The use of biomarkers to explore inflammation and phenotypes during exacerbations of chronic obstructive pulmonary disease

Thesis submitted for the degree of

Doctor of Philosophy

University of Leicester

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September 2011

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<u>Abstract</u>

Exacerbations of chronic obstructive pulmonary disease (COPD) are heterogeneous. In particular the association of airway inflammation with pathogens during exacerbations of COPD remains largely unknown. The identification of biomarkers of COPD aims to characterise and phenotype COPD and COPD exacerbations, explore mechanisms of pathogenesis and provide decisions regarding targeted treatment strategies.

After validating the Meso scale discovery platform, biomarkers in stable state and during exacerbations COPD were explored. COPD exacerbations were associated with increased systemic and pulmonary inflammation and exacerbations associated with bacteria, virus and sputum eosinophils could be identified by sensitive and specific biomarkers. Multivariate statistical analysis identified independent biological exacerbations phenotypes and that these phenotypes could be predicted from stable state biomarkers. Finally a biomarker directed strategy using the peripheral blood eosinophil count to guide systemic corticosteroid therapy during exacerbations of COPD was effective, safe and identified phenotypes which have particular prognostic and therapeutic characteristics.

To conclude, biomarkers can define the heterogeneity of COPD and COPD exacerbations. The identification of COPD phenotypes can potentially move the management towards phenotype specific management and personalised medicine.

Acknowledgements

I would like to thank first and foremost my supervisor Chris Brightling, who provided me with the guidance, support and inspiration to complete this thesis. My thanks also go to my co-supervisor Ian Pavord for his direction, supervision and continual enthusiasm. I would also like to directly thank Sue McKenna and Sarah Terry. Sue's hard work as my research nurse and Sarah's work as my research assistant personally ensured the success of the study. I would like to acknowledge the hard efforts provided to me from Vijay, Carlene and Mitesh at the Institute for Lung Health laboratory. Their valuable assistance in sample collection and processing I am wholeheartedly indebted to. My direct thanks must also go to Hemu and Koirobi at the University of Leicester for much of my bacteria analysis, to Tata at the Centre for Respiratory Infection, Imperial College London, for the processing of all the virus samples and to Annelyse at the Cambridge Institute for Medical Research for all the surfactant protein D work. I would also like to acknowledge my colleagues in AstraZeneca, whose efforts in the MSD biomarker work provided me with much of the data to analyse. My ultimate thanks go to all the patient volunteers that took part for whom there was unreserved and continual dedication to the success of the studies.

My eternal gratitude goes to my mother and father and my siblings Leila, Sarah and Ahmed. And finally, I would like to dedicate this thesis in memory of my best friend Dr Joseph Footitt for his insurmountable energy, support and guidance in all things affiliated to respiratory medicine, respiratory research, bicycle punctures and life; I miss him tremendously.

Publications arising from this thesis

Bafadhel M, Mckenna S, Terry S, Pancholi M, Mistry V, Venge P, Lomas DA, Barer MR, Johnston SL, Pavord ID, Brightling CE. The use of peripheral blood eosinophils to direct corticosteroid treatment of exacerbations of COPD: a randomised placebo controlled trial. *Am J Resp Crit Care Med 2012; In Press (Epub)*

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Bafadhel M, Agbetile J, Mistry V, *et al*. Identification of Aspergillus fumigatus in patients with COPD. *In submission (Chest)*

Abstracts

More than 20 abstracts presented and published in Thorax, European Respiratory Journal and the American Journal of Respiratory Critical Care Medicine.

Prizes awarded

International Congress on Exacerbations of Airway Disease Young Investigator Winner (Miami, 2010); Institute for Lung Health Wendy Stannard Prize Winner (Leicester, 2010); British Thoracic Society Young Investigator runner up (London, 2010); European Respiratory Society Annual Inflammatory Airway Diseases and Clinical Allergy Grant Winner (Amsterdam, 2011)

Statement of work performed

I designed or co-designed all the studies in this thesis and obtained Ethics and Medicines and Healthcare products Regulatory Agency approval for the main observational biomarker study and the eosinophil biomarker prednisolone and placebo directed randomised control trial. For these main studies, I was responsible for all patient recruitment and consent and attended to the patients at each of the stable, exacerbation, follow up and recovery visits, which accounted for approximately over 2000 visits. At each of the visits I personally collected data measurements for the study analysis. For all baseline and exacerbation visits, I took the patient history, performed the clinical examination, undertook spirometry with bronchodilator testing, and undertook blood and sputum sample collection. At these visits I also collected the questionnaire results pertinent to the study. For the remainder of the visits, in conjunction with the research nurse I performed the sputum, blood, spirometry and questionnaire data collection. I undertook approximately 50% of the sputum and blood processing and processed the single ELISA's and biomarkers in the laboratory. I was responsible for all the coordination of the work performed at the collaborating facilities. I designed the database, created the unique bar-coding for all samples processed and was solely responsible for all data entry. Finally, I was responsible for all the statistical analysis of the data; prepared and presented all the abstracts at national and international meetings; and wrote all the original research articles published in peer-reviewed journals that arose from the work in this thesis.

List of Abbreviations

ANOVA	One way analysis of variance
ATS	American thoracic society
AUC	Area under the curve
BAL	Bronchoalveolar lavage
BODE	Body mass, airflow obstruction, dyspnoea and exercise capacity index
BMI	Body mass index
BTS	British thoracic society
CCR	Chemokine receptor
CFU	Colony forming units
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
CRP	C reactive protein
CRQ	Chronic respiratory disease standardised questionnaire
CV	Coefficient of variation
CXR	Chest radiograph
DNA	Deoxyribonucleic acid
D-PBS	Dulbecco's phosphate buffered saline
DTT	Dithiothreitol
ECP	Eoisnophil cationic protein
ECLIPSE	'Evaluation of COPD longitudinally to identify predictive surrogate endpoints' study
EDN	Eosinophil derived neurotoxin

ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPO	Eosinophil peroxidase
ERS	European respiratory society
FE_{NO}	Fraction of exhaled nitric oxide
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GMCSF	Granulocyte macrophage colony stimulating factor
GOLD	Global initiative for chronic obstructive lung disease
HTA	Health technology assessment
ICC	Intra class coefficient
ICD	International classification of diseases
IFN-γ	Interferon gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP-10	Interferon gamma induced protein
IQR	Interquartile range
ITAC	Interferon inducible T cell chemoattractant
κ	Cohen kappa coefficient
KCO	Diffusion capacity of the lung for carbon monoxide corrected for alveolar volume
LPS	Lipopolysaccharide
MBP	Major basic protein

MCP	Monocyte chemotactic protein
MEWS	Modified early warning score
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
MRHA	Medicines and Healthcare products Regulatory Agency
MSD	Meso scale discovery platform
NE	Neutrophil elastase
NHS	National Health Service
NIHR	National institute for health research
NK	Natural killer cells
NNH	Number needed to harm
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
OR	Odds ratio
PCA	Principal Component analysis
PCR	Polymerase chain reaction
PCT	Procalcitonin
PFA	Principal Factor analysis
PPM	Potentially pathogenic micro-organism
PSB	Protected specimen brush
QoL	Quality of life

qPCR	Real time quantitative polymerase chain reaction
r	Pearson correlation coefficient
RANTES	Regulated upon activation, Normal T cell expressed and secreted
RCT	Randomised control trial
RNA	Ribonucleic acid
ROC	Receiver operator characteristic
rs	Spearman rank correlation coefficient
RT-PCR	Reverse trancriptase polymerase chain reaction
RV	Rhinovirus
sAA_1	Serum amyloid protein 1
SELDI-Tof	Surface enhanced desorption ionisation time of flight
SEM	Standard error of the mean
SGRQ	St. Georges respiratory questionnaire
SPD	Surfactant protein D
TARC	Thymus and activation regulated chemokine
TCC	sputum total cell count
Th	T helper cells
T_LCO	Diffusion capacity of the lung for carbon monoxide
TNF	Tumour necrosis factor
TRACE	Time resolved amplified cryptate emission technology
VAS	Visual analogue scale
VC	Vital capacity
WHO	World Health Organization

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1.   Introduction

1.1.   Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is characterised by chronic airflow limitation which is not fully reversible. The most common aetiology in Western countries is chronic cigarette smoke exposure. COPD presents a huge global healthcare problem associated with significant morbidity and mortality. Periods of disease instability are termed exacerbations and are the leading cause of patient distress and healthcare expenditure. COPD encompasses several features based on simple spirometric testing comprising of emphysema, chronic bronchitis and chronic asthma. Early descriptions of COPD date back to 1819, by René Laënnec, the inventor of the stethoscope. In his 'Treatise on the Diseases of the Chest', Laënnec used the term emphysema and described the loss of elastic recoil, air trapping, dilatation of the alveoli and mucosal hyperplasia (Laënnec, 1819). Laënnec reported that emphysema was common and attributed the airspace dilatation pathognomonic of emphysema to the physical obstruction of the bronchioles by mucus plugging. Frederik Ruysh described emphysema almost a century earlier as a "lung that could not be deflated and did not communicate with the main airway" in a man who had been troubled with breathlessness and cough in his illustrated pathological specimen catalogue (Ruysh, 1721; Warren, 2009).

1.2.   Epidemiology

The World Health Organization (WHO) recognises COPD as a major global health burden. It is predicted that by 2020, COPD will be the 3rd leading cause of death worldwide affecting a population of over 80 million (WHO report, 2004). In the United

Kingdom, COPD is diagnosed in 1 million people over the age of 40. An estimated 2 million sufferers are yet to be diagnosed described by the British Lung Foundation as the 'Missing Millions' based on extrapolated incidence data. The cost of COPD to the National Health Service (NHS) is estimated to be over £1 billion per annum (NICE report 2010). The prevalence of COPD continues to increase with significant health and socio-economic impacts and this is likely to reflect a complex picture including earlier diagnosis, greater disease awareness and an ageing population (Lopez, 2006).

1.3. Definition and diagnosis

The current definition of COPD taken from the Global Initiative for Chronic Obstructive Lung Disease (GOLD) is as follows (Rabe, 2007):

'a preventable and treatable disease with some significant extra pulmonary effects that may contribute to the severity in individual patients. Its pulmonary component is characterised by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal response of the lung to noxious particles or gases'.

A diagnosis of COPD is made in the context of a relevant clinical history, examination and spirometric investigation. The clinical history of COPD, which is often insidious, includes that of dyspnoea on exertion, chronic cough and sputum production. The clinical signs include wheeze and diminished breath sounds on auscultation. Formal diagnosis of COPD is made after demonstration of post bronchodilator airflow obstruction, with a forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC) ratio of < 0.7 ($FEV_1/FVC < 0.7$). Severity of COPD is then classified by the degree of airflow limitation; Mild $FEV_1 \geq 80\%$ predicted; Moderate FEV_1 79% - 50%

predicted; Severe FEV$_1$ 49% - 30% predicted; and Very Severe FEV$_1$ < 30% predicted or < 50% predicted plus the presence of chronic respiratory failure (defined as PaO$_2$ < 8.0kPa while breathing room air and/or evidence of cor pulmonale).

Spirometric classification is associated with limitations, particularly a tendency to over-estimate obstruction in a normal ageing lung and under-estimate in younger individuals with true airway obstruction (Roberts, 2006). Furthermore, the current definition of COPD and spirometric criteria for diagnosis may not accurately reflect the complex disease processes occurring in the lung. It does not define the different airway inflammatory patterns that exists, nor infer treatment responsiveness. The heterogeneity that exists within COPD cannot be clearly demonstrated by FEV$_1$ alone and it is now recognised that further tools are necessary to identify groups with which there are similar prognostic or therapeutic characteristics (Han, 2010).

1.4. Pathophysiology

Airflow obstruction is attributed to increased airway resistance, a consequence of structural abnormalities of the lung parenchyma, the small and large airways and the pulmonary vasculature (Hogg, 2004). Observations of Poiseuille's Law confirm that resistance of flow through a tube (or airway) is inversely proportional to the radius^4 of the tube. Hence narrowing and destruction of the small airways in COPD can explain the increase in airway resistance. The underlying cause of these structural abnormalities is related to airway inflammation. This is amplified and uncontrolled following exposure to noxious particles particularly cigarette smoke.

One hypothesis for the pathogenesis of COPD is an imbalance of host defence. Innate immunity is non-specific and comprises of the mucociliary network, polymorphonuclear cells, eosinophils, macrophages, natural killer (NK) cells and mast cells (Hogg, 2004). Innate immunity recognises antigens and activates the adaptive immune system, which has a memory basis following antigen presentation. In COPD there is a predominance of neutrophils, cytotoxic $CD8^+$ T lymphocytes, and macrophages found within the airway and lumen (Saetta, 1993; Di Stefano, 1996; O'Shaughnessy, 1997; Saetta, 1998; O'Donnell, 2006), but eosinophils have also been demonstrated in the airways (Lacoste, 1993; Pizzichini, 1998; Balzano, 1999; Brightling, 2000; Brightling, 2005). The airway inflammation seen in COPD has been shown to persist despite smoking cessation (Rutgers, 2000; Willemse, 2005). In a study of bronchial biopsies (O'Shaughnessy, 1997) and sputum cell counts (O'Donnell, 2004) it was found that neutrophilic airway inflammation was inversely proportional to lung function, further suggesting that inflammation has a large role in the pathogenesis of COPD and the development of airflow obstruction.

1.4.1. Histopathology

Small airway obstruction

Conducting airways less than 2mm in diameter are the major site of airway obstruction in COPD (Hogg, 2004). Within these conducting airways, the accumulation of inflammatory exudates within the lumen, smooth muscle hypertrophy and peribronchiolar fibrosis leads to small airway obstruction and has been associated with the severity of COPD (Hale, 1984; Hogg, 2004). Studies performed by McDonough *et al* have demonstrated that the number of small airways in the lung (measuring 2 to 2.5mm) calculated by multi-detector computed tomography was reduced incrementally

in the COPD GOLD severity stages compared to smoking non obstructed controls and also showed that narrowing of the terminal bronchiole preceded the appearance of microscopic emphysema (McDonough, 2011).

Emphysema

Emphysema is defined as airspace dilatation and destruction distal to the terminal bronchiole, resulting in an increase in lung compliance. Although chronic and excessive smoke exposure is associated with emphysema, the presence of emphysema alone is associated with an abnormal inflammatory response. In a study by Retamales *et al*, it was demonstrated that there is an increase in cellular inflammation in the lungs of emphysematous COPD subjects compared to smoking non obstructive controls (Retamales, 2001). Emphysema can be described further according to the anatomical structures affected and these are termed centrilobular, centriacinar and panacinar. It has been shown that the centrilobular form of emphysema is associated with increased inflammation in the small airways, whilst the panacinar form is usually a finding characteristic of Alpha-1-antitrypsin deficiency (Hogg, 2004).

Chronic Bronchitis

Chronic bronchitis is defined as '*chronic cough with sputum production for at least three months over 2 consecutive years*'. The inflammation is located within the central airways, associated with excessive mucus production and defective mucociliary clearance (Hogg, 2004). Radiological investigation using computed tomography have determined that bronchial wall thickening in subjects with COPD was associated with symptoms of chronic bronchitis (productive cough and sputum production) and frequent

exacerbations requiring antibiotic therapy compared to subjects with predominately emphysematous changes on imaging (Fuijmoto, 2006).

1.4.2. Airway inflammation

Central to COPD pathophysiology in stable disease and during exacerbations is airway inflammation. Numerous COPD studies have identified increased numbers of neutrophils, eosinophils and macrophages (Saetta, 1997; Barnes, 2003; O'Donnell 2004), whilst the definition of COPD now encompasses the abnormal inflammatory response to noxious particles (Rabe, 2007).

Neutrophils

Neutrophilic airway inflammation is considered to be the hallmark of COPD. The neutrophil has an important role in innate immunity. The secretion of serine proteases from neutrophils such as elastase and proteinase-3 may contribute to the pathological processes with mucus hypersecretion and contribution to alveolar destruction (Stockley, 2002; Barnes, 2003). Mature neutrophils are released into the circulation and are localised to sites of inflammation under the influence of interleukin-8 (IL-8) and tumour necrosis factor (TNF-α). Neutrophil survival is increased by the effects of growth factor granulocyte macrophage colony stimulating factor (GM-CSF) (Stockley, 2002; Barnes, 2003; Hogg, 2004). In response to infection neutrophils are attracted to sites of tissue injury and produce IL-8 and myeloperoxidase (MPO). Neutrophil degranulation at these sites releases proteases and reactive oxygen species. Following neutrophil apoptosis the neutrophils are phagocytosed by macrophages. Prolonged survival and failure of apoptosis increases the release of proteases from neutrophils with further chemoattractant stimulation that lead to recruitment, stimulation and survival of the

neutrophil. Neutrophils are elevated in the sputum and bronchoalveolar lavage (BAL) of COPD subjects during stable state (Lacoste, 1993; Keatings, 1996) and this has been shown to be related to airflow limitation (Stanescu, 1996; Balzano, 1999).

Macrophages

Approximately 15% of circulatory monocytes become airway, interstitial and alveolar macrophages. Alveolar and airway macrophages have similar functions. The macrophage is responsible for phagocytosis via innate immunity and in the initiation of adaptive immunity. Macrophages are responsible for production of important pro-inflammatory cytokines such as IL-6, IL-8, TNF-α, interleukin-1β (IL-1β) and monocyte chemotactic protein 1 (MCP-1/CCL2). Alveolar macrophages also secrete elastolytic enzymes, the predominant being matrix metalloproteinase 9 (MMP-9) (Russell, 2002). Macrophages numbers have been found to be increased in sputum, BAL and tissue in COPD (Confalonieri, 1998; Rutgers, 2000).

Eosinophils

Eosinophilic airway inflammation has been shown to predominately occur in asthma and allergic diseases, but controversy exists over its role in the pathogenesis of COPD. Eosinophils originate from pluripotent common myeloid progenitor cells in the bone marrow, with differential termination and release into the peripheral circulation mediated by interleukin-5 (IL-5). Circulating eosinophils spend little time in the peripheral circulation and migrate to tissues under the influence of the specific chemoattractants IL-5, eotaxin-1 and -2 (CCL11 and CCL2) and Regulated upon Activation, Normal T cell Expressed and Secreted (RANTES/CCL5) via binding to chemokine receptor 3 (CCR3) found abundantly on the eosinophil cell surface. The

migration of eosinophils from the circulation to the tissue involves interactions between circulating peripheral blood eosinophils and the vascular endothelium within the target tissue, transmigration across the vascular endothelium and movement from the interstitium to the site of inflammation. Survival of eosinophils within the tissue is regulated by apoptotic inhibition and maintained via the local effects of IL-5 and GM-CSF and direct eosinophil cell secretion of interleukin-4 (IL-4), interleukin-13 (IL-13) and RANTES. Activated eosinophils are further responsible for the production of many cytokines such as IL-4, IL-5, IL-6, IL-8, IL-13 and TNF-α. Stored within the secondary granules are eosinophilic specific basic proteins. These are major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil derived neurotoxin (EDN). Degranulation of eosinophils and identification of pathognomonic Charcot-Leyden crystals within the tissue, followed by the subsequent release of the eosinophilic specific basic proteins has cellular toxicity and is responsible for epithelial cell injury (Wardlaw, 1999).

Eosinophils in COPD

Despite their controversial role in COPD, eosinophils have been detected during stable state in varying degrees from the central and peripheral airways from sputum and BAL specimens respectively and from bronchial biopsies (Lacoste, 1993; Brightling, 2000; Rutgers, 2000). In work by Balzano *et al*, sputum eosinophils were higher in COPD subjects compared to healthy controls and ECP levels were comparable to the asthma subjects. Furthermore, in assessing associations with lung function and airway obstruction, Balzano *et al* found that the differential sputum eosinophil count had a significant inverse relationship with FEV_1 and FEV_1/FVC (Balzano, 1999). Studies have shown a negative association between eosinophilic inflammation and lung

function and that the fastest decline in COPD lung function was found in those with the highest degree of eosinophilic inflammation, which suggests that eosinophils may have a role in chronic inflammation in COPD (Stanescu, 1996; Balzano, 1999). The finding of elevated ECP in the central and peripheral airways (Keatings, 1997; Balzano, 1999) but not in the tissue (Lacoste, 1993), would suggest that the activation of eosinophils occurs within the airway. However the literature is not conclusive for the role of the eosinophil in COPD and work has focussed on cross-sectional analysis only.

1.5.   Exacerbations of COPD

The natural history of COPD is progressive airflow obstruction and lung function decline. Episodes of acute deterioration are characterised by periods of worsening in respiratory symptoms and function and are a common feature of COPD. These episodes are termed exacerbations and impact significantly upon the quality of life of patients. Exacerbations are associated with significantly worse health outcomes (Seemungal, 1998), accelerated lung function decline (Donaldson, 2002), an increased risk of further exacerbations (Burge, 2000; Hurst, 2010) and an increased risk of death (Connors, 1996). In the United Kingdom, COPD exacerbations account for 15% of all acute hospital admissions, 1 million bed days and an annual expenditure of approximately £500 million (NICE report, 2010).

It has proved difficult to gain a consensus agreement for the definition of an exacerbation of COPD, despite their importance and associated health care costs, morbidity and mortality. Studies preceding the Rodriguez-Roisin definition (Rodriguez-Roisin, 2000), were inconsistent and often used a mixture of patient reported symptoms or stages of instituted therapy. In studies that used symptoms to define the exacerbation

these were arbitrarily classified as major and minor, based on the presence of major symptoms of dyspnoea, sputum production and sputum purulence and minor symptoms which included wheeze, nasal discharge, sore throat, fever or cough. In studies conducted in the East London COPD cohort the use of major and minor symptoms were recorded by patients on daily diary cards. An exacerbation was defined as one that comprised of either 2 major symptoms or 1 major symptom and 2 minor symptoms with a fixed time classification of symptoms for at least two consecutive days (Seemungal, 1998). The use of daily diary cards is an adjunct to capturing recorded and unrecorded exacerbations, although the time frame cut off of 2 consecutive days remains arbitrary without further evidence existing to challenge this.

The definition used by the East London COPD cohort were not too dissimilar to the definition used by Anthonisen (Anthonisen, 1987), where the symptom criteria were type 1 if there was dyspnoea, sputum production and sputum purulence; type 2 if two out of three of these major symptoms were reported; and type 3 if one of these major symptoms were reported in addition to at least one of either upper respiratory infection within 5 days (sore throat or nasal discharge), fever without alternative cause, increased wheeze, increased cough, or increase in respiratory or heart rate by 20% to that above the recorded baseline respiratory or heart rate. The Anthonisen exacerbation classification into type 1, 2 and 3 were defined on the basis of responsiveness to anti-microbial therapy (positive response in type 1 and 2 exacerbations). However there was no time inference for exacerbation definition in contrast to studies using diary cards.

The definition proposed by the European Respiratory Taskforce in 1995, did not define an exacerbation *per se* but reported that the causes of an acute exacerbation could be

classified into those that were primary (trachea-bronchial tree infection) or secondary (which non-exhaustively included pulmonary embolism, pneumonia, pneumothorax, cardiac failure and gastro-intestinal bleeding) (Siafakas, 1995). This definition assumed that any alternative or additional co-morbidity would likely drive an increase in symptoms present at stable state and thus included in the definition of an acute exacerbation of COPD.

