122–238
PaO_2 , kPa	8.94	8.14–9.43
PaCO_2 , kPa	5.88	5.32–6.29
Smoking, yr	45	39–51
Inhaled corticosteroid dose, mg/d*	1000	500–2,000
Exacerbation frequency, per yr	2.51	1.28–3.83

Definition of abbreviation: IQR = interquartile range.

* Beclomethasone equivalents.

selected RSV-positive PCR samples confirmed homology in each case with RSV.

Of the samples, 36.8% of winter samples (December–February), 27.1% of spring samples (March–May), 36.7% of summer samples (June–August), and 34.0% of autumn samples (September–November) were RSV PCR positive. The incidence of RSV detection in stable patients showed no significant seasonality ($p = 0.558$). There was no difference in detection rates between spontaneous and induced sputum samples.

Evidence for RSV Persistence

To study whether detection of RSV in sputum was due to persistence of the virus in particular individuals or merely as a result of sporadic infection, we compared the predicted probability of RSV detection occurring in all samples from an individual based on the overall detection rate of 32.8%, to the actual prevalence of patients with RSV throughout the study. The probability that an individual would have RSV in four of four sputum samples based on a random distribution of the virus in this population is 0.0116, or 1.16%; the actual prevalence of patients with RSV in four of four positive samples is 0.2, or 20%, suggesting persistence in certain individuals.

Patients were categorized *a priori* using their stable-sample RSV status in two groups: “low RSV” (in which $\leq 50\%$ of their samples were RSV PCR positive) and “high RSV” (in which $> 50\%$ of samples were positive). There were 18 patients in the high RSV group and 56 patients in the low RSV group. There was no difference in the number of available samples per patient between the two groups (p value = 0.424), or in baseline characteristics or the inhaled corticosteroid dosage between the two groups (see Table 2). Similarly, there were no differences in exacerbation frequency between the groups; 55% in the high RSV and 48% in the low RSV group were frequent exacerbations

(with an annual rate of \geq the cohort median of 2.51 [IQR, 1.27–3.83]).

RSV Detection and FEV₁ Decline

Between the first and last RSV samples, there were 781 FEV₁ readings on the 74 patients, an average of 8.37 per patient. For those in whom RSV was detected in more than 50% of samples; the high RSV group ($n = 18$) showed a decline in FEV₁ of 101.4 ml/yr (95% confidence interval [CI], 57.1–145.8) compared with 51.2 (31.7–70.8) ml/yr in the low RSV group ($\leq 50\%$ samples were RSV PCR positive, $n = 56$). The difference in rate of FEV₁ decline between these two groups was significant ($p = 0.01$; Figure 1).

Of the 56 patients in the low RSV group, 20 (35.7%) were active smokers, and of the 18 patients in the high RSV group, 4 (22.2%) were active smokers. If an adjustment for active smoking status was made in the analysis of the relationship between RSV detection and FEV₁ decline, there remained a significantly faster decline in the high RSV group by an additional 47.9 ml/yr (0.8–87.8; $p = 0.019$). The effect of smoking itself on FEV₁ decline was not significant ($p = 0.205$) in this analysis. In a covariate analysis adjusting for any effects of exacerbation frequency on differences in rate of FEV₁ decline between RSV groups, there remained a faster decline in the high RSV group by 50.6 ml/yr ($p = 0.013$).

To ensure that variation in the number of samples obtained per patient did not affect the relationship between RSV detection and lung function decline, a subgroup analysis of patients with the same number of sputum samples was made using the sample number of four per patient. The relationship between RSV detection and FEV₁ decline remained significant ($p = 0.009$), with a faster decline in FEV₁ of 52.2 (CI, 13.3–91.3) ml/yr in the high RSV group subset.