The current GOLD definition of an exacerbation is as follows (Rabe, 2007):

'an event in the natural course of the disease characterised by a change in the patients baseline dyspnoea, cough, and/or sputum that is beyond normal day-to-day variations, is acute in onset, and may warrant a change in regular medication in a patient with underlying COPD'. This definition was in part derived from the Rodriguez-Roisin definition presented at the turn of the 21st century which incorporated the concept of natural fluctuations in the disease time course and the need to seek medical attention (Rodriguez-Roisin, 2000).

The definition by Rodriguez-Roisin was as follows:

'a sustained worsening of the patient's condition, from the stable state and beyond normal day-to-day variations, that is acute in onset and necessitate a change in regular medication in a patient with underlying COPD'. Rodriguez-Roisin also proposed a generalised sub-classification upon healthcare utilisation and defined this as 'Mild – increased need for medication with which they are able to manage in their own home environment'; 'Moderate – an increased need of medication but medical assistance is sought'; and 'Severe – necessitation of hospitalisation'. Both the Rodriguez-Roisin and GOLD definitions encompass both event-based and symptom-based exacerbations.

Event-based exacerbations are those that result in patient and medical initiated healthcare utilisation whilst symptom-based definitions can also capture milder, unreported exacerbations which have been reported to occur in up to 50% of exacerbations analysed by diary cards (Seemungal, 1998; Seemungal, 2000; Langsetmo, 2008).

Despite efforts to establish a universal respiratory community consensus to define exacerbations, these remain subjective. The affected individual's perception of their symptoms and the medical providers' decision will influence the diagnosis of an exacerbation. Disease severity is also likely to confound the diagnosis of an exacerbation because small variations in symptoms in severe disease are likely to trigger significant changes in symptom reporting and the subsequent initiation of therapy. Additionally complex social and psychological problems associated with chronic disease and the perceptions of disease are likely to strongly influence patients' use of medical services. At present there is a lack of an objective tool to accurately diagnose or direct treatment during an exacerbation of COPD and limitations remain in independently defining exacerbations.

1.6. The aetiology of exacerbations

The role of bacteria

Despite being the most frequently detected pathogens the role of bacteria during exacerbations of COPD remains controversial. In the pathogenesis of exacerbations it has been proposed that bacteria could cause a primary infection leading to an exacerbation or that this event could occur secondary to infection by a virus; both scenarios will lead to increased inflammation. The 'British' hypothesis (MRC report,

1965) embodied the importance of bacteria in the pathogenesis of COPD with chronic mucus secretion and plugging leading to recurrent infections and in turn further airway obstruction. However the counter argument that airway hyper-responsiveness and atopy were indeed a prerequisite for the development of COPD was termed the 'Dutch Hypothesis' (Orie, 1961) and the identification of pathogenic bacteria in stable COPD, has led to some conclusions that bacteria were merely epiphenomena. In addition to the difficulty in defining the role of bacteria during exacerbations are the methods available for their detection, culture and quantification. Furthermore, a unified definition for a bacterial exacerbation, the indication for antibiotic therapy, the designation of a positive clinical and microbiological response, implying causality of bacteria during COPD exacerbations is lacking.

The most commonly used method to detect and quantify bacteria relies on standard culture techniques and semi-quantitative colony forming units (CFU). A study by Stockley *et al* identified pathogenic bacteria in approximately 50% of subjects during outpatient exacerbations using standard culture and CFU. In this study, sputa from 121 moderate exacerbations were examined. The macroscopic description of sputum into mucoid, mucopurulent and purulent was shown to correspond with the presence of pathogens and bacterial load. Only 10% of mucoid samples compared to 90% of mucopurulent/purulent samples were found to have a CFU count $>10^7$. In almost 95% of mucoid exacerbations there was resolution of symptoms without antibiotic therapy (Stockley, 2000). Severe exacerbations requiring hospitalisation have consistently shown that bacteria on standard sputum culture are identified in 50 to 80% of samples (Miravitlles, 1999; Papi, 2006; Seemungal, 2008; Daniels, 2010).

However, a major limitation during sputum sampling is contamination from the oropharynx. To overcome this alternative sampling methods during exacerbations have been investigated. In outpatient COPD exacerbations Monso *et al* (Monso, 1995), used the protected specimen brush (PSB) to quantify bacterial infection during 29 out-patient exacerbations and compared this to 40 subjects at stable state. They determined that 52% of exacerbations had a potentially pathogenic micro-organism (PPM) using PSB culture. Additionally the total bacterial load was higher during an exacerbation. In a study examining hospitalised COPD exacerbations Soler *et al* determined that 45% of subjects had a PPM at exacerbation with a bacterial load of CFU $> 10^2$ using the PSB. Sputum culture had a lower detection rate of 35% compared to the PSB method but there was concordance between the two methods (Soler, 2007).

The occurrence of pathogenic bacteria in 30% of subjects at stable state has led to the hypothesis that a change in bacterial strain change is more likely to evoke an exacerbation (Rosell, 2005). In work by Sethi *et al*, molecular typing of sputa at stable and exacerbations determined that acquisition of a new pathogenic strain was associated with an increased risk of exacerbations (Sethi, 2002), whilst bacterial load was not elevated unless there was a detected strain change (Sethi, 2007). However studies by Sethi *et al* have not looked at changes in airway inflammation or the presence of co-infection, but does highlight that changes in the host-pathogen equilibrium is unlikely to occur spontaneously.

The role of viruses

Modern molecular techniques have improved virus detection leading to an enhanced understanding of the role of viruses in COPD exacerbations. Older culture techniques

were time consuming and required the presence of live virus within the sample. Serological testing is limited by the host immune response to virus presentation. Newer molecular methods have been sought to investigate the role of viruses during exacerbations. Reverse transcriptase polymerase chain reaction (RT-PCR) has been used to identify viruses during exacerbations of asthma and have highlighted that viruses have a significant role and were associated with deterioration in respiratory function and symptoms (Nicholson, 1993; Johnston, 1995). Different methods of upper respiratory tract sampling (nasal swabs, aspirate, lavage, and brush) have shown no differences in virus detection using RT-PCR (Spyridaki, 2009). In particular sputum sampling has not been found to be inferior compared to nasal lavage to detect viruses (Seemungal, 2000; Rohde, 2003).

The virus detection rates during out-patient exacerbations of COPD have ranged between 20% and 60% (Seemungal, 2000; Seemungal, 2001; Wilkinson, 2006; Quint, 2010); detection rates are higher during severe hospitalised exacerbations (Rohde, 2003; Tan, 2003; Papi, 2006). Variations in detection rates between studies are likely to represent differences in sample timing, seasonal variation and polymerase chain reaction (PCR) threshold quantification. Rhinovirus (RV) is the major cause of the 'common cold' and is the most frequently occurring virus in acute exacerbations. Influenza, adenovirus and respiratory syncytial virus have all been detected during exacerbations of COPD (Seemungal, 2001). Detection of RV during an exacerbation has been shown to have worse outcomes compared to exacerbations in which RV was not identified. Wilkinson *et al* studied 39 COPD subjects with paired stable and prospective out-patient exacerbation sampling and showed that RV was detected in 20% of exacerbations and was associated with the greatest decline in lung function

(Wilkinson, 2006). A symptom of a 'cold' recorded on daily diary cards during an exacerbation was associated with an increase recovery time (Seemungal, 2000).

The role of airway inflammation

The inflammatory process in COPD is accentuated during exacerbations. As previously discussed, neutrophils respond to infection and inflammation representing an important response in innate immunity. This augmentation has an unclear role during exacerbations. Studies have demonstrated an increase in neutrophilic inflammation not attributed to bacteria or virus (Bhowmik, 2000; Papi, 2006). The increased neutrophilic response is also demonstrated with increased levels of TNF-α, GM-CSF and IL-8 and the neutrophil activation products neutrophil elastase (NE) and MPO (Hill, 2000; Aaron, 2001; Gompertz, 2001; Qiu, 2003). This neutrophilic response is related to microbial load (Hill, 2000), with MPO causing a macroscopic change in the sputum colour (Stockley, 2000).

The eosinophil is considered a marker of allergy and asthma and its role in stable COPD is controversial. However, eosinophils have consistently been found to be elevated in the sputum, BAL and tissue during exacerbations (Saetta, 1994; Saetta, 1996; Bathoorn, 2009). The increased detection of eosinophils during exacerbations of COPD has not been conclusively attributed to the total cell increase during exacerbations. The finding that there is increased eotaxin and RANTES (Saetta, 1994; Saetta, 1996; Zhu, 2001; Bocchino, 2002) and increased levels of activation with higher concentrations of ECP during exacerbations (Keatings, 1997; Fiorini, 2000; Rohde, 2004) would suggest that eosinophilic airway inflammation in COPD exacerbations is important. Furthermore, reduction in eosinophilic inflammation during stable COPD has been repeatedly shown

to be associated with improvements in important clinical outcomes such as health status, lung function, exercise capacity and reduction in exacerbation frequency (Pizzichini, 1998; Brightling, 2000; Brightling, 2005; Siva, 2007). The presence of a sputum eosinophilia has been proposed to be a sensitive and specific marker of virus-associated exacerbations of COPD independent of bacterial co-culture (Papi, 2006), whilst direct virus challenge testing in asthma and healthy controls has demonstrated elevated levels of epithelial eosinophils (Fraenkel, 1995).

1.7. Treatment of exacerbations

Current guidelines advocate the use of oral corticosteroids and antibiotics for patients with COPD exacerbations. However, the clinical response to treatment varies considerably, the supporting evidence is not conclusive and these therapies have a significant side effect profile. There is currently no reliable method to target therapy, which often results in excessive and inappropriate treatment placing a vulnerable population at further risk.

Systemic corticosteroid therapy

The hypothesis that corticosteroids would be beneficial in COPD exacerbations arose from improvements attained in treating bronchial asthma. The widespread use of corticosteroids as standard therapy during exacerbations has been supported by several randomised controlled trials (RCT) in COPD summarised by a Cochrane systematic review (Walters, 2009).

In an initial review of studies performed between 1950 and 1970, Sahn showed that there was inconsistency in objective improvement from corticosteroid therapy use

during exacerbations and overall study designs were poor. This review concluded that corticosteroids were not beneficial in subjects with COPD, with the caveat that after failure of maximal bronchodilatation, corticosteroids should be considered (Sahn, 1978). The first use of corticosteroids in COPD was presented in 1951 on 4 subjects (Lukas, 1951). In this small observational study the equivalent of 40mg of prednisolone was given for 1 month and showed improvement in vital capacity (VC) in 3 subjects. In a study by Bickerman *et al*, subjective improvements were found in 58% of emphysema subjects given prednisolone for 2 months. Lung function data was only available in 30% of subjects (n=15) and showed a mean increase in VC (Bickerman, 1955). Although not a placebo control study, Franklin *et al* determined that there was an improvement in FEV_1 with prednisolone compared to bronchodilator therapy alone (11% vs. 4%) with further additional benefits when in combination (Franklin, 1958). One of the first controlled trials in hospitalised exacerbations of chronic bronchitis examined the effect of corticotrophin administration versus standard therapy in 24 subjects (Felix-Davies, 1956). In this study, no benefits in physiologic testing (maximum voluntary ventilation) or differences in mortality were found. Within this study, patient selection included subjects with predominant clinical bronchiectasis, there was no power calculation performed for mortality and there was no standardisation performed for additional interventions at hospitalisation.

The earliest RCT was a double blind randomised prednisolone cross-over study, performed in 10 subjects with emphysema. This demonstrated that there was no improvement in VC in 8 out of 10 subjects taking prednisolone (Beerel, 1963). However 2 subjects behaved atypically to the rest of the population with marked improvements in VC. In this study by Beerel *et al*, it is worth noting that the population

studied were clinically and physiologically described as emphysematous and enrolled during stable disease. However there is no further description of the 2 responsive subjects and how they differed from the non-responsive population. In particular there is no reference to a childhood history of asthma or whether eosinophilic inflammation was measured in this population.

These earlier studies were small, with a varied inclusion of diagnoses, different methodologies and were performed at stable state. More recently, improvements in study design with larger RCTs have defined the use of corticosteroids worldwide. From the mid-90's work has been undertaken to investigate the use of intravenous or oral corticosteroids, in moderate and severe exacerbations and optimal treatment duration. The first RCT to study prednisolone or placebo therapy in out-patient COPD exacerbations was performed by Thompson *et al* (Thompson, 1996). FEV_1 was found to improve significantly during prednisolone therapy with a mean difference between groups of 50mL. There was a trend to improvement in symptoms of breathlessness and a reduction in treatment failure. An exacerbation was defined as an acute worsening dyspnoea or cough for 24 hours that necessitated a hospital visit. This study was not powered to infer benefits in healthcare outcomes and more patients in the placebo group were on maintenance triamcinolone, a long acting corticosteroid, which may have biased the final outcomes when comparisons from baseline were performed. Indices of eosinophilic inflammation were presented for baseline only with increased peripheral blood eosinophils in the prednisolone group; which may be an unrecognised corticosteroid responsive sub-group.

Three large studies investigating outcomes in hospitalised exacerbations of COPD (Davies, 1999; Niewoehner, 1999; Maltais, 2002) showed that the use of prednisolone compared to placebo was associated with an early improvement in FEV_1 of 100mL (Niewoehner, 1999) 160mL (Maltais, 2002) and 185mL (Davies, 1999). Following completion of treatment there were no differences in FEV_1 between the study or control group in any of the studies. In an intention to treat analysis, Davies *et al* showed that prednisolone reduced the length of stay from 9 to 7 days (Davies, 1999), whilst Niewoehner *et al* showed that initial length of stay (associated with the COPD exacerbation) was reduced from 9.7 to 8.5 days in the prednisolone group. However, due to diagnoses other than COPD there was a total increase in hospital stay by 3.2 days in the prednisolone treated group (Niewoehner, 1999).

In the study by Davies *et al* the peripheral blood eosinophil count on admission to hospital was twice as high in the placebo compared to the prednisolone group. This finding would account for the differences in prednisolone or placebo responses. The statistical method used to determine reduction in length of stay between the prednisolone and placebo was the inferior Mann-Whitney test (Davies, 1999). The Veteran Affairs study by Niewoehner *et al*, evaluated two different durations of prednisolone therapy compared to placebo in hospitalised exacerbations of COPD. The primary outcome was defined as equivalence in treatment failures between prednisolone and placebo treatment. The study treatment failure rate was 50%, but the primary outcome for either dose duration of prednisolone over placebo was not observed. Adverse events were found to be significantly higher in subjects treated with prednisolone compared to placebo with hyperglycaemia the most frequently occurring adverse event (Niewoehner, 1999).

The largest out-patient COPD exacerbation RCT of prednisolone was performed by Aaron *et al* in 2003 (Aaron, 2003). The primary outcome was the rate in treatment failure in 147 COPD patients experiencing an exacerbation of COPD that warranted treatment but not admission. There was a non-significant trend to treatment failure reduction at 30 days in the prednisolone group compared to placebo (27% vs. 43%, p=0.05). Lung function improved in both groups but significantly more so in the prednisolone group (35% vs. 15%), but there was no associated improvements in disease reported health status. This study was not powered to identify significant differences in mortality.

A comprehensive systematic Cochrane Review has been performed to determine the efficacy of systemic corticosteroid therapy during acute exacerbations of COPD (Walters, 2009). This review studied published RCTs and standardised interventions such as antibiotics, theophylline and bronchodilator therapy. Eleven reported RCTs studies were identified. Three studies were published in abstract form only (Rostom, 1994; Wood-Baker, 1998; Chen, 2005) and data that could be used was received from Wood-Baker *et al* and Chen *et al* only. The findings of this review from 10 RCTs of approximately 1000 COPD patients demonstrated a consistent improvement at early time points (72 hours) for FEV_1, symptoms of breathlessness and short term treatment failure rates. However, at later time points, the benefits were negated and there were no mortality benefits. The Cochrane review concluded that there was significant variation in the definition of an exacerbation and inconsistencies in corticosteroid dose and duration. The use of corticosteroids was consistently found to be associated with adverse events and the number need to harm (NNH) was 5; patients treated with prednisolone were 5 times more likely to develop hyperglycaemia. Data from RCTs of

corticosteroid use during exacerbations including those analysed in the Cochrane review are summarised in a table in Appendix 1.

To conclude it is evident that the current literature shows heterogeneity in responses to corticosteroids during exacerbations of COPD, limited by the variation in study design and definition of an exacerbation. Although there are improvements in lung function, reductions of treatment failure and length of hospital stay, the data does not identify those that are responders and non-responders, an important distinction to reduce risk of harm in a vulnerable population. Efforts to identify those that are likely to have benefits from corticosteroids will have significant impact upon management of COPD exacerbations.

Antibiotic therapy

Antibiotic therapy should be given for treatment of a bacterial infection. However, defining a bacterial infection during an exacerbation can prove to be difficult whilst the widespread use of antibiotic therapy is responsible for microbial resistance and antibiotic related infections. Evidence from a systematic Cochrane review has advocated the use of antibiotic therapy if there is an increase in sputum purulence or sputum production (Ram, 2006). However the efficacy for antibiotic treatment during exacerbations is not clear, with confounding factors such as standardisation of concurrent treatment, mode of delivery of medication and the associated use of corticosteroids.

The use of antibiotic therapy during exacerbations was initially re-visited in a double-blind randomised cross over study performed by Anthonisen *et al* in 1987. The evidence

supporting a benefit for antibiotics during COPD exacerbations up to this point had not been conclusive (Tager, 1975). In this study 173 COPD subjects were enrolled over 3.5 years and antibiotic or placebo therapy was given at the onset of an exacerbation for 10 days and any further exacerbation events were given cross-over therapy. Exacerbations were graded according to Anthonisen symptom criteria; Type 1 if there was dyspnoea, sputum production and sputum purulence; Type 2 if two out of three of these major symptoms were reported; and Type 3 if one of these major symptoms were reported in addition to at least one of either upper respiratory infection within 5 days (sore throat or nasal discharge), fever without alternative cause, increased wheeze, increased cough, or increase in respiratory or heart rate by 20% to that above the recorded baseline respiratory or heart rate. Although no power calculations were performed, the primary outcome was defined as treatment success. Antibiotics compared to placebo were associated with successfully treated type I and type II exacerbations, with the main driver of improvement occurring in Type I exacerbations. No differences in the duration of the exacerbation were detected. Despite the limitations in study power, the application of the exacerbation severity based on the original microbiological response, this data established the use of antibiotic therapy for type I and -II exacerbations of COPD (Anthonisen, 1987).

A RCT by Nouira *et al* investigated the efficacy of antibiotics in severe exacerbations, specifically requiring mechanical ventilation. Subjects were given oral antibiotics or placebo therapy for 10 days. Corticosteroid therapy was not given. The study showed that antibiotic therapy reduced mortality; the need for further antibiotics and the length of mechanical ventilation. The study methodology did not differentiate between non-invasively or invasively ventilated subjects (Nouira, 2001). However, the findings

demonstrated greater benefit of antibiotic therapy in a more severe population suggesting a study bias.

To investigate the benefits of antibiotics in addition to oral corticosteroids in severe exacerbations a recent placebo controlled RCT by Daniels *et al* was performed. In this study 250 exacerbations were investigated. All patients were treated with bronchodilator therapy and oral corticosteroids and were then assigned to receive 7 days of either doxycycline or placebo. The primary end-point was defined as the resolution of symptoms at day 30. Power calculations to detect a difference of 15% in treatment success between antibiotic and placebo therapy for Anthonisen type I and –II exacerbations required a minimum of 264 exacerbations to be studied. Statistical power was not reached and the primary outcome of the study was negative; the authors concluded that in severe hospitalised COPD exacerbations doxycycline was not superior to placebo. Sub group analysis failed to identify a benefit of doxycycline therapy on FEV_1 or a treatment response in subjects with a PPM positive sputum bacterial culture. However, a C reactive protein (CRP) level above 50 mg/L was associated with a positive treatment effect in the antibiotic arm compared to placebo (Daniels, 2010).

The role of antibiotics was further analysed by Rothberg *et al* in a large retrospective meta-analysis. In this study 85,000 patients admitted with a COPD exacerbation in 400 centres were studied to assess the effects of early antibiotic therapy on treatment failure; defined as death, invasive ventilation and re-treatment. Almost 80% were in the early antibiotic treatment group. The results showed that there was a reduction in the mortality and invasive ventilation rates and a reduction in treatment failure by 13% (Rothberg, 2010). However limitations in study methodology include selection bias,

inadequate study design to analyse the primary outcome and to investigate the potential relevance of early versus late (day 3) treatment.

The Cochrane review, has demonstrated that antibiotic therapy during an exacerbation was associated with short-term benefits in mortality (RR 0.23; 95% CI 0.10 to 0.52) and treatment failure (RR 0.47; 95% CI 0.36 to 0.62); however there was an increased risk of antibiotic associated diarrhoea (Ram, 2006). This systematic review was diverse in patient inclusion, clinical settings, treatment prescriptions and non-standardisation of concomitant therapy. A modest 900 patients from ten RCTs were included. The inclusion of the Nouira study which was set in ICU was solely responsible for the significant outcome of reductions in short term mortality and therefore may be responsible for bias (Nouira, 2001). Exclusion of the Nouira study in the review resulted in a non-significant mortality benefit of antibiotics during COPD exacerbations with a RR of 0.26 (95% CI 0.07 to 1.01). A summary of the included studies are presented as a table in Appendix 2.

Of the 10 RCTs in the Cochrane review, only 6 specifically commented on treatment failure. Analysis of all 6 studies resulted in statistical heterogeneity which precluded the combined analysis. Therefore only 4 of these 6 studies were included in the main statistical analysis favouring antibiotic therapy. When all 6 studies were included in the analysis, it was demonstrated that antibiotics were not superior to placebo in treatment failure reductions (RR 0.91; 95% CI 0.70 to 1.18). Another meta-analysis by Puhan *et al* concluded that in mild to moderate exacerbations of COPD there was no benefit in antibiotic treatment over placebo (Puhan, 2007). These results suggest that in an outpatient population antibiotic therapy may not be beneficial.

Despite the available meta-analyses and the RCTs, the role of antibiotic therapy during exacerbations of COPD remains unclear. In those studies that reported side effects, (Nouira, 2001; Rothberg, 2010; Daniels, 2010) there was an increase in the rate of antibiotic related diarrhoea, although no attempt has been made to assess the impact of *Clostridium difficile* infection in this population. Several studies have shown that antibiotics are of benefit to patients hospitalised with COPD exacerbations. However, no clear benefit has been demonstrated in out-patients with mild to moderate exacerbations. These conclusions have not been based on Anthonisen symptom criteria; however these symptom criteria remain a major driver for antibiotic therapy in present clinical practice. This illustrates a failure to translate research findings into clinical practice and demonstrates the lack of validated tools to identify patients who will benefit from antibiotic therapy. Similar findings have been identified in corticosteroid trials. Studies have included different methodologies and both heterogeneous therapies and outcome markers making combined analysis difficult. There has been a lack of definitive markers of treatment response in the studies. Additionally, studies investigating antibiotic therapy have often lacked longitudinal data with no sputum culture in the stable state. Tools to objectively identify patients who will respond to antibiotic or corticosteroid therapy during COPD exacerbations are currently lacking, but are urgently sought for both research and clinical purposes.