TABLE 2. CHARACTERISTICS OF THE 74 PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE AS CHARACTERIZED BY PATTERN OF RESPIRATORY SYNCYTIAL VIRUS DETECTION BY POLYMERASE CHAIN REACTION

	Low RSV Patients* ($n = 56$)		High RSV Patients* ($n = 18$)		P Value (Wilcoxon)
	Median	IQR	Median	IQR	
Age, yr	67.4	61.7–71.8	68.7	62.2–71.7	0.748
FEV ₁ , L	0.95	0.77–1.37	1.09	0.73–1.38	0.735
FEV ₁ , % predicted	39.2	29.6–57.8	42.9	29.0–59.4	0.759
FEV ₁ , % reversibility	9.1	2.1–13.1	3.70	0.0–12.8	0.231
FVC, L	2.37	1.68–2.90	2.60	2.00–3.28	0.297
FEV ₁ /FVC, %	44.1	35.5–53.1	41.3	26.9–54.7	0.696
PEF, L/min	152	120–238	186	147–230	0.661
Pa _{O₂} , kPa	9.00	8.13–9.62	8.47	8.27–9.22	0.387
Pa _{CO₂} , kPa	5.91	5.32–6.31	5.81	5.32–6.11	0.713
Smoking, yr	46	37–52	42.5	39–50	0.555
Inhaled corticosteroid dose, mg/d [†]	1000	500–1,800	1000	600–2,000	0.600
Exacerbation frequency, per yr	2.40	1.32–3.87	2.76	1.04–3.37	0.821
	%		%		p Value χ^2
Sex, males	60		60.8		0.976
Chronic dyspnea	44.8		52.7		0.780
Chronic wheeze	34.3		32.4		0.211
Chronic cough	41.0		37.8		0.506
Chronic sputum production	41.8		27.8		0.988
History of smoking	98.2		100.0		0.568
Smoking at recruitment	41.8		27.8		0.288

For definition of abbreviation, see Table 1.

* Low RSV defined as $\leq 50\%$ of sputum samples were RSV polymerase chain reaction (PCR) negative, and high RSV as $> 50\%$ sputum samples were RSV PCR positive.

[†] Beclomethasone equivalents.