1.8.   The role of biomarkers during exacerbations

The National Institute of Health biomarker definitions working group (Atkinson, 2001) proposed that a biomarker should be defined as '*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*'.

The application of biomarkers ranges from diagnostic capabilities, disease prognostic indicators and therapeutic responsiveness. Successful biomarkers will need to show stability, repeatability, modification to reflect disease processes and to be validated in similar disease cohorts. COPD demonstrates diversity in clinical, pathological and physiological processes; furthermore COPD exacerbations are varied with respect to aetiology, inflammation and a lack of robust evidence supporting treatment regimes. The identification of biomarkers that relate to clinical sub-types of COPD and COPD exacerbations are likely to have significant beneficial impacts on scientific and clinical practices.

Large longitudinal COPD studies have investigated biomarker panels during stable state and exacerbations. In an observational study Hurst *et al* measured 36 plasma biomarkers in paired stable and exacerbation samples (Hurst, 2006). They were unable to determine a sensitive or selective biomarker for COPD exacerbations. CRP in addition to a major exacerbation symptom had the highest area under the curve to determine an exacerbation; a similar finding to that observed by Bozinovski *et al* (Bozinovski, 2008). The 'Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints' study (ECLIPSE), investigated a wide range of biomarkers in over 2000 COPD subjects and healthy controls for a duration of 3 years (Vestbo, 2008). Biomarkers were measured at stable state and were functional, physiological, radiological, genetic and biological. The study aimed to define the heterogeneity of COPD and to determine markers of disease progression. Cross sectional examination of this data to date has determined that the clinical manifestations of COPD are variable (Agusti, 2010) and have confirmed that in patients with a history of exacerbations there is an increased risk of future exacerbations (Hurst, 2010). The COPDGene study is a currently active large

multi-centre observational study identifying genetic biomarkers associated with COPD (Regan, 2010). Susceptibility genes will be investigated in a series of characterised COPD subjects and healthy smokers with genome-wide association studies to be performed to identify genetic variants in COPD.

1.8.1. Biomarkers to guide systemic corticosteroid therapy

Eosinophils

Sputum eosinophils are most widely recognised as an indicator of corticosteroid responsiveness in airway diseases. This has been extensively studied in asthma, where the presence of eosinophils has been shown to confer corticosteroid responsiveness (Little, 2000). The targeted reduction of sputum eosinophils has been shown to decrease exacerbations (Green, 2002) whilst blockade with an anti-IL-5 antibody reduced exacerbation frequency (Haldar, 2009) and corticosteroid burden (Nair, 2009) in severe asthma.

The earliest study to demonstrate that a sputum eosinophilia confers corticosteroid responsiveness in subjects with COPD was performed by Shim *et al*. Here subjects with a significant smoking history, respiratory symptoms consistent with chronic bronchitis and spirometrically confirmed airflow obstruction were entered into a randomised placebo controlled cross-over study (Shim, 1978). Sputum eosinophil count was classified as either rare, clumps or elevated. Both an elevated sputum eosinophil count and the presence of clumps were found to be predictive of a bronchodilator FEV_1 response following 7 days of 30mg of prednisolone. Pizzichini *et al*, in a single blind placebo controlled crossover study investigated whether a sputum eosinophilia of $\geq 3\%$ could predict an improvement in FEV_1 and disease specific health quality in COPD

(Pizzichini, 1998). A cut off of 3% was derived from results obtained in their earlier work (Pizzichini, 1997). Following prednisolone therapy there was a mean FEV_1change of 0.11 L and 0.01 L in the eosinophilic and non eosinophilic COPD subjects respectively. An improvement in quality of life (QoL) and dyspnoea symptoms was also found in the eosinophilic group following prednisolone therapy. Furthermore a study of patients with radiological emphysema by Fujimoto *et al* demonstrated that the baseline sputum eosinophil count correlated with the FEV_1 response to systemic corticosteroid treatment (Fujimoto, 1999). Finally, Brightling *et al* confirmed and replicated these findings in a larger double blind placebo controlled cross-over trial and found that after stratification of the whole group into tertiles, the greatest benefit occurred in subjects with the highest degree of eosinophilia (Brightling, 2000).

These studies have demonstrated that a sputum eosinophilia indicates a positive corticosteroid response to important clinical outcomes in COPD patients. However, these studies have only investigated the corticosteroid response in the stable state. This association has not been studied during exacerbations.

Nitric oxide

The fraction of exhaled nitric oxide (FE_{NO}) is a measurement of nitric oxide (NO) produced by cells in the large and peripheral airways. Endogenous NO is generated by three isoenzymes of NO synthase (NOS) from the substrate L-arginine. Endothelial NOS (eNOS) is an important regulator of blood flow in the endothelial bronchial and circulatory cells; neuronal NOS (nNOS) is an antagonist of acetylcholine; and inducible NOS (iNOS) is stimulated by inflammation (Barnes, 2010). Measurement of FE_{NO} is non-invasive and has been found to be a surrogate marker of eosinophilic airway

inflammation (Berry, 2005). FE_{NO} has been measured in stable COPD (Corradi, 1999; Papi, 2000; Rouhos, 2011) and shown to be reproducible over 24 hours (Rouhos, 2011). Active smoking status is associated with lower levels of FE_{NO} (Rytila, 2006; Louhelainen, 2009) and is negatively correlated with smoking pack year history (Corradi, 1999). During exacerbations of COPD FE_{NO} has been shown to be elevated compared to the stable state (Kersul, 2011) and associated with important clinical outcomes such as length of hospital stay (Antus, 2010). Furthermore during the stable state in COPD, a partial reversal of airflow obstruction in response to inhaled corticosteroids was found to correlate with FE_{NO} (Papi, 2000) and an elevated baseline FE_{NO} was predictive of a response in FEV_1 to inhaled corticosteroid (Kunisaki, 2008). However changes in FE_{NO} levels in response to systemic corticosteroid therapy have not been assessed during COPD exacerbations.

1.8.2. Biomarkers to guide antibiotic therapy

Sputum purulence

Sputum purulence is the most well established clinical biomarker with the strongest statistical evidence to predict bacterial infection during exacerbations of COPD. Neutrophilic inflammation leads to production of MPO which results in a macroscopic sputum colour change. The purulence has been shown to be both selective and sensitive for positive bacterial culture and increased bacterial load (Stockley, 2000; Soler, 2007). A standardised sputum colour chart was found to correlate strongly with levels of sputum IL-8 and MPO in a dose dependant manner (Stockley, 2001). However, the presence of pathogenic bacteria in the stable state may confound the use of sputum purulence as an independent biomarker.

C reactive protein

CRP was first identified in 1930 and believed to be a non somatic form of a pneumococcal protein. In their original description Tillett and Francis (Tillett, 1930) demonstrated that a molecule could be detected in high levels from the sera of patients with a bacterial infection. This molecule was subsequently named CRP and was found to be increased in patients with fever and pneumococcal infection but the levels decreased in association with a successful clinical response. A persistently elevated CRP was identified in patients that failed to respond to treatment and died from pneumococcal infection.

CRP is produced in the liver during acute infection, inflammation and tissue damage under the influence of IL-6 (Pepys, 2003). However it is non-specific marker. CRP levels also increase with age, cardiovascular mortality and the metabolic syndrome (Chambers, 2001; Frohlich, 2000; Pepys, 2003; Danesh, 2004). Compared to healthy controls, CRP was found to be increased in stable COPD (Mannino, 2003; Gan, 2004; Broekhuizen, 2006; Pinto-Plata, 2006). This was associated with important clinical outcomes including disease severity, exercise capacity and mortality (de Torres, 2006; de Torres, 2008). The potential to use CRP as a biomarker for bacterial infection in COPD is confounded by increased levels in the stable state. Nonetheless, during exacerbations associated with sputum purulence or those in which a PPM was identified the CRP levels were increased (Stockley, 2000). Following antibiotic treatment the CRP concentration was shown to fall irrespective of the identification of a PPM on standard culture during an exacerbation (Dev, 1998).

Procalcitonin

Procalcitonin (PCT) is a sensitive marker of bacterial sepsis, reaching peak levels in serum 6-12 hours after infection. A study in patients in intensive care identified that PCT was superior to both IL-6 and IL-8 in identifying patients with either sepsis or Systemic Inflammatory Response Syndrome (SIRS) (Harbarth, 2001). Several studies have demonstrated that PCT can be used as a biomarker to guide antibiotic therapy in lower respiratory tract infections (Christ-Crain, 2004; Christ-Crain, 2006; Schuetz, 2009). In the first of these studies by Christ-Crain *et al* patients with symptoms and signs of an acute lower respiratory tract infection were randomised to either standard treatment or PCT guided treatment (Christ-Crain, 2004). Here a PCT level above 0.25ng/mL was used to guide antibiotic therapy. There was a significant reduction in antibiotic prescriptions in the PCT guided arm, without an increase in adverse events or treatment failures. Two further studies exclusively in COPD patients have also used PCT to direct antimicrobial therapy; these have both demonstrated a reduction in antibiotic prescriptions (Christ-Crain, 2006; Stolz, 2007). Interestingly these findings have been replicated in a large multicentre non inferiority study with over 1000 subjects presenting to hospital with an acute lower respiratory tract infection (Schuetz, 2009).

1.8.3. Biomarkers to guide anti-viral therapy

There are currently no anti-viral therapies recommended for the treatment of COPD exacerbations, however potential drug treatments are in development. The identification of a specific biomarker for virus induced COPD exacerbations would be beneficial both for the development of anti-viral therapies and in guiding treatment of exacerbations in the future.

Sputum eosinophils

In a study investigating the role of infection in severe exacerbations of COPD Papi *et al* determined that the absolute sputum eosinophil count was the most sensitive and specific marker of a virus related exacerbation (Papi, 2006). However in a RV challenge model of COPD exacerbations, there was no increase in the percentage sputum eosinophil count identified (Mallia, 2011). This may reflect a milder COPD population who were skin prick negative, but there was no data provided on the absolute sputum eosinophil counts.

Interferon-γ inducible protein

Interferon-γ inducible protein (IP10/CXCL10) has been shown to be specific for RV infection in both *in-vitro* and *in-vivo* studies (Spurrell, 2005). IP10 was found to be elevated in exacerbations of COPD (Quint, 2010) and asthma (Wark, 2007) associated with RV. This may represent a potential biomarker for this important group of viruses.

1.9. Defining COPD heterogeneity using phenotypes

As discussed previously COPD and exacerbations are heterogeneous in nature. There is considerable overlap in the diagnoses of emphysema, chronic bronchitis and small airway obstruction associated with diversity in inflammation and response to treatment. Currently COPD is defined by symptoms with obstructive spirometry associated with the progressive loss of FEV_1 over time; however these measurements are not usually made at clearly defined intervals. This method of diagnosis may not be able to integrate the complex pathological changes that occur within the vasculature, parenchyma and

the large and small airways. Outcomes of clinical trials have been based on reversing the progressive decline in FEV_1 seen in COPD and have largely been unsuccessful. It is likely that improvements in defining the heterogeneity of COPD will aid understanding of disease processes, pathological mechanisms and potential therapeutic strategies.

A potential alternative approach to defining COPD would be based on the classification of COPD phenotypes and an objective method to define these would be on the basis of phenotype specific biomarkers. The classification of a phenotype is based on groups with similar characteristics and classically is defined as an '*observable characteristic of an organism as determined by genetics and environmental influences*'. There are however limitations with this definition of a phenotype; specifically there is no differentiation based on specific disease characteristics. The definition is generic and difficult to translate to phenotypes that are likely to exist in COPD and exacerbations. An alternative phenotype definition in COPD was proposed by Han *et al* (Han, 2010) and defined as a '*single or combination of disease attributes that describe differences between individuals with COPD as they relate to clinically meaningful outcomes, such as symptoms, exacerbations, response to therapy, rate of disease progression, or death*'.

One of the earliest concepts to provide characterisation of phenotypes in COPD was the original description by Dornhorst of two extreme clinical phenotypes. These were the non-cyanotic dyspnoeic emphysematous patient with muscle wasting ('pink puffer') and the cyanotic congestive chronic bronchitic with right heart failure ('blue bloater') (Dornhorst, 1955). Further work by Filley *et al* observed that there was a cohort of subjects that reported breathlessness and weight loss as a predominant symptom whilst identification of heart failure was unusual. Conversely in other subjects the presence of chronic productive cough symptoms was often associated with secondary

polycythaemia and right heart failure (Filley, 1968). In their original study the investigators could only define one third of subjects from those screened as either 'pink puffers' or 'blue bloaters'. This illustrated that the majority of patients did not fit the newly defined phenotypes further emphasising disease heterogeneity in the population. Measurement of cardiac and physiological parameters in the study identified differences between the 'pink puffer' and 'blue bloater' groups. These included a reduced rate of oxygen delivery to tissues and reduced cardiac output during exercise in the 'pink puffer' subjects. These two pivotal studies were the first attempt to characterise phenotypes in COPD and provided a platform to relate to important clinical outcomes.

Recent studies have used unbiased mathematical methods in an attempt to characterise COPD and identify new phenotypes. These methods have used clinical variables to define COPD phenotypes; however there was significant overlap between individual groups limiting the utility of this approach.

Pistolesi *et al* studied approximately 300 COPD subjects with variable airflow obstruction and used symptoms, lung function and computed tomography to characterise COPD (Pistolesi, 2008). In this study the investigators identified 2 groups of patients. Group A had minimal cough and sputum production, were thinner and had radiographic changes predominantly showing parenchymal destruction consistent with emphysema. Group B patients reported chronic cough and had radiographic changes compatible with emphysema and bronchial wall thickening. Group A patients were described as an *emphysematous* clinical phenotype and Group B patients were termed a *bronchial* clinical phenotype. Further work by Weatherall *et al* used cluster analysis from clinical, physiological and radiological variables to phenotype a wide range of

airways disease including asthmatic and COPD patients. They identified three separate phenotypes; subjects with features of i) emphysema alone; ii) asthma, chronic bronchitis and emphysema overlap; and iii) chronic bronchitis symptoms in non smokers (Weatherall, 2009). In a study by Burgel *et al* cross sectional clinical data from a cohort of COPD subjects recruited into a national COPD study were analysed using principal component analysis. This method was used to reduce the number of entered variables and was followed by cluster analysis to investigate clinical COPD phenotypes (Burgel, 2010). In this study the investigators identified 4 clinical phenotypes; type 1 was composed of young subjects with severe airflow obstruction, frequent exacerbations, poor quality of life and a low body mass index (BMI); type 2 included older subjects, with mild airflow obstruction, infrequent exacerbations and normal or high BMI while type 3 and type 4 had moderate airflow obstruction and differed only by age, co-morbidities and symptoms.

These three studies all appear to identify phenotypes which reflect the original description of 'pink puffers' and 'blue bloaters' by Dornhorst. The use of statistical tools designed to overcome bias including cluster and factor analysis is likely to aid the identification of phenotypes. However the limitation of using only clinical and physiological characteristics resulted in identification of previously recognised sub-groups of COPD. The phenotypes described in the 21st century make a modest contribution to the field and differ minimally from those described by Dornhorst in the 1950's. In particular there is a paucity of literature with respect to phenotypes in acute exacerbations of COPD and studies using biological mediators including serum and sputum cytokines for example are urgently required.

The evidence for corticosteroids use during exacerbations of COPD is not conclusive and has not identified sub-groups which may benefit from this treatment. The evidence for antibiotics in COPD exacerbations indicates that antimicrobial therapy is beneficial in patients with increased sputum production and purulence. Exacerbations are typically self-reported and subjective events in which no satisfactory objective biomarker has yet been identified. Although likely to exist, exacerbation phenotypes have yet to be fully characterised. As illustrated in this introduction, there is considerable heterogeneity in clinical and aetiological presentations of exacerbations and furthermore evidence to guide specific therapy is lacking. At present no tool exists to predict which patients will respond to a specific therapy leading to an urgent need to identify phenotypes of COPD exacerbations and biomarkers to guide this therapy.

1.10. Study hypothesis

In this work the following hypotheses will be tested:

- That biomarkers measured in the sputum and serum of COPD patients can be used to accurately define individual phenotypes of COPD exacerbations and that these correspond to clinically important exacerbation phenotypes which reflect exacerbation aetiology, predominately associated with bacteria, virus or sputum eosinophil detection

- That the use of multivariate statistical techniques and the measurements of biomarkers during exacerbations of COPD can determine new phenotype characteristics of exacerbations independent of previously established clinical variables such as symptoms and lung function

- That where individual exacerbations phenotypes are identified, these can be predicted by the measurement of biomarkers in subjects during the stable of COPD

- That a biomarker can be identified and used effectively and safely to direct a treatment strategy during an exacerbation of COPD

2.   Methods

To investigate the stated hypothesis three independent studies were performed with individual inclusion and exclusion criteria. These criteria and a description of the study population are included in the relevant chapters. Parameters measured in all the studies are included in this methods section while those only performed in the separate studies are described in the corresponding chapter.

2.1.   Lung function

Lung function was performed in accordance with the joint American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines (Brusasco, 2005; Miller, 2005; MacIntyre, 2005; Wanger, 2005). Spirometry was performed on all visits with recordings obtained pre and post 400µg salbutamol bronchodilatation. The best out of three consecutive blows to record the FEV_1 and the FVC was then used.

2.2.   Sputum

Spontaneous or induced sputum was collected from subjects during visits throughout the study. Both methods of sample collection have been shown to be similar for the differential cell counts (Bhowmik, 1998).

2.2.1. Sputum induction protocol

In those subjects who were unable to spontaneously expectorate sputum, the following sputum induction protocol was performed. The procedure was fully explained with the following instructions:

- Guidance on posture: sit upright during the nebulisation procedure and lean forward during expectoration

- Guidance on effective expectoration: instructions for coughing and moving sputum successfully into specimen container

- Guidance on contamination reduction: instructions to blow nose and to rinse mouth prior to expectoration

The procedure requires all subjects to have FEV_1 measured before and after pre-treatment with 400μg inhaled salbutamol to minimise bronchoconstriction. Nebulised saline (5mL at 3, 4, and 5%) was given in sequence via an ultra-sonic nebuliser (UltraNeb, DeVilbiss, Sunrise Medical, USA) for 5 minutes. After each inhalation, subjects were asked to blow their nose and rinse their mouth prior to coughing and expectoration of sputum. FEV_1 was measured after each inhalation to assess for bronchoconstriction and to assess safety for procedure continuation. The process was terminated if there was a greater than 20% drop in FEV_1, significant symptoms or successful sputum expectoration. The sputum induction protocol used is shown in figure 2.1. In COPD subjects, sputum induction has been shown to be safe in subjects with a FEV_1 of $\geq$ 0.5 L (Brightling, 2001). All sputum samples were processed within 2 hours of collection in a Class II biological safety cabinet.

Figure 2.1; Sputum induction protocol

MEASURE FEV$_1$

↓

400μg inhaled or 2.5mg NEBULISED SALBUTAMOL

↓

MEASURE FEV$_1$

↓

ADMINISTER 3% NEBULISED SALINE FOR 5 MINUTES

↓

BLOW NOSE and RINSE MOUTH

SPUTUM
EXPECTORATED

↓

MEASURE FEV$_1$

STOP PROCEDURE

≥10%, <20% FALL in FEV$_1$

<10% FALL in FEV$_1$

≥20% FALL in FEV$_1$

OR SYMPTOMATIC

REPEAT WITH 4% and 5% SALINE
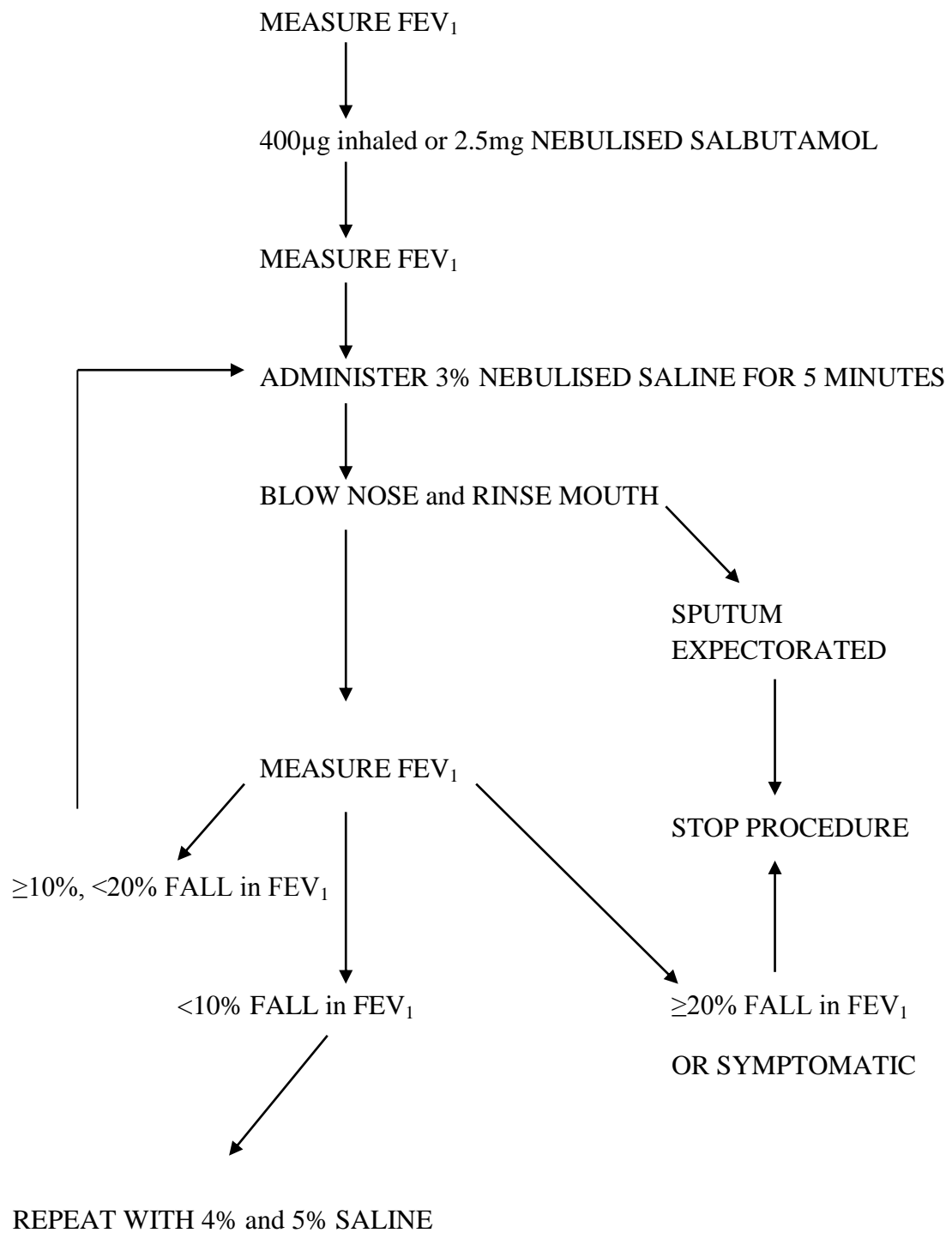
2.2.2. Sputum processing

The collected sputum sample was emptied into a petri dish and placed on a dark background to aid visualisation of sputum plugs. Sputum plugs were selected from the saliva and were then gathered into a large condensed mass in small circular movements. Sputum plugs were then removed for analysis of bacteria and virus.

The remainder of the selected sputum was weighed and incubated with 8 times the volume/weight of Dulbecco's phosphate buffered saline (D-PBS) (Sigma, Poole, Dorset). The sputum sample was dispersed by gentle aspiration into a Pasteur pipette and placed onto a bench rocker for 15 minutes on ice and then centrifuged at 790g for 10 minutes at $4\,^{o}$C. This was followed by removal of 4 times the volume/weight of this D-PBS supernatant with storage in 300μL aliquots at -80 ^{o} C for further mediator analysis. The remainder of the D-PBS sputum suspension was incubated with 0.2% dithiothreitol (DTT) (Sigma, Poole, Dorset), placed on a bench rocker on ice for 15 minutes and filtered through pre-wet 48μm gauze. 100 µL of this filtrate was removed for quantification of colony forming units and an additional 500 µL was removed for bacteria quantitative real time PCR (qPCR) analysis. A further 10μL of the filtrate was removed to assess the total cell count and cell viability using a Neubauer haemocytometer. The haemocytometer was flooded with 10μL of the filtrate mixed with 10μL of 0.4% trypan blue (Sigma, Poole, Dorset) and all cells were counted in the four corner squares of the haemocytometer to include viable, non-viable and squamous cells.

The total number of cells and total cell counts were calculated using the following formula:

- Total number of cells (x10^6) = [mean number of cells counted/square × 2 × filtrate volume (mL)] / 100

- Total cell count (x10^6/g sputum) = [mean number of cells counted/square × 2 × filtrate volume (mL)] / 100 × selected sputum weight

The remainder of the filtrate was then further centrifuged for 10 minutes at 790g at 4 ^{o} C. The DTT supernatant was removed into 300μL aliquots and stored at -80 ^{o} C for further mediator analysis.

Following DTT supernatant removal, the cell pellet was re-suspended in a small volume of D-PBS and adjusted to make a cell suspension of 0.50 – 0.75 x10^6 cell/mL with D-PBS for cytospin preparation. 75 μL of cell suspension was placed in cytocentrifuge cups and spun at 450 rpm for 6 minutes. The slides were then air dried for 15 minutes at room temperature and stained with Rowanowski stain (0.5g Eosin, 1.5g Azure-B-thiocyanate, 10nM HEPES buffer pH7.2, DMSO). A differential cell count was obtained by counting > 400 non squamous cells on the prepared slide. The sputum processing pathway design is demonstrated in figure 2.2.

Figure 2.2; Sputum processing protocol

SELECTED SPUTUM PLUGS
→ VIRUS PCR

→ BACTERIA CULTURE

WEIGHED SPUTUM PLUG

8x D-PBS SOLUTION ADDED

DISPERSE SPUTUM, BENCH ROCKER ON ICE (15min), CENTRIFUGE (790g, 10min)

→ 4x VOLUME SUPERNATANT REMOVED AND ALIQUOTED (D-PBS)

4x 0.2% DTT SOLUTION ADDED

DISPERSE SPUTUM, BENCH ROCKER ON ICE (15min), FILTER THROUGH GAUZE

→ 100μL REMOVED FOR CFU counts

→ 500μL REMOVED FOR BACTERIA qPCR

→ 10 μL REMOVED FOR CELL COUNT

CENTRIFUGE (790g, 10min)

→ SUPERNATANT REMOVED AND ALIQUOTED (DTT)

RESUSPEND IN D-PBS AND ADJUST FOR CYTOSPIN FORMATION

2.3. Blood collection and processing

A volume of 10mL of venous blood was collected by venepuncture and collected into serum gel activator (coated with silica particles to enable clotting) and EDTA plasma (coated with K2 to prevent clotting) prepared containers. These were left to stand upright for 1 hour and then centrifuged at 1700rpm for 10mins at room temperature. Serum and plasma was then separated and following a further centrifugation step (2300rpm for 10mins at room temperature) were divided into serum and plasma aliquots for biomarker analysis. Venous blood was also taken to measure full blood count, differential cell count and CRP.

2.4. Atopy testing

Skin prick testing was used to assess atopy to the aeroallergens *dermatophagoides pteronyssinus*, dog, cat, and grass pollen; and a comprehensive assessment of fungal atopy to *Alternaria alternata, Aspergillus fumigatus, Botrytis cinerea, Cladosporium herbarum* and *Penicillium chrysogenum* (Alk-Abello, Denmark), with negative and histamine controls. A positive response to an allergen was recorded when there was a wheal > 3mm bigger than the negative control. Total IgE levels (assay detection limits 2-5000 kU/L, normal reference range 0–114) and allergen specific IgE antibody levels to cat, dog, timothy grass, *dermatophagoides pteronyssinus, Aspergillus fumigatus* (assay detection limits 0.01-100 kU/L, normal reference range 0–0.34) and *Aspergillus fumigatus*-IgG levels (assay detection limits 0.02-200mg/L, normal reference range 0–40) were measured using the ImmunoCap 250 system (Phadia, UK).

2.5.   Questionnaires

St George's Respiratory Health Questionnaire

The St Georges Respiratory Questionnaire (SGRQ, University of London, UK) is a validated respiratory health impairment 2 part questionnaire measuring recollection of symptoms, assessment of current activity and disease impact. Scores are expressed as a percentage of overall impairment with a score of 0 indicating the best possible health status and a score of 100 indicating the worst possible health status (Jones, 1992). The mean (95% confidence interval) SGRQ total score using in healthy subjects is 6 (5 to 7). A difference of 4 in the total SGRQ score is recognised as the minimum clinically important difference.

Chronic Respiratory Disease Standardised Questionnaire

The Chronic Respiratory Disease Standardised Questionnaire (CRQ) was used to measure disease specific health. Scores ranging from 1 to 7 with a higher score, indicate a better quality of life, were calculated for the domains of dyspnoea, fatigue, emotion and mastery after an interviewer led administration of 20 questions (Guyatt, 1988). A total score was then calculated as an average of the 4 domains. A difference of 0.5 in CRQ score is recognised as the minimum clinically important difference.

Visual analogue scale

The visual analogue scale (VAS) for the domains of cough, breathlessness, sputum production and sputum purulence was used to record symptoms (Brightling, 2001). Each subject was asked to draw on a 100mm line with 'no symptoms' at one end and 'the worst symptoms ever' at the other for each symptom domain (Appendix 3). A total score for VAS symptoms was taken as the cumulative of the individual domains.

2.6. Statistical analysis

Analysis of cross sectional data

For comparison of unpaired or paired parametric or non-parametric groups, the Student T-test, Paired T-test, Mann-Whitney test and Wilcoxon matched pairs test was used respectively. For comparison of three groups or more for parametric and non-parametric variables the one-way analysis of variance (ANOVA) or Kruskal-Wallis test was used and the χ^2 test for proportions. Correlations were assessed by the Pearson correlation coefficient (r) and Spearman rank correlation coefficient (rs) for parametric and non-parametric data.

Analysis of longitudinal data

Longitudinal and follow up data was analysed using the Cohen Kappa coefficient (κ), and intra class coefficient (ICC). The κ coefficient measures inter-observer agreement for categorical data, taking into account the agreement that may occur by chance (in contrast to simple percentage agreement). The ICC closely resembles the Pearson correlation coefficient but differs in that data is centred and scaled for a pooled mean and standard deviation and agreement of 1 does not confer a linear association. Logistic and multiple regression analysis were used to assess relationships of independent variables on dependant categorical and continuous variables respectively.

Receiver operator characteristic curve analysis

To determine the diagnostic accuracy of the biomarkers, receiver operator characteristic (ROC) curves and the area under the curve (95% confidence interval) (AUC 95% CI) were calculated. A method used extensively in engineering, statistics and medicine, the ROC analysis is a graphical plot of true positive rates on the y-axis and false positive

rates on the x-axis to determine values or cut-offs for sensitivity and specificity of a test. In essence, a binary classification of true positive, false positive, true negative and false negative is displayed as a continual distribution and graphically represented to determine thresholds. Originally conceived by British Naval officers during the Second World War, radar receiver operators were assessed on their ability to correctly detect signal (aircraft) from noise, with alterations in operator and radar gain leading to differences in correct and incorrect reporting. In its application, two populations are generated, one that has the 'disease' and the other 'disease free'. Within in each 'disease' and 'disease free' population, the test of interest generates numerical results for each subject, which can then be plotted on a histogram as overlapping normal distribution curves as illustrated in figure 2.3.

Figure 2.3; Schematic example of true negative and true positive histogram plots for 'test' results of interest on x-axis and frequency of occurrence on y-axis and derivation of receiver operator characteristic curves
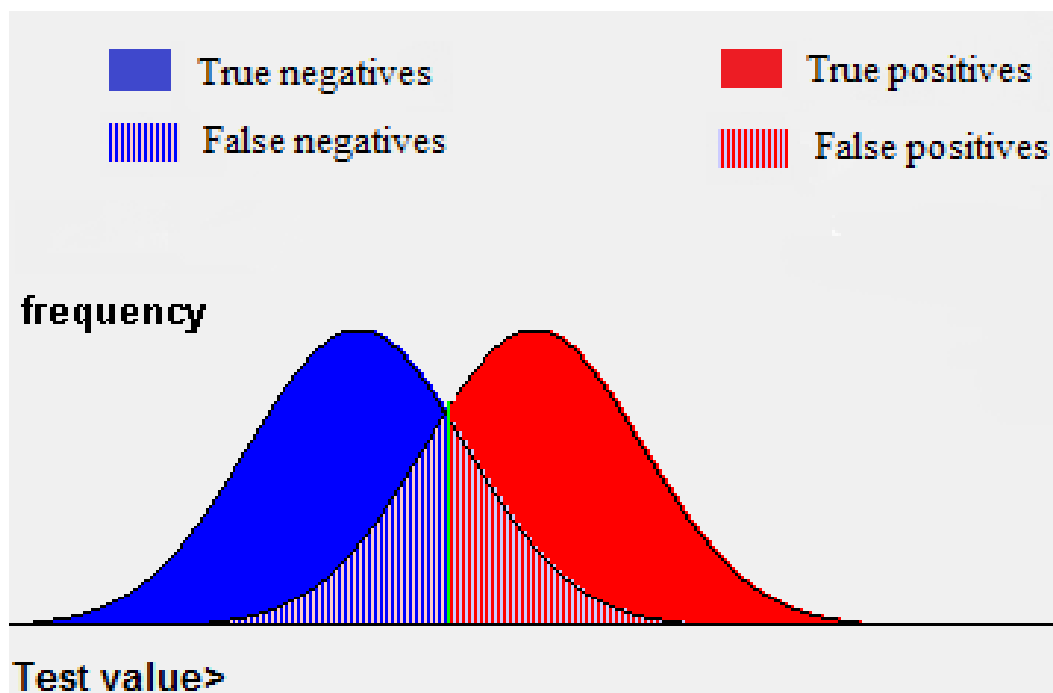
The ROC curve analysis allows for numerous 'test value' thresholds to obtain different false positive and true positive rates. The area under the ROC curve is an illustration of the overlap between the ability of the 'test' to determine the 'disease' and the 'disease free' population. As seen in figure 2.3, the greater the separation between the two populations in the histogram the greater the ability of the 'test' to discriminate between true positives and true negatives. A ROC curve plots the results, 1 minus specificity (false positive rate) and sensitivity (true positive rate) on the x and y axis respectively as illustrated in figure 2.4. The AUC (range 0.5 to 1.0) is a reflection of the 'test' to accurately predict the 'disease' or 'disease free' group. An AUC of 1.0 represents a perfect test. An AUC of 0.5 is a poor test unable to differentiate between the 'disease' and 'disease free' groups, effectively equivalent to flipping a coin (and shown as the diagonal line on the ROC curve in figure 2.4).

The optimal cut-off point at which the histogram populations overlap with equal weighting of sensitivity and specificity is termed the Youden index. This can also be indentified on the ROC curve as the point on the curve closest to the maximum sensitivity and specificity (equivalent to the point closest to the top left hand corner of the ROC curve).

Figure 2.4; Example of receiver operator characteristic (ROC) curve, with x-axis demonstrating false positive rate and y-axis plotting the true positive rate
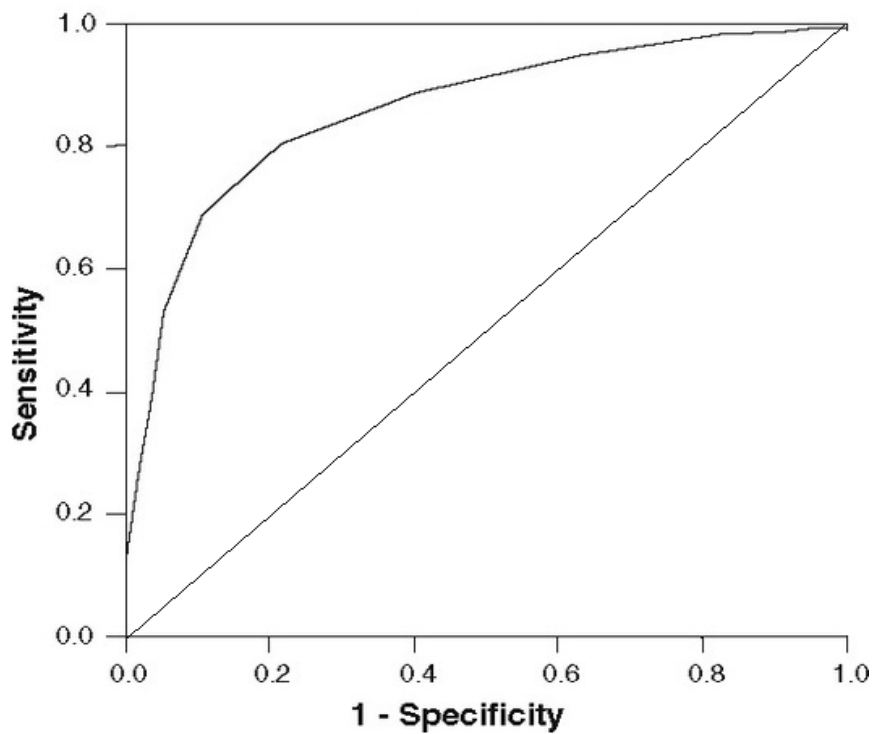
Data presentation

Throughout this thesis, parametric data is presented as mean (standard error of the mean) unless mean (range) or mean (95% confidence interval) is more appropriate. Log transformed data is presented as geometric mean (95% confidence interval). Non parametric data is presented as median (interquartile range); the interquartile range is presented as the difference between the 75th and 25th centile. Differences between groups are presented as mean difference (95% confidence interval of mean difference), fold difference (95% confidence interval of fold difference) and median difference (interquartile range of difference) as appropriate for parametric, log transformed and non parametric data respectively.

A p-value of <0.05 was taken as the threshold of significance for all statistical analyses.

3. Assessment of sputum mediator recovery using two sputum processing methods

Abstract

Inspection of sputum inflammatory cells provides great insight into characterisation of airway diseases including COPD. Current sputum processing techniques use dithiothreitol to break disulphide bonds within the sputum, but some mediators remain difficult to measure. Available validated platforms that measure mediators in high-throughput using small quantities of sample are lacking. In this study I validate the Meso scale discovery platform (MSD) to measure multiple mediators from sputum processed with dithiothreitol and sputum first treated with Dulbecco's phosphate buffered saline. The number of mediators that can be successfully measured using the MSD increases from 10/26 to 19/26 using these sputum techniques respectively.

Introduction

It has been recognised that COPD is associated with systemic and pulmonary inflammation. The use of sputum induction was established by Pin *et al* during the 1990's in a procedure described in asthmatics (Pin, 1992). Prior to this the method, measurement of indices of airway inflammation was primarily by analysis of BAL and bronchial biopsies. However the use of bronchoscopy to obtain samples was limited by its associated discomfort, inconvenience and risks. In asthma, sputum induction has been shown to be repeatable (Pin, 1992; Pizzichini 1996; Kelly 2000), treatment responsive (Pavord, 2000) and has characterised important cellular phenotypes (Simpson, 2006). There was concern regarding the safety of sputum induction in COPD patients which precluded its general use. Rytila *et al* found that although the sputum induction procedure was tolerated, there was significant bronchoconstriction in COPD subjects and the use of pre-induction β_2 agonist bronchodilator therapy was advocated (Rytila, 2000). In the study by Rytila *et al* subjects with an $FEV_1 < 1.0$ L were induced with 0.9% saline whilst subjects with an $FEV_1 \geq 1.0$ L the induction was performed using 3% hypertonic saline. Further experiments using sputum induction confirmed that the procedure was well tolerated and safe in COPD subjects with an $FEV_1 > 0.5$ L using 3%, 4% and 5% hypertonic saline and that the cellular indices were repeatable (Brightling, 2001).

Current sputum processing methods have used dithiothreitol (DTT), a strong reducing agent as a mucolytic and adjunct to breaking disulphide bonds within the sputum (Pizzichini, 1996). DTT is an unstable compound when exposed to air and its activity is limited to pH values > 7.0. Although DTT is useful for sputum processing it is recognised that the biological activity of cytokines, which may inherently rely on the

disulphide bonds for bioactivity, maybe reduced in the DTT sputum processing technique whilst endogenous proteases may restrict and underestimate total cytokine levels (Kelly, 2002). Detection of many cytokines and chemokines in asthmatics has proven difficult using enzyme-linked immunosorbent assays (ELISA), especially Th2 cytokines (Kelly, 2002) because of the DTT effect. In a study by Woolhouse *et al* mediator recovery from the sol phase of sputum from subjects with chronic bronchitis or bronchiectasis treated with DTT or saline were not different for the two methods used for the following mediators; IL-1β, IL-6 and IL-8. However TNF-α levels were reduced in DTT treated sputum. Woolhouse *et al* also showed that there was a poor correlation between the concentrations of mediators measured using either the DTT or saline processing method, despite using standard assays for cytokine measurements (Woolhouse, 2002). In a comprehensive review of different sputum processing techniques Kelly *et al* examined differences in mediator recovery using whole or selected sputum, fluid or sol phase and different concentrations of DTT (Kelly, 2002). Largely confined to studies performed in asthmatic subjects, the review by Kelly *et al* on behalf of the ERS provided key points and standardisation in methods to evaluate mediator recovery. In this chapter I have assessed the validity of measuring mediators using the Meso scale discovery platform (MSD, Maryland, USA) in sputum supernatants from COPD subjects using the DTT sputum processing technique and the D-PBS sputum processing method described in chapter 2.2.2.

Methods and results

Spiking experiments were performed in duplicate according to current recommendations (Kelly, 2002). Briefly, known concentration of cytokine and chemokine mediators were spiked into selected COPD sputum plugs processed with

DTT or D-PBS and compared to unprocessed DTT or buffer (unprocessed buffer set as the spiking standard control experiment). Samples for the spiking experiments were obtained from 5 subjects with a physician diagnosis of COPD and a post bronchodilator FEV_1/FVC ratio of <0.7. Sputum was collected from COPD subjects following sputum induction as described in chapter 2.

Mediator recovery from MSD platform

Mediator recovery from unprocessed buffer following spiking of a known mediator concentration is shown in figure 3.1. This first experiment reflected the recovery of the mediators using the MSD platform. It showed that 20 out of 26 mediators demonstrated a recovery from unprocessed buffer of between $60 - 120\%$. CCL11 was found to have a recovery of >150% in the spiking experiment from the buffer. Mediator recovery from buffer processed with DTT following spiking is shown in figure 3.2. The same mediators using DTT in buffer were recovered and CCL11 continued to demonstrate a recovery of > 150%. This second experiment demonstrated that DTT reduced the mean recovery of measured mediators but remained within acceptable recovery limits for 20 out of 26 mediators.

Mediator recovery from sputum

Mediator recovery from sputum processed with DTT following spiking is shown in figure 3.3. 18 mediators out of 26 mediators had a recovery between $60 - 120\%$. The mediators CCL3 and IL-17 were no longer in the suitable recovery range from sputum processed with DTT.

Figure 3.1; Mediator recovery from unprocessed buffer in standard spiking experiments using the Meso scale discovery platform. Asterisked (*) mediators had a satisfactory recovery of between 60 – 120%. Mediators indicated by † had a recovery of > 120% or <60%.