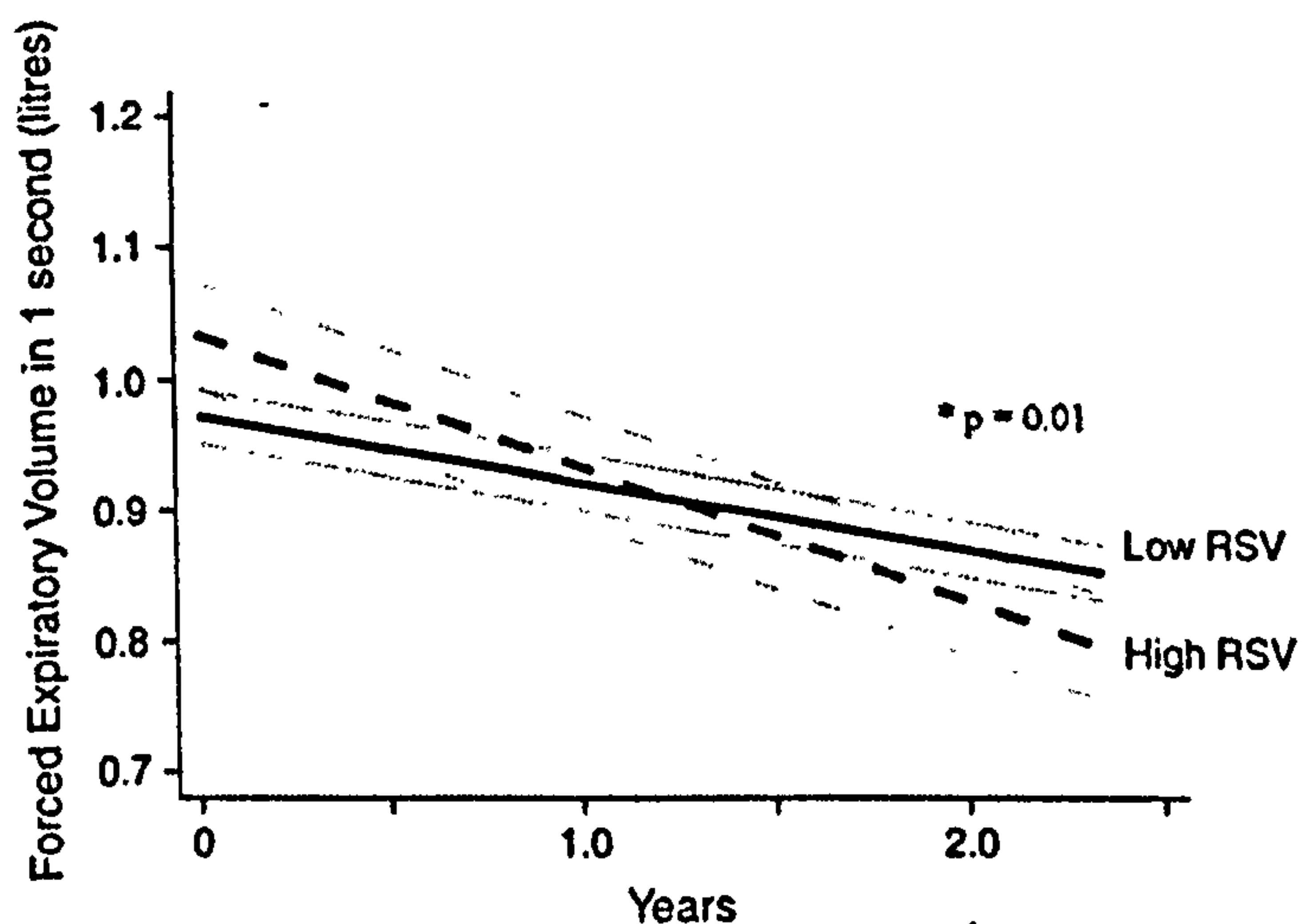


Figure 1. Relationship between respiratory syncytial virus (RSV) detection pattern and FEV₁ decline over study period. Heavy lines represent decline in FEV₁ with standard errors (SEs) for (1) low RSV ($\leq 50\%$ of sputum samples were RSV polymerase chain reaction [PCR] positive; $n = 56$; 51.2 ml/yr; SE, 10.1; confidence interval [CI], 31.7–70.8), intercept 0.97 (SE, 0.42; CI, 0.90–1.05; continuous lines), and (2) high RSV ($> 50\%$ sputum samples, RSV PCR positive; $n = 18$; 101.4 ml/yr; SE, 0.017; CI, 57.1–145.8), intercept 1.03 (SE, 0.08; CI, 0.88–1.19; dashed lines).

Airway Bacteria and RSV

Total bacterial load was available for 213 of the 241 samples. The overall prevalence of bacterial pathogens (PPMs) was 66.1%. The mean (SD) airway bacterial load was greater in the high RSV patients ($10^{8.12} [0.48]$ log cfu/ml), compared with low RSV patients ($10^{7.76} [0.58]$ log cfu/ml; $p = 0.024$). There was no significant association between the detection of RSV and isolation of individual PPMs, or with all potential pathogenic organisms as a group ($p > 0.17$ in all cases).

Airway Inflammatory Markers and RSV Persistence

Detection of RSV was associated with higher levels of airway inflammation as measured by sputum IL-6, IL-8, and MPO ($p < 0.001$ in all cases). The relationships between RSV detection category and airway inflammatory markers are shown in Figure 2.

Levels of individual airway inflammatory markers above the median were associated with a trend to faster FEV₁ decline. High levels of IL-6 were associated with additional FEV₁ decline of 6.7 ml/yr (CI, 11.8–25.3), with high IL-8; 12.2 ml/yr (–6.4–30.9) and with high MPO 6.9 ml/yr (8.3–22.1), but these did not reach significance ($p > 0.18$ in all cases). To determine if the association between RSV detection and lung function decline was independent of airway inflammation, inflammatory markers were included in the model of analysis. The differences between FEV₁ decline in high and low RSV groups were not statistically independent of associations with airway inflammation. Additional decline in high RSV group with IL-6 as a covariate: 42.9 ml/yr (–12.6–98.4, $p = 0.13$); for IL-8, 39.9 ml/yr (–16.0–95.9, $p = 0.16$); and for MPO, 28.0 ml/yr (–23.8–79.8, $p = 0.29$).