Mediator recovery using MSD following standard spiking experiments (unprocessed buffer)
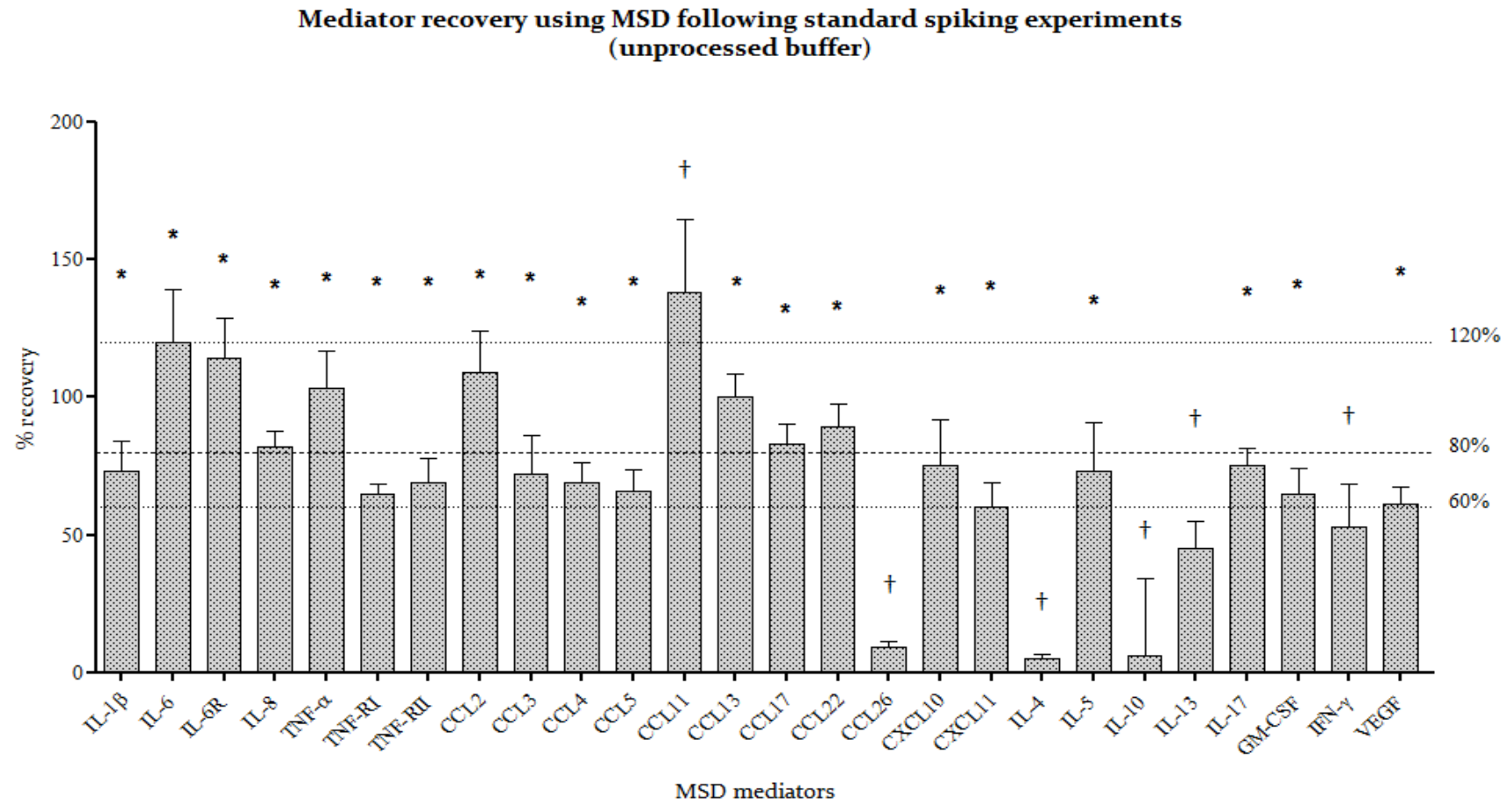
Figure 3.2; Mediator recovery from DTT processed buffer in standard spiking experiments using the Meso scale discovery platform. Asterisked (*) mediators had a satisfactory recovery of between 60 – 120%.
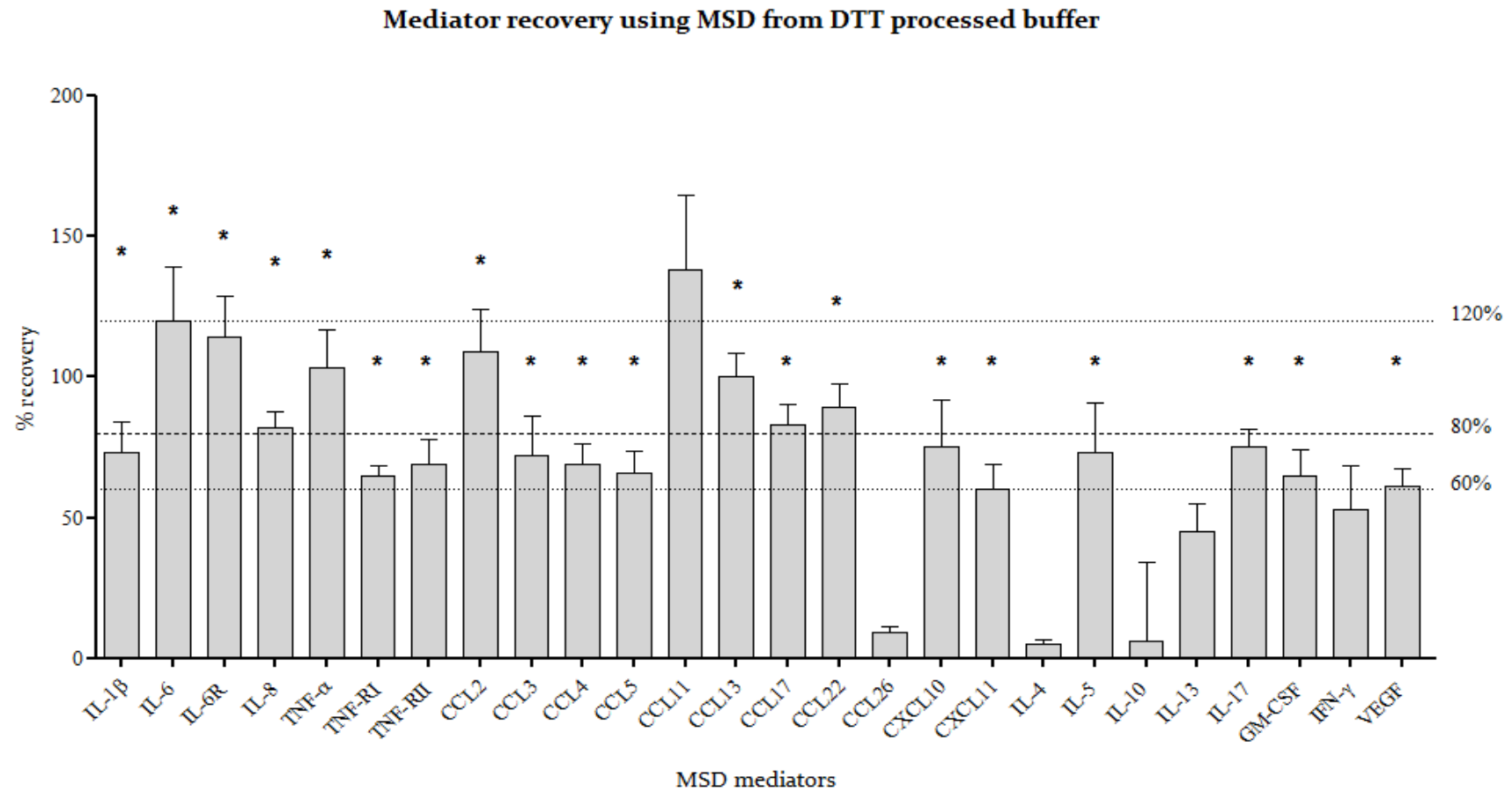
Figure 3.3; Mediator recovery from DTT processed sputum in standard spiking experiments using the Meso scale discovery platform. Asterisked (*)

mediators had a satisfactory recovery of between 60 – 120%.

Mediator recovery using MSD from sputum processed with DTT
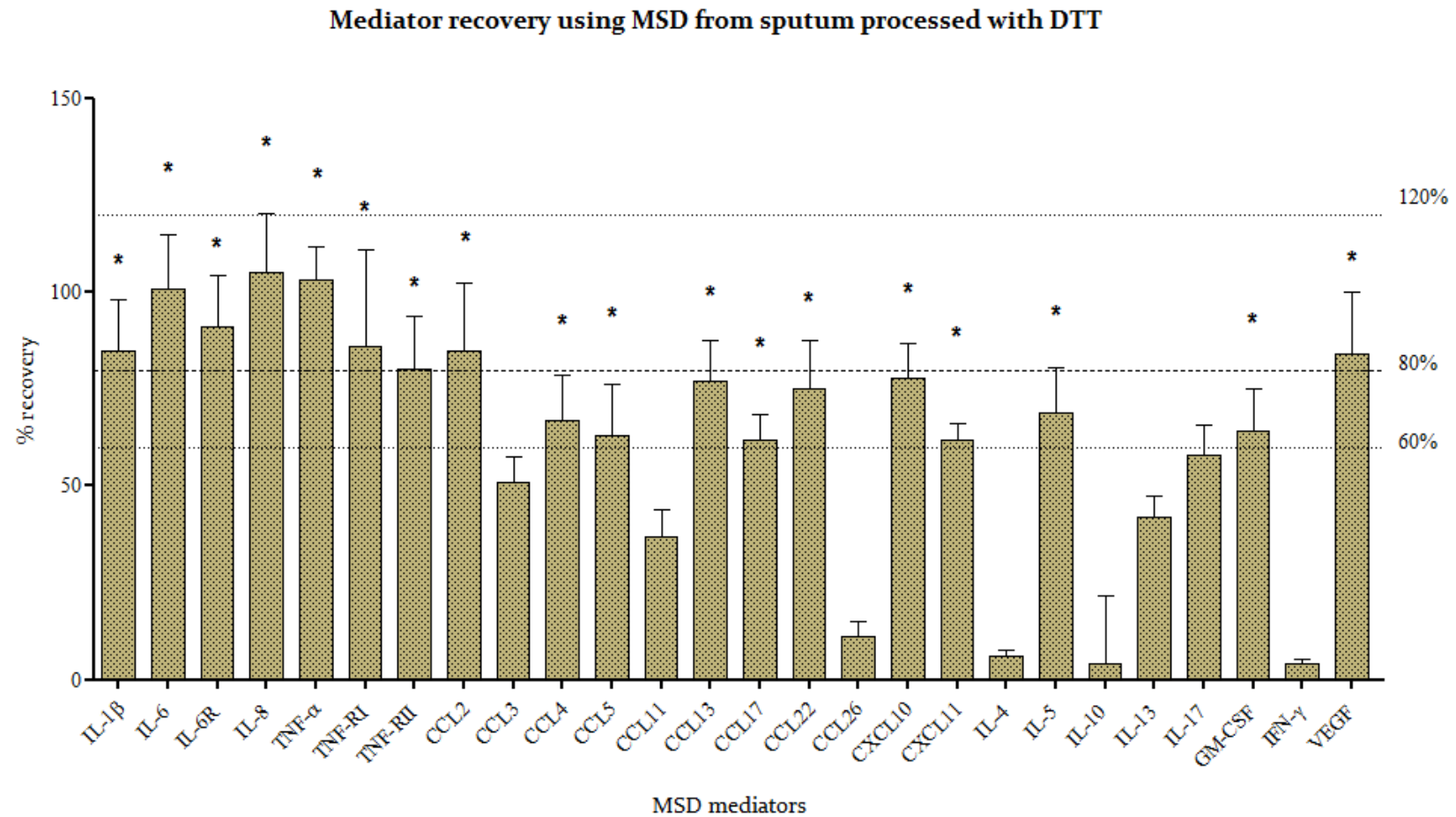
Figure 3.4; Comparison of mediator recovery from DTT processed sputum and DTT processed buffer using the Meso scale discovery platform. Asterisked (*) mediators had a satisfactory recovery of 80% satisfying ERS recommendations.

Mediator recovery comparison from DTT processed buffer (☐) and sputum (▦)
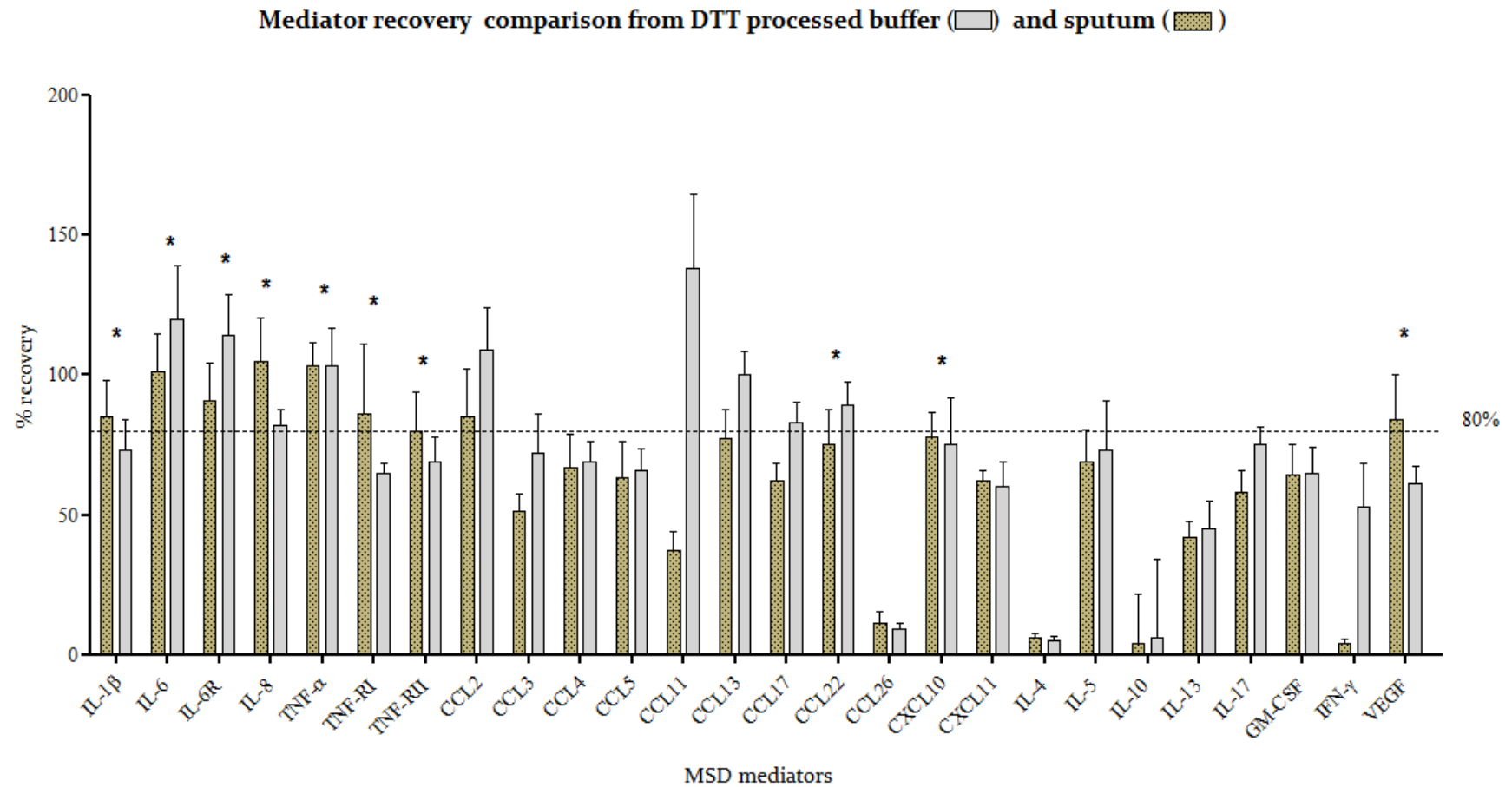
Comparison of mediator recovery from sputum processed with DTT and buffer processed with DTT identified that 10 out of 26 mediators had an overall recovery of >80% which were deemed appropriate by ERS guidelines (Kelly, 2002). This is shown in figure 3.4 (calculated as mean recovery from DTT treated sputum/mean recovery from DTT treated buffer x 100).

Finally, the spiking experiments were performed in D-PBS treated sputum (figure 3.5). This identified mediator recovery of between 60% - 120% in 23 out of 26 mediators and improved recovery in comparison to DTT processed sputum. Comparison of D-PBS processed sputum and D-PBS processed buffer are presented in figure 3.6. However, of these 23 mediators, 4 (IL-4, IL-10, IL-13 and IFN-γ) were not suitably recovered following the control spiking experiment performed in figure 3.1. Table 3.1 lists the mediators recovered according to ERS recommendations between the two sputum processing methods.

Sputum total and sputum differential cell counts between DTT and D-PBS processing methods

To assess the potential effect on cytospin slides of differences between the DTT and D-PBS sputum protocols, the differential and total cell counts was compared in 20 subjects with COPD. There were no significant differences in the total cell or differential counts between the DTT and D-PBS sputum processing methods (mean (SEM) % neutrophils 78 (5) versus 80 (5); geometric mean [95% confidence interval] % eosinophils 1.3 [0.7 to 2.7] versus 1.0 [0.6 to 1.9]; geometric mean [95% confidence interval] total cell count 4.0 (1.7 to 9.8) versus 3.8 (1.6 to 9.0) x10^{6}cells/g sputum).

Figure 3.5; Mediator recovery from D-PBS processed sputum using the Meso scale discovery platform. Asterisked (*) mediators had a satisfactory recovery of > 80%. Mediators indicated by † did not demonstrate suitable recovery from standard spiking experiments

Mediator recovery using MSD from D-PBS processed sputum
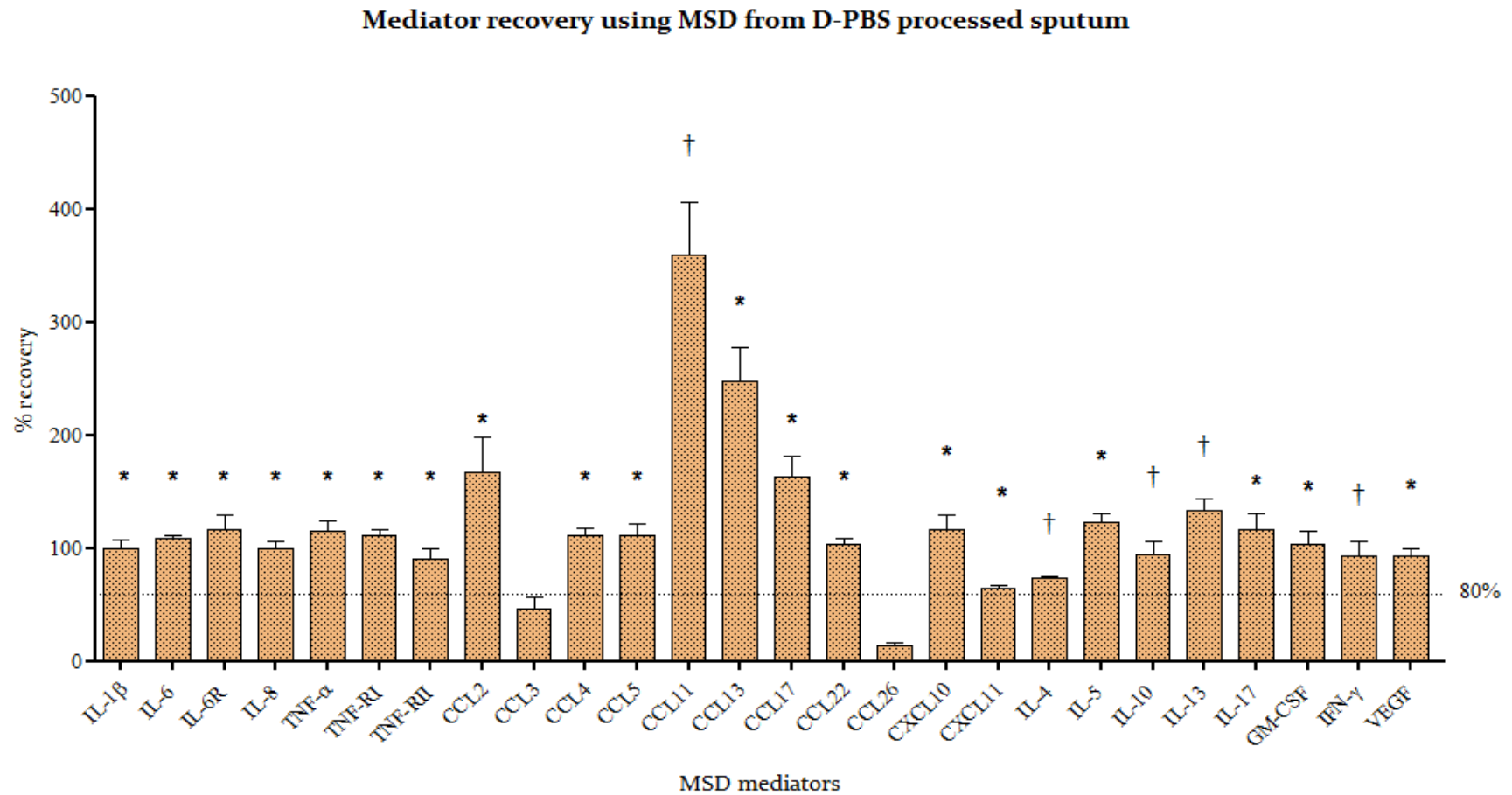
Figure 3.6; Comparison of mediator recovery from D-PBS processed sputum and D-PBS processed buffer using the Meso scale discovery platform.

Asterisked (*) mediators had a satisfactory recovery of 80%. † Mediators with unsuitable assay recovery
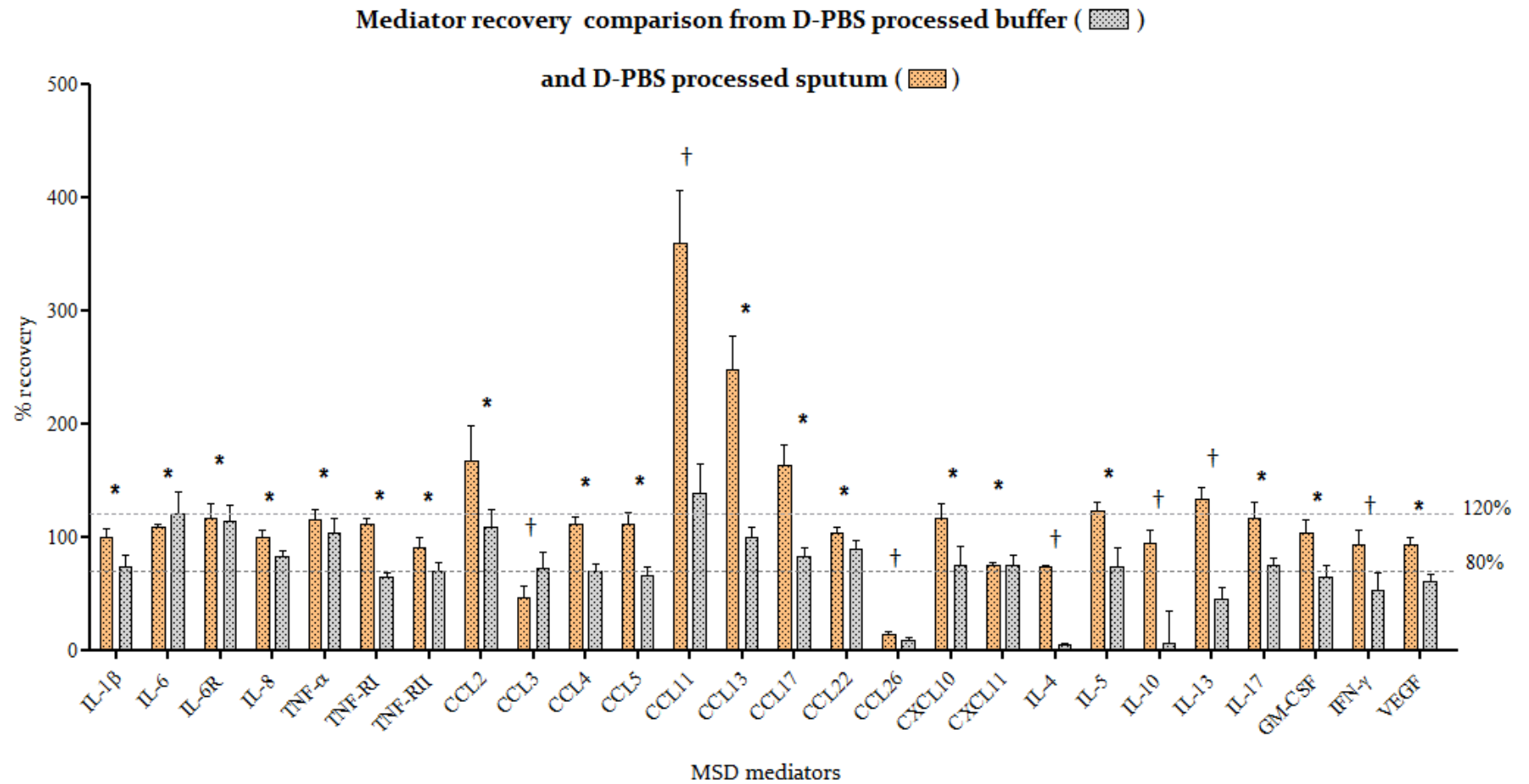
Table 3.1; MSD mediators and acceptable recovery using two sputum processing methods

MSD Mediators	Mediators recovered from DTT sputum	Mediators recovered from D-PBS sputum
IL-1β, IL-6, IL-6R, IL-8	IL-1β, IL-6,IL-6R, IL-8	IL-1β, IL-6, IL-6R, IL-8
TNF-α, TNF-RI, TNF-RII	TNF-α, TNF-RI, TNF-RII	TNF-α, TNF-RI, TNF-RII
CCL2, CCL3, CCL4, CCL5, CCL11[†], CCL13, CCL17,		CCL2, CCL4, CCL5, CCL13, CCL17
CCL22, CCL26[†], CXCL10, CXCL11	CCL22, CXCL10	CCL22, CXCL10, CXCL11
IL-4[†], IL-5, IL-10[†], IL-13[†], IL-17		IL-5, IL-17
GM-CSF, IFN-γ[†], VEGF	VEGF	GM-CSF, VEGF

†CCL11, CCL26, IL-4, IL-10, IL-13, IFN-γ not valid using MSD assay

Discussion

In this chapter I have shown that MSD can be used to reliably measure multiple mediators from the sputum of COPD subjects. I have demonstrated in a series of experiments that satisfactory measurement of 10 mediators can be performed in samples processed using the DTT protocol in the sputum of COPD subjects. The mediator recovery was improved using a D-PBS wash step in the sputum processing protocol and recovery was acceptable in 19 mediators. Furthermore, I have shown that the D-PBS sputum processing method did not affect the total cell count or sputum cellular differential counts when cytospin slides were prepared.

Mediator recovery experiments from the Meso scale discovery platform

The MSD platform employs a multi-array technology using electrochemiluminescence detection and patterned arrays for measurements of mediators from biological samples. In this chapter, spiking experiments were used to assess the performance of the MSD platform to measure a number of mediators from the sputum of COPD subjects. In the first experiment presented in figure 3.1, a known concentration of mediator is spiked into unprocessed buffer as a control experiment. This showed that 20 out of 26 mediators had a recovery of between 60% - 120% and therefore that the MSD was a valid assay for these 20 mediators. There were 5 mediators (CCL26, IL-4, IL-10, IL-13 and IFN-γ) that did not achieve 60% recovery in this control experiment. This demonstrated that the MSD platform was not satisfactory for these mediators as results from sputum samples may be underestimated. The cause for this reduction in the control experiment is not determined in this study; but may be the result of 'physical mediator losses'. This is a reflection of non-specific binding of cytokine to sites such as plastic containers or filters. Also CCL11 was found to have a recovery of >150%. This unexpectedly high mediator recovery means that MSD is an unsuitable assay for this cytokine and may reflect additional absorbance from non-specific sites or imperfections in the antibody binding to the plate. Therefore the use of MSD to measure CCL11 in sputum samples is likely to result in an overestimate of cytokine concentration.

The effects of DTT on mediator recovery from the Meso scale discovery platform

The second experiment illustrated in figure 3.2 was performed to assess the effects of DTT on the MSD platform. The results of the experiment showed that the same 20 mediators were recovered in the control experiment without DTT. Interestingly the concentrations of mediators measured in both experiments were broadly similar. This

data suggest that analysis of cytokines using MSD in combination with DTT is reliable. However when sputum is processed with DTT the number of mediators that can be reliably measured is reduced. In figure 3.3 the mediator recovery from COPD sputum that was processed with DTT showed that the mediators CCL3, CCL11, CCL26, IL-4, IL-10, IL-13, IL-17 and IFN-γ were no longer reliable as the concentrations were reduced by the processing methods.

These experiments demonstrate for the first time the performance of the MSD platform in measuring 26 mediators from the sputum of COPD patients. In keeping with the results from previous studies, DTT in the sputum processing technique affected the accurate measurement of Th1 and Th2 cytokines (Kelly, 2002). Accurate recording of sputum IL-4 concentration in asthmatics has been shown to be difficult; sputum IL-4 levels were unexpectedly similar between asthmatics of different severities and controls in a study by Kim *et al* and this is likely to reflect the effects of DTT and on the ELISA used (Kim, 2010). Spiking experiments have shown that in sputum processed with DTT IL-5 recovery was poor (Kelly, 2000) and that addition of protease inhibitors enhanced this (Kelly, 2001). Furthermore, in studies by Berry *et al* and Erin *et al*, IL-13 levels have been found to be difficult to measure following the addition of DTT (Berry, 2004; Erin, 2008). Few studies have investigated the measurements of Th1 and Th2 cytokines in the sputum of COPD subjects. In a study by Saha *et al*, IL-13 was only detected in 6 out of 34 COPD patients from sputum processed using DTT. The authors concluded that this may represent an underestimation of the IL-13 concentration in COPD patients (Saha, 2008).

In the final experiment, mediator recovery from sputum processed with D-PBS was measured using the MSD platform and the results illustrated in figures 3.5 and 3.6. D-PBS sputum processing was compared to DTT processed sputum and there was an improvement in the recovery of mediators. Here 19 rather than 10 of the 26 mediators fulfilled a recovery of >80% as recommended by the ERS consensus statement (Kelly, 2002). Several studies have examined the concentration of mediators in the fluid phase of sputum (Pizzichini, 1996; Kelly, 2000; Kelly, 2001; Kelly, 2002) but to date the measurement of only a few of these mediators has been validated in COPD (Woolhouse, 2002; Saha, 2008). In this chapter I have assessed the MSD and a novel sputum processing method to measure mediators that may play a role in inflammation in COPD. Interestingly using the D-PBS sputum method, mean recovery of IL-4, IL-10, IL-13 and IFN-γ following spiking was higher than in the DTT processed sputum. The control experiment identified that measurements of these mediators was likely to be underestimated due to the MSD platform. The results from the sputum processed with D-PBS suggest that this method results in superior detection of Th1 and Th2 cytokines and therefore the use of sputum processed with D-PBS will enable further studies in COPD at stable state and during exacerbations.

Cellular examination between two sputum processing methods

A pivotal study in sputum processing techniques was performed by Pizzichini *et al*, this was the first to use DTT which acts as a mucolytic and was shown to enable accurate and reproducible measurements of sputum cellularity and soluble mediators (Pizzichini, 1996). In this chapter I have shown that the addition of a D-PBS wash step did not affect the quality of the cytospin preparation, the total cell count or differential cell count and had the added benefit of improving cytokine and chemokine detection. In the

experiments reported in this chapter sputum plugs were collected from COPD subjects by standard sputum induction techniques. Studies examining mediator concentrations in sputum have often been collected by sputum induction, despite the ability of many COPD subjects to produce spontaneous sputum samples during both the stable state and exacerbations. However, in a study by Bhowmick *et al* examination of sputum cell counts and mediator concentrations collected by spontaneous expectoration or sputum induction were not found to be different for these two collection methods in the same COPD subjects (Bhowmick, 1998). In all the data presented in this thesis the same method of sputum plug selection was used for processing. Studies have demonstrated that the selection of sputum plugs reduces salivary contamination when compared to methods using whole sputum. Salivary concentrations of mediators such as ECP, tryptase and fibrinogen are lower compared to analysis using selected sputum plug techniques, resulting in dilutional effects when whole sputum samples are used. Measurements of soluble mediators have been shown to be repeatable in selected sputum techniques however this has only been validated in asthmatic subjects (Pizzichini, 1996; Gershman, 1996; Spanevello, 1998).

Limitations

In this chapter I have attempted to assess the performance of a novel sputum processing method to measure mediators in sputum induced in COPD subjects during the stable state. In order to compare two sputum processing methods, recovery between 60 and 120% was identified as satisfactory in the control experiment; whilst the intra-assay variability was not assessed. This could be a potential drawback and further assessments need to be undertaken to demonstrate validity and repeatability using the MSD. This chapter has not presented any results of sputum collected spontaneously or during

exacerbations. Therefore this represents a potential limitation with this method of analysis using MSD. However Bhowmick *et al* have demonstrated that there were no differences in sputum cell counts and IL-8 cytokine levels between spontaneously expectorated and induced sputum (Bhowmick, 1998).

Sputum is a complex mixture of mucus, DNA, degraded cells and their products, secreted proteases and binding proteins; this heterogeneous make-up may result in inconsistencies in the measurement of soluble compounds. At exacerbation it is likely that there is increased complexity in the sputum. High levels of viscid DNA and proteases have been detected in patients with infected bronchiectasis and cystic fibrosis (Linnane, 1998). Thus the validation of the MSD method for mediator recovery during exacerbations would be beneficial. Furthermore, measurement of mediators by MSD using the D-PBS sputum processing technique in different severities of COPD, during exacerbations and in non-obstructed controls would be advantageous.

Conclusions

The D-PBS sputum processing method improves the recovery of measureable cytokines and chemokines from COPD subjects using the MSD platform and does not affect the cytospin preparation.

Throughout the remainder of the studies performed in this thesis, the mediator measurements using the MSD were performed on sputum processed with D-PBS as described in chapter 2. Only the mediators that had recovery > 80% satisfying ERS recommendations were subsequently used in the biomarker analysis; these are shown in table 3.1.

4.   Comparing the utility of C reactive protein and procalcitonin to identify bacterial infections and determine thresholds to guide antibiotic therapy

Abstract

Antibiotic overuse in respiratory illness is common and is believed to be associated with drug resistance and hospital acquired infections. Biomarkers that can accurately identify bacterial infections in respiratory disease may reduce antibiotic prescription. The utility of the biomarkers PCT and CRP in patients with pneumonia, exacerbations of asthma or COPD were therefore studied to identify a PCT or CRP threshold to guide antibiotic therapy. There were 319 patients who were hospitalised with pneumonia, exacerbations of asthma or COPD. In all studied patients serum PCT and CRP concentrations were correlated (rs = 0.56, p<0.001). Patients with pneumonia had increased PCT and CRP levels (median (IQR) 1.27 ng/mL (2.36), 191 mg/L (159)) compared to those with exacerbation of asthma (0.03 ng/mL (0.04), 9 mg/L (21)) and COPD (0.05 ng/mL (0.06), 16 mg/L (34)). The area under the ROC curve (95% CI) for distinguishing between patients with pneumonia (antibiotics required) and exacerbations of asthma (antibiotics not required), for PCT and CRP was 0.93 (0.88 to 0.98) and 0.96 (0.93 to 1.00) respectively. A CRP value of greater than 48mg/L had a sensitivity of 91% (95%CI 80% to 97%) and specificity of 93% (95% CI 86% to 98%) in identifying patients with pneumonia. Data from this study suggests that PCT and CRP levels can both independently distinguish pneumonia from exacerbations of asthma and COPD. CRP levels could be used to guide antibiotic therapy and reduce antibiotic overuse in hospitalised patients with acute respiratory illness.

Introduction

Treatment of respiratory tract infections with antibiotics is common and associated with the development of drug resistance and hospital acquired infections (Steinman, 2003; Bauer, 2009; Hawkey, 2009; Dubberke, 2009). Strategies to reduce antibiotic prescriptions can reverse this trend but are currently lacking (Frank, 1997). Community acquired pneumonia and acute exacerbations of asthma and COPD represent a large proportion of healthcare utilisation with a high burden of antibiotic use (Braman, 2006; Rabe, 2007; Lim, 2009). Despite the recommendation that antimicrobials should not be given during acute exacerbations of asthma (Graham, 1982; Glauber, 2001; BTS, 2008) and evidence to suggest that they provide little benefit in mild to moderate COPD exacerbations (Ram, 2006; Puhan, 2007; Puhan, 2008) antibiotic prescription remains common. In primary care, it is estimated that 50% of all antibiotics prescribed are for an acute respiratory illness (Roumie, 2005).

Recently strategies using biomarkers to reduce antimicrobial therapy have been employed. In a selected group of patients with COPD exacerbations, PCT reduced antibiotic prescription by 40% (Stolz, 2007). In a population based study in Spain CRP was shown to be increased in patients with community acquired pneumonia when compared to age-matched healthy controls (Almirall, 2004), whilst persistently elevated levels of CRP have been associated with complications and treatment failure in pneumonia (Smith, 1995; Almirall, 2004; Coelho, 2007; Menendez, 2009; Menendez, 2009). However, there is little evidence to compare the effectiveness of PCT to CRP in accurately diagnosing a bacterial infection of the lower respiratory tract. The identification of a biomarker to accurately guide antibiotic therapy is likely to lead to reduction in unnecessary antimicrobial therapy and is therefore urgently required.

The hypothesis studied in this chapter is that the biomarkers PCT and CRP can be used to accurately distinguish when antibiotic therapy is indicated in hospitalised patients presenting with an acute respiratory illness. Additionally that the PCT and CRP cut off values can be identified that will enable reductions in antibiotic prescription rates.

Methods

This study was nested within a larger National Institute for Health Research Health Technology Assessment (NIHR HTA) trial. The '3 Winters' study was a prospective observational diagnostic accuracy study investigating rapid near patient methods for diagnosis and identification of respiratory infections. Patients with an acute respiratory illness (pneumonia, exacerbation of asthma, exacerbation of COPD) were recruited from the acute medical admission unit within the University Hospitals of Leicester NHS Trust during the winter months of 2006-2008.

Patients

Adults hospitalised with an International Classification of Diseases-10 (ICD-10) (Bramer 1998) diagnosis discharge code of community acquired pneumonia or an exacerbation of asthma or COPD were studied. Patients with pneumonia were identified as those with clinical signs and symptoms suggestive of a lower respiratory tract infection and evidence of consolidation on plain chest radiograph (CXR). Patients with an acute exacerbation of asthma or COPD were identified as those with an appropriate past medical history and acute respiratory symptoms without consolidation on CXR or an alternative diagnosis. Patients with COPD or asthma with consolidation on CXR were excluded from either study group. The study was approved by the Leicestershire,

Northamptonshire and Rutland Ethics Committee and all patients gave informed written consent.

Measurements

At study entry, demographic and clinical data were recorded. Data regarding antibiotic use before and during the hospital admission, length of hospital stay and clinical markers of illness severity using the modified early warning score (MEWS) (Subbe, 2001) and the British Thoracic Society pneumonia severity score (CURB-65) (Lim, 2003) were collected from case notes. All patients had a CXR which was reviewed on admission by a physician and subsequently reported by a consultant radiologist. All patients had venous blood collected at study entry and qualitative detection of *S. pneumoniae* urinary antigen using the Binax assay. Where available, sputum for microbial culture was processed to identify *S. pneumoniae, M. catarrhalis, H. influenzae, P. aeruginosa* and *S. aureus* by standard culture. PCT and CRP assays were performed by personnel who were blind to the patient diagnosis.

Procalcitonin

Serum PCT was measured in duplicate using the time resolved amplified cryptate emission analyser (TRACE, Brahms UK). Concentrations below the limit of detection level of 0.02ng/mL were assigned zero. Threshold concentrations of 0.1 and 0.25 ng/mL were evaluated in this study as these have been validated in previous trials to guide antibiotic therapy (PCT <0.1ng/mL bacterial infection very unlikely and antibiotic use strongly discouraged; PCT <0.25ng/mL bacterial infection unlikely and antibiotic use discouraged; PCT >0.25ng/mL bacterial infection likely and antibiotic use suggested) (Christ-Crain, 2004; Christ-Crain, 2006; Stolz, 2007; Schuetz, 2009).

C reactive protein

Serum CRP was measured using the Abbott Architect ci8200 analyser (Abbott laboratories, Abbott Park, USA). An antigen agglutination antibody reaction between CRP in the samples and polyclonal anti-CRP antibody was detected as an absorbance change; the magnitude of change was related to the CRP level. CRP concentrations were then calculated from the standard calibration curve. The reportable range for CRP was 3 to 300 mg/L.

Statistical Analysis

Statistical analyses were performed using PRISM Version 4 (GraphPad, San Diego, CA) and SPSS version 16 (SPSS Inc., Chicago, IL). Parametric data was expressed as mean and standard error of the mean and non-parametric data was described as median and inter-quartile range. One-way analysis of variance and the Kruskal-Wallis test were used for across and between group comparisons of parametric and non-parametric data respectively; post-hoc analysis was performed using Tukey's or Dunn's comparison Tests. Correlations were assessed by Pearson correlation coefficient (r) and Spearman rank correlation coefficient (rs) for parametric and non-parametric data. The Clopper-Pearson method was used to calculate confidence intervals for proportions. To determine the diagnostic accuracy of the biomarkers, ROC curves and area under the curve with 95% confidence intervals for asthma exacerbation patients versus pneumonia patients were plotted. The pneumonia group was used as a gold standard for antibiotic prescription, while patients with asthma exacerbation were allocated the gold standard for no antibiotic prescription. The Youden index was used to identify the optimal cut off point on the ROC curve with equal weighting of sensitivity and specificity and to

determine the potential reduction in antibiotic therapy in hospitalised patients with COPD. A p-value of <0.05 was taken as the threshold for statistical significance.

Results

There were 319 patients with available sera for study analysis; 62 with pneumonia, 96 with acute exacerbation of asthma, and 161 with exacerbations of COPD. The trial consort diagram is presented in figure 4.1.

Figure 4.1; Trial consort profile

Diagnosis: **Asthma** (N=165)	Diagnosis: **COPD** (N=288)	Diagnosis: **Pneumonia** (N=110)
6 – CXR unavailable	3 – CXR unavailable	2 – CXR unavailable
63 – Sera unavailable	124 – Sera unavailable	46 – Sera unavailable
Acute sample available for **96** patients	Acute samples available for **161** patients	Acute samples available for **62** patients
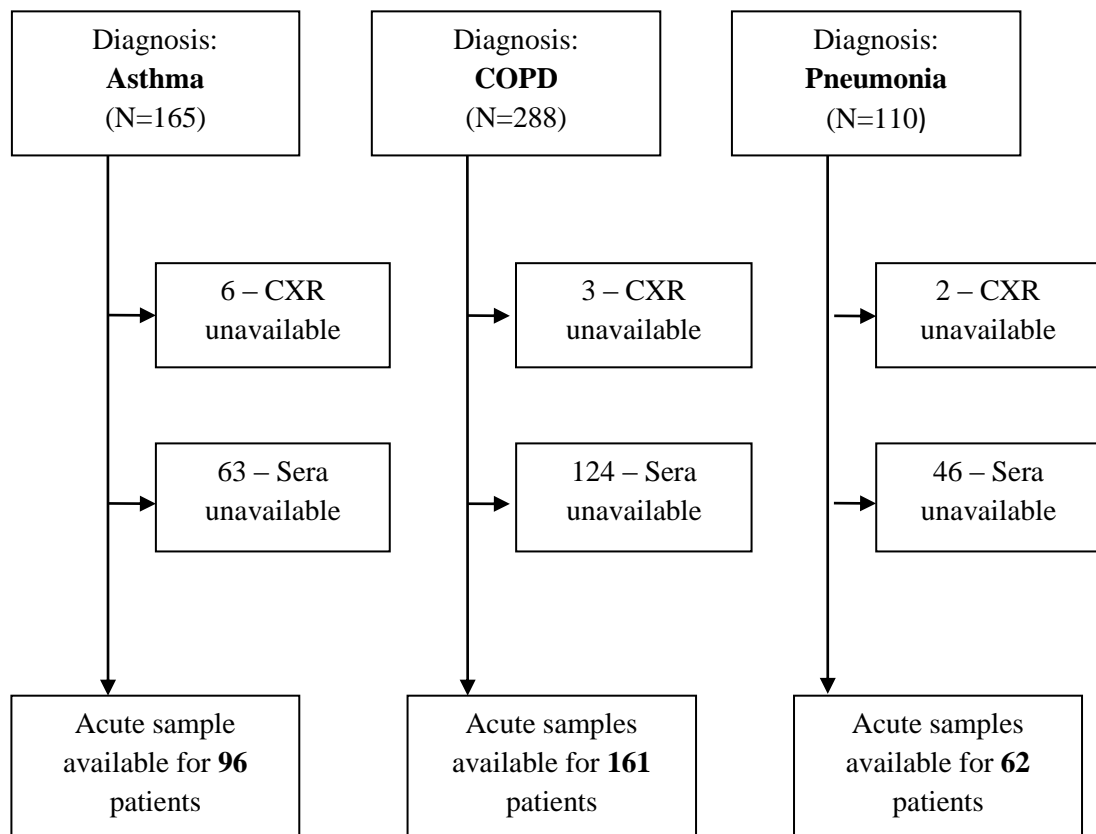
Sputum culture positive for PPM on admission was detected in 29% and 44% of pneumonia and COPD patients respectively. Urinary pneumococcal antigen was

positive in 29% of pneumonia patients. The clinical characteristics of all patients at admission are tabulated in table 4.1

Table 4.1; Demographic and clinical data from patients admitted with pneumonia, exacerbation of asthma, or exacerbation of COPD.

	Pneumonia	Asthma exacerbation	COPD exacerbation	p-value
Number, n	62	96	161	-
Male, %	**63**	**37**	**48**	**<0.01**
Age *	**63 (24 to 93)**	**41 (18 to 84)**	**69 (39 to 93)**	**<0.01**
Antibiotics given, %	**100**	**57**	**76**	**<0.01**
Intravenous antibiotics given, %	**74**	**10**	**18**	**<0.01**
Antibiotics prior to admission, %	25	35	35	0.34
S pneumoniae urinary antigen positive, %	**29**	**2**	**5**	**<0.01**
Length of stay, days *	**6 (1 to 40)**	**3 (1 to 19)**	**5 (1 to 31)**	**<0.01**
Temperature, °C †	**37.1 (1.1)**	**36.8 (0.9)**	**36.6 (0.8)**	**<0.01**
Respiratory rate, n †	22 (7)	22 (7)	23 (6)	0.95
Systolic blood pressure, mmHg	**120 (3)**	**132 (2)**	**134 (2)**	**<0.01**
Pulse rate, bpm	98 (2)	100 (2)	95 (1)	0.08
Modified early warning score *	3 (0 to 7)	3 (0 to 7)	2 (0 to 8)	0.06
Blood leukocytes (x10^9 cells/L) †	**14.1 (7.5)**	**10.8 (4.3)**	**10.1 (4.9)**	**<0.01**
Blood neutrophils (x10^9 cells/L) †	**12.4 (7.2)**	**7.7 (4.4)**	**7.5 (4.7)**	**<0.01**
CRP (mg/L) †	**191 (159)**	**9 (21)**	**16 (34)**	**<0.01**
PCT (ng/mL) †	**1.27 (2.36)**	**0.03 (0.04)**	**0.05 (0.06)**	**<0.01**

Data presented as mean (SEM), unless stated. * mean (range), †median (interquartile range)

Patients with pneumonia had higher PCT and CRP levels than those with COPD (p<0.0001) or asthma (p<0.0001) (figure 4.2 and figure 4.3).

Figure 4.2; Box-and-whisker plots for patients admitted with exacerbation of asthma, COPD and pneumonia for PCT. The horizontal bar represents the median; the box length represents the interquartile range. Outliers are identified by ^{O} (1.5 times the interquartile range), and * (3 times the interquartile range).