Multivariate Analysis of RSV and Other Factors Affecting FEV₁ Decline

After allowance for total bacterial load, smoking status, and starting FEV₁, high RSV patients had an FEV₁ decline of 114.7 ml/yr (95% CI, 42.4–186.8) compared with the decline of 56.6 ml/yr (26.2–87.0) seen in low RSV patients ($p < 0.05$ for the

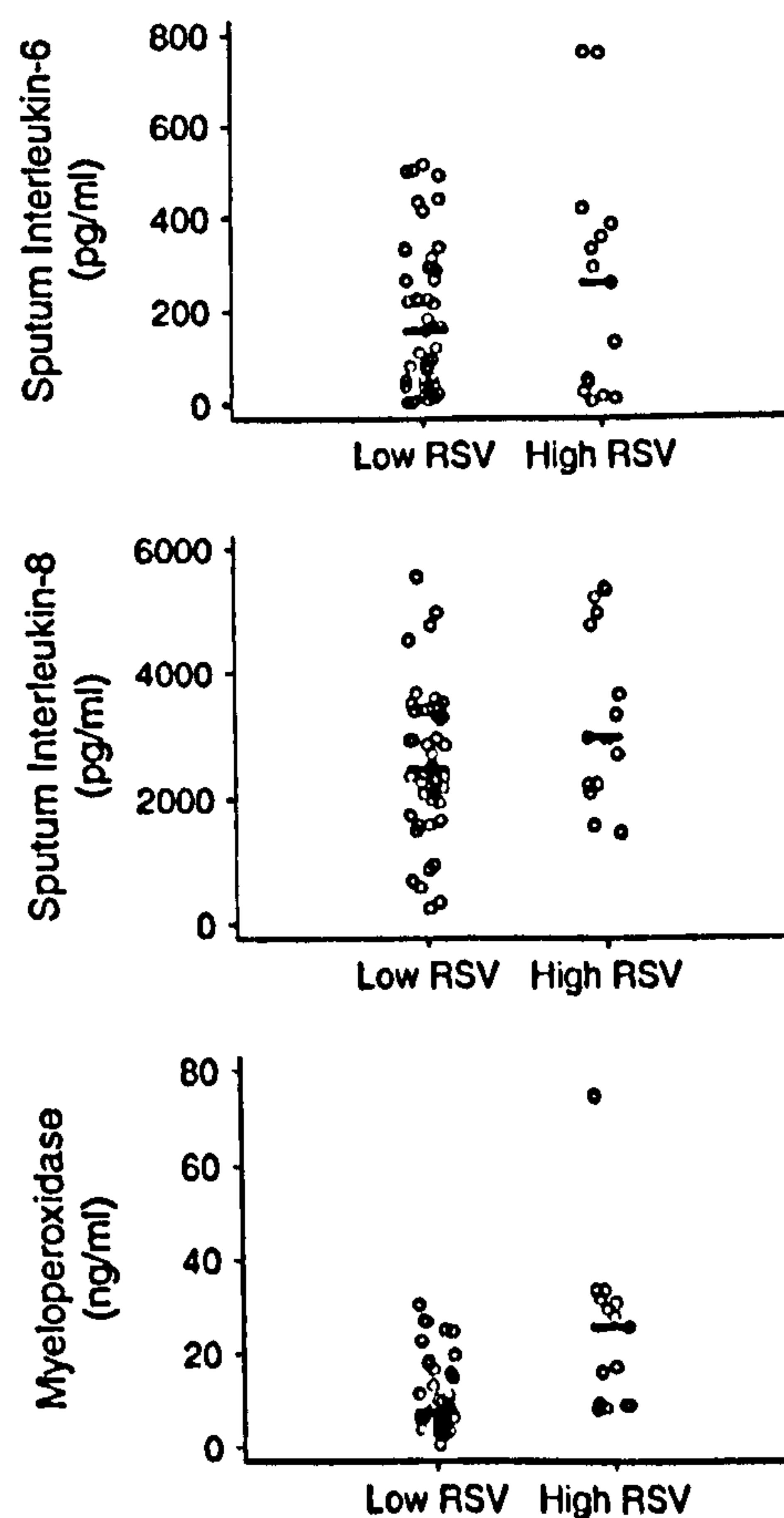


Figure 2. Comparison of sputum interleukin 6 (IL-6; pg/ml), IL-8 (pg/ml), and myeloperoxidase levels (ng/ml) for low RSV and high RSV patient groups ($p < 0.001$, for difference between groups in all cases using cross-sectional regression analysis assuming a Poisson distribution in the dependent variable to allow for repeated measures). Sputum IL-6 (182 [178–185] and 253 [244–261] pg/ml), IL-8 (2,571 [2,558–2,585] and 3,229 [3,197–3,262] pg/ml), myeloperoxidase (10.3 [7.5–13.2] and 23.0 [16.7–29.3] ng/ml) for low RSV and high RSV patient groups, respectively.

difference between the two groups). If the FEV₁ data were expressed as a percentage of the predicted FEV₁, high RSV patients had a significantly faster decline of 1.94%/yr (0.2–3.63) in addition to the decline of 2.1%/yr (0.9–3.3) in low RSV patients ($p = 0.03$). If exacerbation frequency was included in the multivariate analysis, the faster decline in the high RSV group remained significant independent of any effects of exacerbations (19.3 [2.2–36.3] ml/yr, $p = 0.027$). A multivariate analysis with airway inflammation (MPO), smoking status, starting FEV₁, bacterial load, and exacerbation frequency revealed a nonsignificant finding of an additional 6.9-ml/yr FEV₁ decline (–15.4–29.2, $p = 0.545$) relative to the low RSV group.

DISCUSSION

This is the first longitudinal prospective study to investigate the role that RSV may play in the etiology and progression of stable COPD. We show that RSV RNA can be detected in the sputum of many patients with COPD in the stable state, and its detection is associated with higher levels of airway inflammation, greater airway bacterial loads, and an accelerated decline in lung function.

RSV is an established cause of acute respiratory illness in children, and RSV bronchiolitis is associated with the development of persistent wheeze in later childhood (22); however, it

is not clear whether this association is causal. The role of viral infection in the etiology of airway obstruction in adults is even less well established. In a different setting, Retamales and colleagues reported that adenovirus E1A protein was expressed in respiratory epithelial cells in patients with emphysema, and that the quantity of E1A expression correlated with disease severity and inflammatory cell numbers (5). However, to date, longitudinal studies to determine the role of latent viral infection in COPD disease progression have been lacking. The data from this study suggest that RSV may play a role in the pathogenesis of airway inflammation and subsequent deterioration in lung function in adults with COPD.

Persistent RSV infection is well known in children with T-cell immunodeficiency, and has been demonstrated in the lungs of guinea pigs (23) and mice (24) for up to 150 d after experimental infection. In these animal models, RSV persistence was associated with continued infectivity and chronic airway inflammation despite an appropriate systemic humoral (23, 24) and T-cellular immune response (24).

In both acute and chronic models of infection, the key sites for RSV-induced inflammation in the lung are the small airways, in which epithelial damage and increased mucous production result in small airway obstruction and hyperinflation (25). It is of note that the small airways are also the primary site for the persisting inflammation and airway obstruction, which are characteristic of COPD (26, 27). Biopsies of subjects with COPD have demonstrated that the small airways are infiltrated with inflammatory cells, in particular CD8⁺ cells, neutrophils, and airway macrophages (27, 28). In particular, the presence of CD8⁺ T cells and B lymphocytes organizing into follicles was associated with disease progression (27). The findings of this study may suggest CD8⁺ T-cell populations, characteristic of COPD airway biopsies, may be recruited to the lung due to persistent viral infection, but an impaired immune response that is incapable of eliminating virus infection permits ongoing replication at low levels.

RSV detection was associated with heightened airway inflammation in terms of increased levels of IL-6, IL-8, and MPO. It is possible that RSV has direct proinflammatory effects on the airway, which may contribute to faster decline in lung function. The observed association between RSV detection and FEV₁ decline remained significant if possible confounders such as airway bacterial load, exacerbation frequency, smoking status, and baseline FEV₁ were included in the analysis. However, if airway inflammation was included in the covariate or multivariate model, no significant effect of RSV on lung function independent of airway inflammation was seen. One explanation is that RSV, inflammation, and decline are causally linked; however, it is also possible that inflammation predisposes the airway to viral persistence and that RSV detection is therefore an epiphenomenon. The direct relationship between airway inflammation and lung function decline was not significant in this analysis. This may be due to other noninflammatory processes such as airway remodeling (27) and effects on cellular apoptosis (29) or via other arms of the inflammatory cascade (30). However, over a longer follow-up period in a similar patient population, direct relationships between lung function decline and airway inflammation have been demonstrated (4), and therefore a similar relationship may have been found with prolongation of the follow-up period of this study. An alternative explanation of our findings is that patients with more aggressive COPD and faster disease progression have impaired acquired or innate immune responses, allowing RSV to persist. To determine the causal role of RSV infection, one would need to attempt to eradicate RSV with vaccines or antiviral drugs now under development.