Procalcitonin
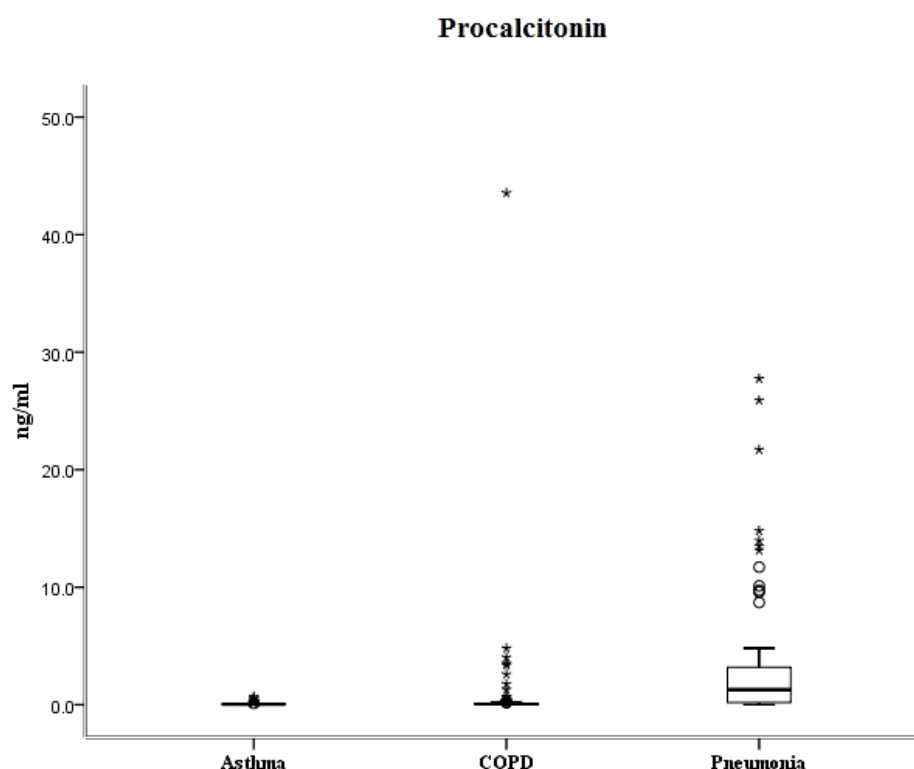
The concentration of PCT was below the limit of detection in 24%, 6% and 2% of asthma, COPD and pneumonia patients respectively. All of the pneumonia patients received antibiotic therapy, whilst 57% and 76% of asthma and COPD patients received antibiotics respectively.

Figure 4.3; Box-and-whisker plots for patients admitted with exacerbation of asthma, COPD and Pneumonia for CRP. The horizontal bar represents the median; the box length represents the interquartile range. Outliers are identified by ^{O} (1.5 times the interquartile range), and * (3 times the interquartile range).

C Reactive Protein
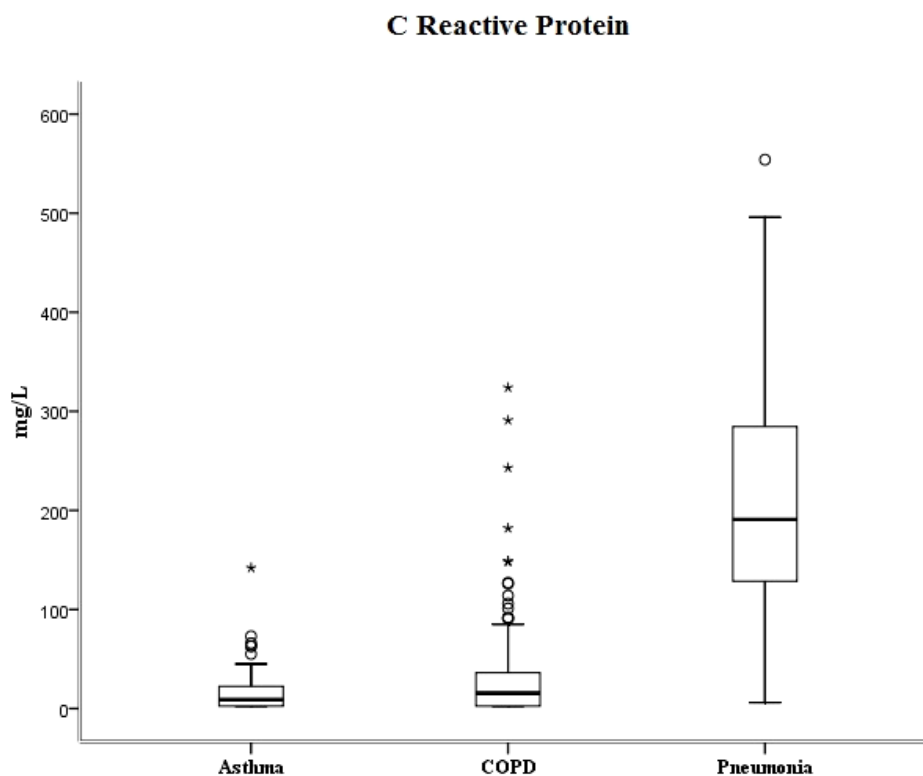
In all patients studied PCT and CRP levels were strongly correlated (rs=0.56, p<0.0001). The area under the ROC curve (95% confidence interval) for the clinical parameters of peripheral neutrophil counts, temperature on admission and MEWS score for distinguishing between patients with pneumonia (where antibiotics are definitely required) and exacerbations of asthma (where antibiotics are definitely not required) were 0.77 (0.69 to 0.85), 0.65 (0.56 to 0.74) and 0.54 (0.44 to 0.63) respectively (figure 4.4).

Figure 4.4; Receiver operator curve distinguishing between patients with pneumonia (where antibiotics are definitely required) and exacerbations of asthma (where antibiotics are definitely not required) for peripheral neutrophils, temperature, and modified early warning score.
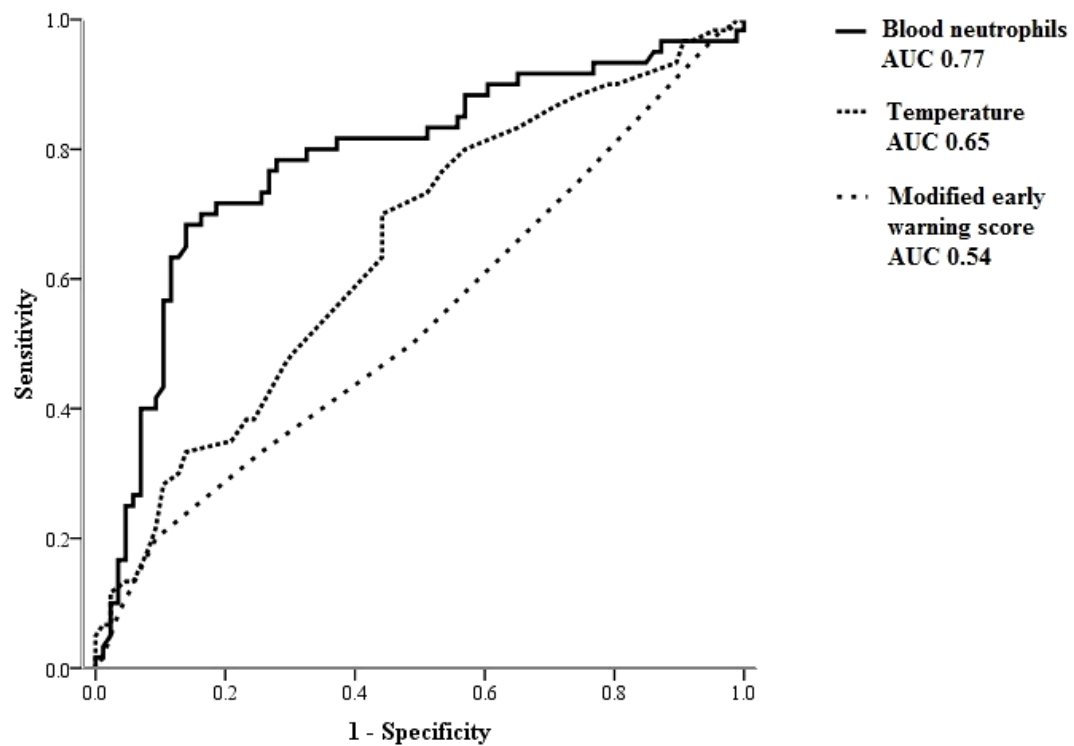
The area under the ROC curve (95% confidence interval) for PCT and CRP was 0.93 (0.88 to 0.98) and 0.96 (0.93 to 1.00) respectively. The product of PCT and CRP (PCT×CRP) yielded a value of 0.98 (0.96 to 1.00) (figure 4.5).

Figure 4.5; Receiver operator curve distinguishing between patients with pneumonia (where antibiotics are definitely required) and exacerbations of asthma (where antibiotics are definitely not required) for PCT, CRP and PCT×CRP product
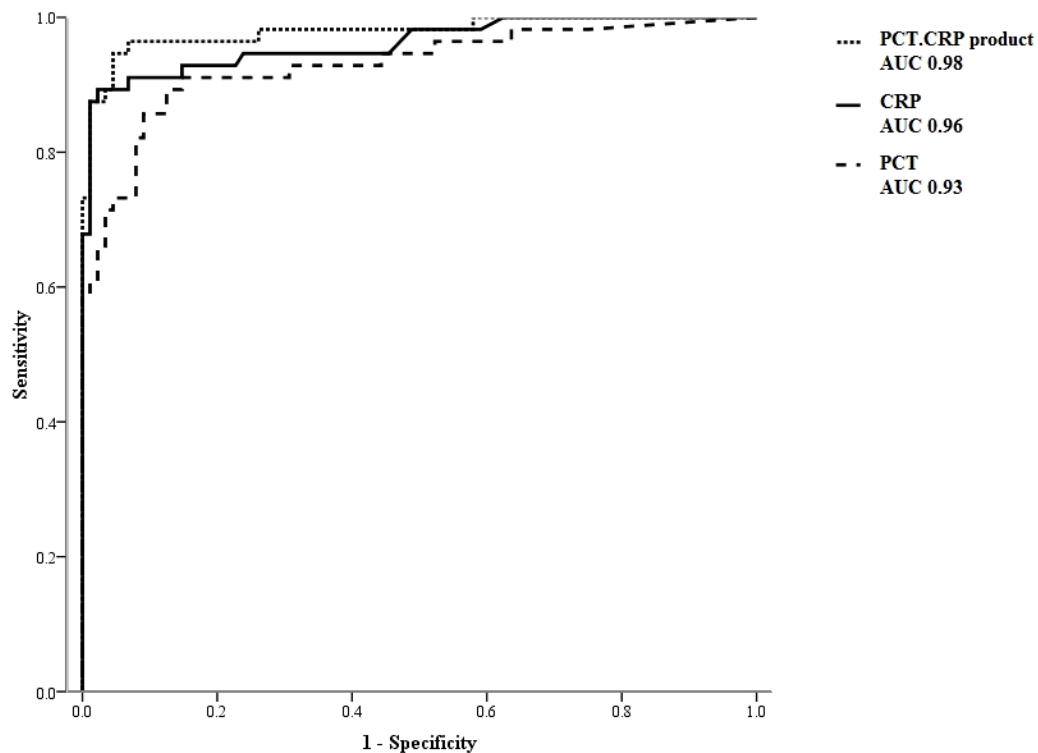
Using the ROC curve to identify the optimum CRP threshold value; a level of greater than 48mg/L had a sensitivity of 91% (95% CI 80% to 97%) and specificity of 93% (95% CI 86% to 98%) to identify patients with pneumonia. The optimal threshold value for PCT was 0.08ng/mL and a level greater than this had a sensitivity of 89% (95%CI 78% to 95%) and specificity of 78% (95%CI 72% to 82%) to identify patients with pneumonia.

33% of all patients received antibiotics prior to admission. The area under the ROC curve (95%CI) was not different between those patients who had received prior antibiotic therapy and those who had not for both CRP (0.96 (0.92 to 1.00) versus 0.94 (0.89 to 0.99) and PCT 0.96 (0.89 to 1.00) versus 0.92 (0.83 to 1.00).

The proportion of patients who would have received antibiotics if biomarker directed therapy was employed using the threshold values for PCT of 0.1 and 0.25ng/mL and for CRP of 10, 30, and 49 mg/L, is shown in table 4.2.

Table 4.2; Proportion of patients receiving antibiotic therapy on admission and proportion (95%CI) that would have received antibiotic therapy if biomarker directed therapy were used at different threshold values for PCT and CRP

| | | Proportions who would have been treated if biomarker-directed therapy were used | | | | |
| | Proportions actually treated | PCT ng/mL | | CRP mg/l | | |
		0.1	0.25	10	30	48
Asthma, %	57	9	4	43	15	7
(95% CI)		(4 to 16)	(1 to 11)	(33 to 54)	(9 to 24)	(2 to 14)
COPD, %	76	24	7	59	32	21
(95% CI)		(17 to 31)	(4 to 13)	(51 to 67)	(25 to 40)	(15 to 28)
Pneumonia,%	100	82	73	95	91	91
(95% CI)		(70 to 91)	(60 to 83)	(85 to 99)	(80 to 97)	(80 to 97)

Using a PCT threshold value of greater than 0.25ng/mL to guide antibiotic therapy, antibiotic use would have been reduced from 57% to 4% in patients with exacerbation of asthma; from 76% to 7% in patients with exacerbation of COPD; and from 100% to 73% in those with pneumonia. Using a CRP threshold value of greater than 48mg/L to guide antibiotic therapy, antibiotic use would have been reduced from 57% to 7% in patients with asthma; from 76% to 18% patients with exacerbation of COPD; and from 100% to 91% in patients with pneumonia.

Discussion

In this study I have shown that the biomarkers CRP and PCT are elevated in patients with pneumonia compared to patients with exacerbations of asthma and COPD. Using ROC curve analysis, these biomarkers are better predictors of pneumonia compared to clinical methods such as temperature, peripheral blood neutrophil count or the modified early warning score. Thresholds that are sensitive and specific were determined for PCT and CRP which hypothetically could be used to reduce the proportion of antibiotic prescription during exacerbations of COPD.

Antibiotic prescriptions

In this study, a high antibiotic prescription rate in asthmatic patients was observed and this is contrary to current recommendations (Graham, 1982; BTS, 2008). In addition to this there was also a high level of antibiotic use in exacerbations of COPD. There is conflicting evidence regarding antibiotic therapy during exacerbations of COPD. In a Cochrane systematic review, 10 RCTs were analysed for 850 COPD exacerbations (Ram 2006). Although a significantly heterogeneous group of studies with respect to antibiotic choice, duration, administration and a lack of standardisation of concomitant therapy, the Cochrane systematic review concluded that antibiotic therapy was associated with a reduction in short term mortality, treatment failure (defined as no resolution or deterioration in symptoms or death) and sputum purulence. Exacerbations in this Cochrane review were symptom based and were defined as those with increased sputum purulence, increased sputum volume, increased breathlessness, increased wheeze, or fluid retention. However, there was no benefit found in the primary outcome

of antibiotics in COPD exacerbations to reduce the duration of hospital admission, admission to intensive care or lung function. Only 2 of the RCTs were out-patient based, whilst the remainder were hospitalised exacerbations of COPD. Several further meta-analyses published by Puhan *et al* (Puhan 2006, 2007) have shown that community based and therefore mild to moderate exacerbations of COPD do not warrant antibiotic therapy. A large retrospective meta-analysis of hospitalised exacerbations by Rothberg *et al* (Rothberg 2010) has shown that antibiotics given early (within 3 days of the hospital admission) are associated with favourable outcomes and reductions in mortality and treatment failure. Evidence from Puhan and Rothberg *et al* would suggest that antibiotics should be provided during severe hospitalised exacerbations of COPD. The consistent finding however throughout these studies is that antibiotics were associated with significant adverse events and increased rates of *C. difficile* and antibiotic related diarrhoea.

Procalcitonin and C reactive protein

In this study I have demonstrated that PCT and CRP sensitive and specific thresholds calculated with ROC curves can correctly identify patients with an acute respiratory illness that are likely to have a bacterial infection. The biomarkers PCT and CRP were superior to currently available modified early warning scores or clinical indices of bacterial infection such as temperature and peripheral leukocyte or neutrophil counts. Pneumonia was used as the gold standard for necessitating antibiotic prescription, whilst an asthma exacerbation without CXR evidence of consolidation was used as the gold standard for omitting antibiotic therapy. This in turn derived thresholds for CRP and PCT which could be used to direct antibiotic therapy during COPD exacerbations.

Further investigations of these thresholds provided hypothetical scenarios of proportions of patients that would be treated with antibiotics and identified a marked reduction in antibiotic use if these thresholds were adopted.

PCT is a precursor of the hormone calcitonin and is a marker of a bacterial inflammatory response, stimulated by bacterial products such as LPS and in response to the circulating pro-inflammatory cytokines IL-1 and TNF-α. PCT has been shown to effectively discriminate between septic shock and the systemic inflammatory response syndrome (SIRS) in intensive care patients (Harbarth, 2001). The exact mechanism of PCT synthesis remains unclear but it is produced from numerous cells involved in inflammation (Monneret, 1999). There is a clinical need to reduce unnecessary antibiotic use during COPD exacerbations in both primary and secondary care; a biomarker directed method could be used. This has been performed successfully with PCT in large clinical trials. Christ-Crain *et al* have used PCT to guide antibiotic therapy for patients with symptoms suggestive of a lower respiratory tract infection, including patients with COPD (Christ-Crain, 2004; Christ-Crain, 2006); whilst Schuetz *et al* and Stolz *et al* have replicated these findings in larger clinical studies (Stolz, 2007; Schuetz, 2009). These studies have shown that a biomarker driven algorithm can reduce antibiotic prescription significantly and that this can be achieved without an increase in adverse events or treatment failures.

In this chapter, I have shown that CRP is useful in differentiating pneumonia from asthma and determined cut-off values to guide when antibiotic prescription would be

recommended. There is currently little evidence using CRP as a guide to antibiotic therapy, although a recent study of the utility of biomarkers in predicting bacteraemia in patients with community acquired pneumonia suggested a superior capacity of PCT when compared to CRP (Muller, 2010). However in exacerbations of COPD comparisons of CRP and PCT have shown that CRP is a better predictor of treatment responses (Daniels, 2010). Furthermore, these findings concur with those of other recent studies showing that CRP can distinguish patients with pneumonia from those with exacerbations of COPD or heart failure (Joffe, 2009; Justo, 2009). The results presented in this chapter suggest that CRP could be used to guide antibiotic prescription in patients hospitalised with acute lower respiratory tract symptoms and this is a similar finding observed in a large cohort of patients with lower respiratory tract symptoms in primary care (Cals, 2009). Interestingly the results of the CRP ROC curves presented in this chapter have a similar accuracy to studies investigating infected and non infected patients in the intensive care unit (Chan, 2004; Povoa, 2005).

Using the ROC curve, different thresholds of CRP and PCT were interrogated to identify when antibiotic therapy should be given. The proportions of patients with pneumonia, asthma or COPD that would have been treated (or not) using a biomarker directed strategy were then calculated. As anticipated it was shown that lower levels of PCT and CRP were more sensitive biomarkers to determine pneumonia; CRP was found to be superior. From the ROC curve a CRP cut off of 48 mg/L was found to be the most sensitive and specific level for accurately defining pneumonia (when antibiotics are recommended) or asthma (when antibiotics are not recommended). This CRP value would have reduced antibiotic prescriptions by 76% in exacerbations of

COPD. The presence of consolidation on CXR is the gold standard for defining pneumonia and in this situation a biomarker is not required. However, where consolidation is not present on the CXR in patients with acute respiratory illness the use of biomarkers such as CRP to guide antibiotic therapy should be used, this includes COPD exacerbations. In a study by Daniels *et al* sub-group analysis determined that a CRP of 50mg/L during an exacerbation of COPD was associated with a favourable treatment response following 7 days of doxycycline therapy. The treatment response in this study by Daniels *et al* was defined as complete resolution or improvement of signs and symptoms associated with the exacerbation (Daniels 2010). These corroborative findings performed in an independent severe COPD exacerbation cohort provide further evidence that a biomarker directed strategy using a CRP cut off of 50mg/L could be clinically efficacious and a randomised control trial is warranted.

Limitations

One possible limitation of this study is the use of the ICD-10 classification of disease coding to define diagnosis at enrolment. Whilst coding can be inaccurate, additional clinical and radiographic evidence to improve diagnostic accuracy and confidence that the clinical groups represented those encountered in clinical practice were employed. A further limitation is the lack of aetiological data to distinguish patients with a viral or bacterial cause for the lower respiratory tract illness. However, it has been previously shown that bacteria are detected in less than half of pneumonias whilst isolation of viruses *per se* does not exclude bacterial co-infection and the need for antibiotics (Holm, 2007). By applying a clinical diagnosis to this cohort and using blinded comparisons, PCT and CRP were evaluated. CRP was found to have at least equal

accuracy for the detection of pneumonia and could be used in a similar way to PCT to guide antibiotic therapy in hospitalised patients with lower respiratory tract infection.

Conclusions

The threshold levels generated in this chapter to guide antibiotic therapy could lead to safe reductions in antibiotic use among patients hospitalised with exacerbations of airways disease. Using the threshold level for CRP of greater than 48mg/L to guide antibiotic therapy in acute respiratory illness, reductions in antibiotic therapy would be significant. Future studies using these biomarkers and their corresponding threshold values should be used to direct antibiotic therapy in COPD exacerbations.

5. Biomarkers to identify clinical phenotypes of acute exacerbations of chronic obstructive pulmonary disease

Abstract

Exacerbations of COPD are heterogeneous with respect to inflammation and aetiology and how this impact on treatment responses is unknown. Biomarker expression was investigated during COPD exacerbations to determine potential biomarkers that recognise clinical COPD exacerbation phenotypes associated with bacteria, viruses or eosinophilic airway inflammation. Patients with COPD were observed over 1 year at stable and exacerbation visits. Biomarkers were measured in sputum and serum. Viruses and bacteria were assessed in sputum by polymerase chain reaction and routine diagnostic bacterial culture. Biomarkers that differentiated clinical exacerbation phenotypes were investigated.

Of all exacerbations studied 55%, 29% and 28% were associated with bacteria, virus or sputum eosinophilia respectively. The biomarkers that best identified these clinical phenotypes were sputum IL-1β (area under ROC curve 0.89 (95% CI 0.83 to 0.95), serum CXCL10 0.83 (0.70 to 0.96) and percentage peripheral eosinophils 0.85 (0.78 to 0.93) respectively.

The heterogeneity of COPD exacerbations can be defined using biomarkers. Sputum IL-1β, serum CXCL10 and peripheral eosinophils are biomarkers of bacteria, virus or eosinophil associated exacerbations of COPD.