Although RSV may have proinflammatory effects, it is also possible that it acts by modulating the response of lung cells to

other inflammatory stimuli, including bacterial lipopolysaccharide (31), or by promoting neutrophil adhesion, thereby augmenting lung damage (32). Bacterial colonization of the lower airway in patients with COPD is well described (2, 3, 33, 34) and provides a stimulus to airway inflammation and disease progression (2). The additional presence of RSV in the lower airway may augment bacterially driven inflammation. It is also possible that chronic viral infection of the lower airway occurs due to increased susceptibility in individuals with existing bacterial colonization, although we found no direct association between the isolation of PPMs and detection of RSV. It is feasible therefore that eradication of airway bacteria may result in local repair and improvement of local defenses against viral persistence.

The relative importance of airway bacterial infection in COPD has been shown to increase with disease severity (34). This study was performed with a group of patients with moderate to severe disease and therefore the findings that RSV can be detected and is associated with accelerated disease progression in this population may not necessarily be extrapolated to patients with milder disease. Further studies across the full spectrum of disease as well as in smoking and nonsmoking control subjects are required to better understand the role of this pathogen in adults.

The use of sputum to detect the presence of RSV in the stable state allows repeated sampling of patients with advanced disease, which alternative techniques, such as bronchoscopic sampling, do not manage. The use of sputum sampling in a longitudinal cohort study inevitably results in a variable number of samples obtained per patient, and this problem is further compounded by the use of quality control in processing to exclude inadequate samples. However, this quality-control step is a vital one, because variations in sampling methodology are likely to be responsible for the differences in detection of RSV in patients with stable COPD seen in one study to the next (13, 35). Although PCR detection assays may appear, on paper, to be highly sensitive, it is apparent that the type of biological sample taken (sputum rather than swab), the rapidity of sample processing and storage, and the nucleic acid extraction techniques used all impact on the likelihood of detection of RSV RNA by PCR (35).

The use of highly sensitive PCR assays may detect viral nucleic acids; however, this does equate to detection of intact and pathogenic virus. Furthermore, our PCR technique did not distinguish between RSV type in this study and it is not known whether the virus isolated from an individual is genetically stable and representative of chronic infection rather than recurrent reinfection. Further studies are required to confirm the exact nature of viral persistence in the lower airway of patients with COPD.

This study did not examine the detection rates for RSV among normal control subjects, but the PCR detection techniques used in this study have also been used previously. In a number of these other studies, healthy control groups have been studied and low detection rates have been found. Indeed, we have studied adult control groups in two recent studies; the detection rates for RSV in both of these was zero (36, 37), suggesting that RSV detection in the stable state may be a factor in patients with moderate to severe COPD, but not in healthy control subjects.

In conclusion, we have shown that RSV RNA can be detected from lower airway samples of some patients with COPD in the stable state, with evidence of persistent detection in certain individuals. RSV RNA detection was associated with greater airway inflammation and accelerated disease progression in these patients. These findings suggest that RSV may play a role in the natural history of stable COPD. However, further investigation into the nature and consequences of viral persistence are

required to confirm whether RSV is a potential therapeutic target in this important patient group.

Conflict of Interest Statement: T.M.A.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. G.C.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.L.J. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.J.M.O. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.A.W. has received honoraria for lectures at meetings and/or attendance at advisory boards from the following companies: Glaxo and then GlaxoSmithKline (GSK), Boehringer Ingelheim, Astra Zeneca, Bayer, Novartis, Aventis Pasteur. She has received research grants totaling approximately \$300,000 from GSK for studies of various aspects of COPD exacerbations. She has received a grant of approximately \$450,000 for a study of tiotropium in COPD and \$25,000 from Astra Zeneca for a health economic study, and \$300,000 from Aventis Pasteur for a study of viral epidemiology of COPD.

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