Introduction

Acute exacerbations of COPD are associated with substantial morbidity and mortality and are typically associated with increased neutrophilic and to a lesser extent eosinophilic airway inflammation (Saetta, 1994; Bhowmik, 2000). Respiratory virus and bacterial infections have been implicated in causing the majority of exacerbations (Seemungal, 2001; Papi, 2006; Sethi, 2008) but how these infections alter lower airway inflammation and relate to treatment response is not completely understood. During stable state a sputum eosinophilia is associated with corticosteroid responsiveness (Shim, 1978; Pizzichini, 1998; Brightling, 2000), whilst the presence of a high bacterial load and sputum purulence has favourable outcomes with antibiotics (Stockley, 2000; White, 2003; van, 2004; Ram, 2006). These findings would suggest that it is possible to identify clinically important COPD exacerbation phenotypes. However at present exacerbation phenotypes have not yet been defined and clinicians do not have suitable tools to reliably classify these. It is crucial to define COPD exacerbation heterogeneity to effectively target therapy as systemic corticosteroids and antibiotics, which are the mainstay of exacerbation treatments have marginal efficacy (Davies, 1999; Niewoehner, 1999; Aaron, 2003; Puhan, 2007; Puhan, 2008; Rothberg, 2010). Additionally these therapies are associated with significant adverse events in a vulnerable population and so effectively targeting therapy may limit these. The hypothesis studied in this chapter is that during COPD exacerbations associated with bacteria, viruses or sputum eosinophilia biomarker to define these clinical exacerbation phenotypes can be identified.

Methods

Patient selection and recruitment

Patients with a physician diagnosis of COPD, defined according to GOLD (Rabe, 2007) were recruited from general respiratory clinics at the Glenfield hospital, Leicester and from local advertising to enter the study. The full study inclusion criteria were:

- Age > 40 years

- Post 400mcg salbutamol bronchodilator FEV_1/FVC ratio <0.7

- ≥ 1 exacerbation in the previous year requiring corticosteroids and/or antibiotic therapy, including subjects that required hospitalisation for an exacerbation of COPD

The exclusion criteria were:

- Inability to produce sputum following the induced sputum procedure

- A current or previous history of asthma

- Currently active pulmonary tuberculosis

- Any other clinically relevant lung disease other than COPD

- Pregnancy or lactation

- The presence of a clinically relevant illness (past or present) with which in the opinion of the investigator would not be in the best interest of the patient or influence the results of the study

The presence of co-morbidities, reported atopy to common aeroallergens, or significant reversibility on lung function testing was not an exclusion factor *per-se.* All patients

recruited provided written informed consent and could voluntarily withdraw from the study at any time. The study was approved by the local ethics committee.

Study design

Stable visits

Patients were invited to attend an initial screening and consent visit and then enrolled into the prospective observational 1 year study for stable 3 monthly visits and exacerbation visits. At study entry demographic data including age, medical history, medications; including dose of inhaled and oral corticosteroid, detailed smoking history and previous exacerbation history was recorded. Patients had their height and weight recorded and a CXR (if a CXR was not performed in the previous 12 months). Stable visits including the baseline visit was performed a minimum of 8 weeks after an exacerbation. The study outline for stable visits is presented in figure 5.1.

Figure 5.1; Study visit schedule during stable state

Scheduled Visits

Visit 1

Consent

Within 1 month

↓

Visit 2

Demographics, CXR, Spirometry & Reversibility, PFT Sputum, Blood Questionnaires

Every 3 months

↓

Visit 3, Visit 4, Visit 5, Visit 6

Spirometry & Reversibility Sputum, Blood Questionnaires
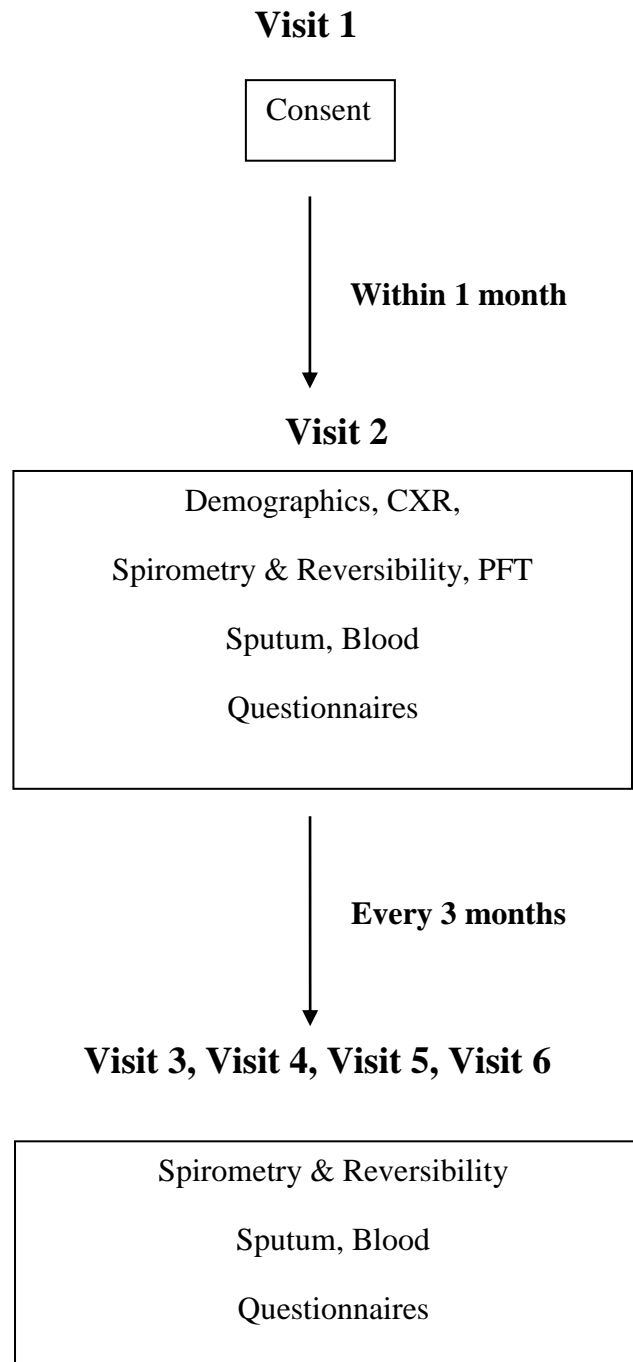
Exacerbation visits

All patients were given daily diary cards to complete (Appendix 4). This was a daily record of minor and major symptoms based on studies from the East London COPD cohort (Seemungal, 1998). Major symptoms were increased breathlessness, sputum volume or sputum purulence. Minor symptoms were nasal discharge, wheeze, cough or sore throat. The baseline diary symptoms were recorded and all patients were asked to contact the research team if there was an increase in major or minor symptoms over two consecutive days. Exacerbations were defined according to Anthonisen criteria and healthcare utilisation, namely as an increase in 2 major symptoms or 1 major symptom and 2 minor symptoms from patient reporting or daily diary card triggering the patient to contact the department and one that necessitated a change in therapy by a physician (Anthonisen, 1987; Rodriguez-Roisin, 2000). Patients were all clinically assessed (including CXR, temperature recording and blood gas analysis if clinically indicated) to exclude other causes of breathlessness. Patients with an exacerbation of COPD were then treated according to guidelines (Halpin, 2004).

The study outline for exacerbation visits is presented in figure 5.2.

Figure 5.2; Study visit schedule during exacerbations

Exacerbations

0 weeks

Clinical history and examination

Spirometry & Reversibility

Sputum, Blood

Questionnaires

2 weeks

Spirometry & Reversibility

Sputum, Blood

Questionnaires

6 weeks

Spirometry & Reversibility

Sputum, Blood
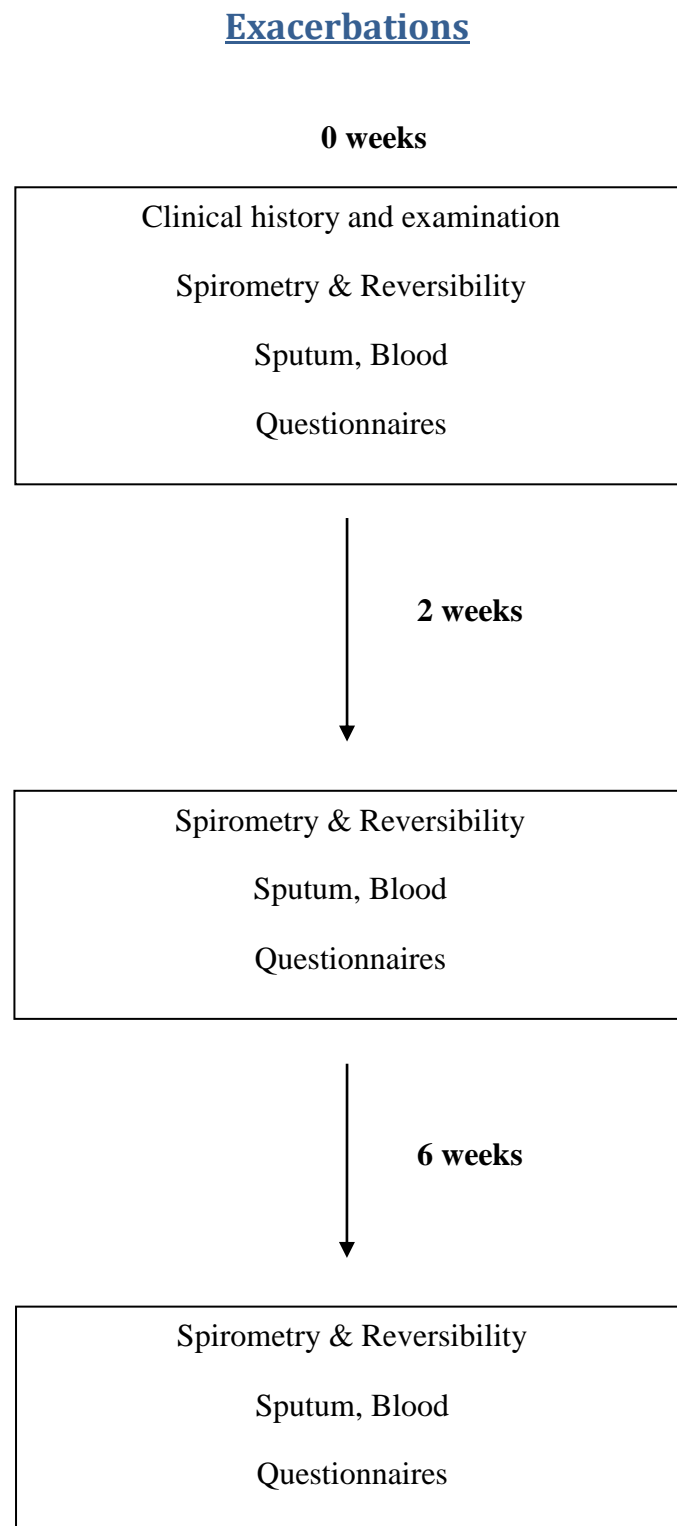
Questionnaires

At all visits, patients underwent pre and post 400μg salbutamol bronchodilator spirometry (Vitalograph, UK), induced or spontaneous sputum collection and measurements of symptoms and health quality assessments using the VAS, the SGRQ and the CRQ.

Sputum was collected and processed to produce cytospins for cell differential counts and supernatant for fluid phase measurements. Venous blood was collected and analysed for differential cell counts and separated into serum and plasma for further mediator analysis.

Measured sputum and serum biomarkers

A wide panel of biomarkers were measured using the MSD platform and single ELISA assays in the sputum and blood of patients at baseline stable and exacerbation visits. Exacerbation data recording and sampling was only performed in patients that had not received prior oral corticosteroids and/or antibiotics.

Serum and sputum samples were analysed using the MSD platform according to the manufacturer's instructions. In brief, 25μL of the cytokine assay diluents was added to the plate and incubated for 30 minutes. This was followed by the addition of 25μL of serum or sputum D-PBS supernatant and incubated for 2 hours. The plate was then washed 3 times with diluted wash buffer and 25μL of detection antibody was added. After a further incubation period of 1 hour and a repeated wash step, 150μL of read buffer was added and the plate was read.

C reactive protein assay

CRP was measured as described in chapter 4.

Procalcitonin

PCT was measured as described in chapter 4.

Neopterin

Quantitative determinations of neopterin were performed using a competitive ELISA (Neopterin ELISA, IBL Hamburg, Germany). 10µL of each standard, control, serum, and sputum supernatant were transferred to respective wells of the Microtiter plate (coated with anti-rabbit IgG). 100µL of enzyme conjugate was transferred to each well along with 50µL of neopterin antiserum. The plate was incubated for 90 minutes at room temperature on an orbital shaker in the dark. The plate was then washed three times and 150µL substrate added and then incubated for a further 10 minutes at room temperature. Finally the reaction was stopped by adding 150µL stop solution into each well. The optical density was measured at 450nm. The intra-assay imprecision was <7% of the coefficient of variation (CV) and the inter-assay imprecision was <13% of the CV.

Surfactant protein D (SPD)

Serum surfactant protein D (SPD) was measured from 100µl of serum in triplicate using a mouse monoclonal antibody in-house ELISA. Plates (Corning Inc., Costar 3590) were coated overnight at 4°C with rabbit polyclonal anti-SPD antibody at 1.6µg/mL in coating buffer. The wells were then washed with buffer (PBS, 0.05% v/v Tween20) and blocked for 2 hours with 300 µl/well of blocking buffer (PBS, 3% w/v BSA, 0.05% v/v

Tween20). Plates were then washed and standards and samples were diluted in buffer and incubated for 2 hours. After washing, the wells were incubated with mouse anti-SPD monoclonal antibody (1:1000 in blocking buffer). After a further wash step, the wells were incubated with rabbit anti-mouse horseradish peroxidise (HRP) antibody (1:20000 in blocking buffer) for 1 hour. The reaction was developed for 10 min with tetramethylbenzidine (TMB) substrate solution (Sigma-Aldrich Co, Dorset, UK), and stopped with 1M H_2SO4. HRP activity was measured in a plate reader (Molecular Devices, Thermo-max microplate reader) at 450 nm.

Eosinophilic cationic protein

Total serum ECP was measured using a commercial ELISA (Diagnostics Development, Uppsala Sweden). Briefly 100 µL of sample and standard were added to pre-coated anti-human ECP polyclonal rabbit antibody and incubated for 60 minutes. After 3 wash steps, 100 µL of secondary antibody solution was added and incubated. Conjugate solution was added followed by incubation and addition of the substrate. Finally the reaction was stopped and read using the plate reader. The intra-assay imprecision was <5% of the CV and the inter-assay imprecision was <12% of the CV.

A definitive list of all measured markers and their detection limits is presented in table 5.1

Table 5.1; Lower and upper detection limits for all measured biomarkers using the MSD platform and single ELISA

Mediator	Lower limit of detection		Upper limit of detection	
	Serum	Sputum	Serum	Sputum
IL-1β (pg/mL)	1	5	10,000	20,000
IL-8 (pg/mL)	1	3	10,000	20,000
IL-6 (pg/mL)	6	4	10,000	10,000
TNF-α (pg/mL)	0.4	5	10,000	10,000
TNF-RI (pg/mL)	8	27	10,000	20,000
TNF-RII (pg/mL)	8	10	10,000	20,000
CCL26 (pg/mL)*	103	29	10,000	10,000
CCL17 (pg/mL)	7	6	10,000	10,000
IL-4 (pg/mL)*	0.5	5	10,000	10,000
IL-5 (pg/mL)	0.4	1	10,000	10,000
IL-13 (pg/mL)*	8	4	10,000	10,000
CCL2 (pg.mL)	8	8	10,000	10,000
CCL4 (pg/mL)	2	37	10,000	20,000
IL-10 (pg/mL)*	0.5	1	10,000	10,000
IL17A (pg/mL)*	2	6	10,000	10,000
IFN-γ (pg/mL)*	0.1	0.6	10,000	10,000
CXCL10 (pg/mL)	0.1	16	10,000	10,000
CXCL11 (pg/mL)	1	3	10,000	10,000
CCL3 (pg/mL)*	11	19	10,000	10,000
CCL13 (pg/mL)	2	8	10,000	10,000
GM-CSF(pg/mL)*	0.1	2	10,000	10,000
IL-6R (pg/mL)	-	10	-	20,000
CCL5 (pg/mL)	-	2	-	10,000
VEGF (pg/mL)	8	8	10,000	10,000
Neopterin (nmol/L)	0.7	0.2	111	28
Serum Amyloid A_1 (sAA_1) (ng/mL)	18	-	200,000	-
Procalcitonin (PCT) (ng/mL)	0.02	-	5000	-
Surfactant protein D (SPD) (ng/mL)	40	-	3500	-
C reactive protein (CRP) (mg/L)	5	-	500	-
Eosinophilic cationic protein (ECP) (ug/L)	0.47	-	600	-

* Sputum mediators below limit of detection >50% of the time or not recovered from standard spiking experiments in chapter 3. These sputum mediators not analysed further.

Detection of bacteria

Routine culture

Selected whole sputum for routine bacterial culture was processed according to the Health Protection Agency National Standard Method BSOP 57 (Health Protection Agency, 2009). Sputum was viewed macroscopically and described as mucoid, mucopurulent or purulent. An equal volume of 0.1% of DTT was added to the sputum and after gentle agitation was incubated for 15 minutes at 37°C to assist in homogenisation. A further dilution step of the homogenised sputum, whereby 10 µL was added to 5mL of sterile distilled water was performed. Using a sterile loop, 10µL (therefore at a dilution of 10^{-3}) was inoculated using the streaking method to a chocolate and blood agar media plate. The chocolate and blood agar plates were pre-prepared with a bacitracin and optochin disc respectively. Each plate was incubated in 10% CO_2 for 48 hours and read daily. Reporting for all samples was standardised as follows: No growth; No significant growth; and qualitative reporting for clinically significant pathogens as light, moderate and heavy growth for each dominant organism grown (*Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus, Streptococcus pneumoniae*, and *Pseudomonas aeruginosa*).

Colony forming units

Semi-quantitative bacterial analysis was performed by CFU estimation in accordance to previously described methods (Pye, 1995). 900µL of sterile D-PBS solution was placed in 5 sterile eppendorfs labelled as 10^1, 10^2, 10^3, 10^4, 10^5 and serial dilutions of the 100µL DTT filtrate removed during the sputum processing procedure were made. Three 20µL drops were placed from each serial dilution onto chocolate and blood agar media. Each plate was then incubated for 24 hours in 5% CO_2 at 37°C. After incubation,

counts were made from the dilution with <100 CFU and averaged for each of the droplets to determine a total CFU load.

Quantitative real time polymerase chain reaction assay

Total bacterial DNA was extracted from 500µL of homogenised sputum using the commercial QIAmp DNA Mini Kit assay (QIAGEN Ltd, Hilden Germany). Briefly DNA extraction was performed using a 2-step process. Firstly this involved lysis with 20mg/mL lysozyme followed by incubation at 37°C for 30 minutes. Further lysis was performed with Proteinase K (600mAU/mL solution) at 55°C for 30 minutes and 95°C for 15 minutes. The lysate obtained was buffered and added to the QIAamp spin column and centrifuged briefly for optimal adsorption of the DNA to the column's silica gel membrane. This was followed by three wash steps. Finally DNA was eluted in 200µL of DNAse, RNAse free distilled water and stored at -20°C.

DNA standards for RT-PCR were prepared from pure culture DNA extracted from overnight cultures of *E. coli, H. influenzae, M. catarrhalis, S. aureus* and *S. pneumoniae*. 10-fold dilutions were prepared ranging from 10^2 to 10^7.

Quantification of the total bacterial load, *H. influenzae* and *S. aureus* was performed using the SYBR Green assay (PE Applied Biosystems, Warrington, UK), which employs fluorescent binding to the minor groove of the DNA double helix following polymerisation. The TaqMan (Applied BioSystems, UK) assay was used to quantify *M. catarrhalis* and *S. pneumoniae*. Each assay run was performed with two negative controls (distilled RNase DNase water) and all sputum samples were examined in duplicate.

The target genes and primers for each of the RT-PCR assays are as shown in table 5.2.

Table 5.2; Target genes and primers for each of the qPCR bacteria detection assays

Target organism	Target gene	Primers	Sequence
Total bacteria	16S rDNA	338F	5' ACTCCTACGGGNGGCNGCA 3'
		515R	5' GTATTACCGCNNCTGCTGGCAC 3'
S. pneumoniae	Pneumolysin	S.pneumoniae F [(Greiner, 2001)]	5' AGCGATAGCTTTCTCCAAGTGG-3'
		S.pneumoniae R	5' CTTAGCCAACAAATCGTTTACCG 3'
		S.pneumoniae probe	5'**Cy5**-ACCCCAGCAATTCAAGTGTTCGCG-**BHQ2** 3'
H. influenzae	Outer membrane protein P6	P6 F [(Stralin, 2005)]	5' TTGGCGGWTACTCTGTTGCT 3'
		P6 R	5' TGCAGGTTTTTCTTCACCGT 3'
S. aureus	Thermonuclease	S.aureus (nuc) F [(Fang, 2003)]	5' GCGATTGATGGTGATACGGTT 3'
		S.aureus (nuc) R	5' AGCCAAGCCTTGACGAACTAAAGC 3'
M. catarrhalis	Outer membrane protein CopB	M. catarrhalis F [(Greiner, 2003)]	5' GTGAGTGCCGCTTTACAACC 3'
		M. catarrhalis R	5' TGTATCGCCTGCCAAGACAA 3'
		M. catarrhalis probe	5'**JOE**TGCTTTTGCAGCTGTTAGCCAGCCTAA-**TAMRA** 3'
M. pneumoniae	16S rRNA	Myco-1	5'AAGGACCTGCAAGGGTTCGT 3'
		Myco-2	5' CTCTAGCCATTACCTGCTAA 3'
C. pneumoniae	Outer membrane protein ompA	APNOL	5'ATTAAGAAGCTCTGAGCATA 3'
		APNOU	5'AATTCTCTGTAAACAAACCC 3'
		APN 1	5'AGCCTAACATGTAGACTCTGAT 3'
		APN 2	5' TGCCAACAGACGCTGGCGT 3'

Mycoplasma pneumoniae and *Chlamydophila pneumoniae* were detected using PCR (Creer, 2006).

Detection of virus

Viral RNA for PCR analysis was extracted from sputum, using in-house assays for examination of the following viruses; *rhinovirus, respiratory syncytial virus*, *human parainfluenza virus 1-3*, *adenovirus*, *influenza virus A and B*, *coronavirus 229E and OC43, human metapneumovirus* and *human bocavirus*. *Picorna* virus analysis was performed by a reverse transcription method followed by PCR.

Definition of bacteria, virus and sputum eosinophil associated exacerbations of COPD

Bacteria associated exacerbations were defined as a positive bacterial pathogen detected on routine culture (*H. influenzae, M. catarrhalis, S. pneumoniae, S. aureus* or *P. aeruginosa*) and/or a total aerobic CFU count $\geq 10^7$. Bacterial detection methods using qPCR were not used to define bacteria associated exacerbations in this study. A virus associated exacerbation was defined as one that had a positive sputum viral PCR, whether in isolation or in combination with a positive bacterial pathogen on routine culture. A sputum eosinophil associated exacerbation was defined as the presence >3% of the non-squamous cells following cytospin slide preparation.

Statistical Analysis

Statistical analysis was performed using PRISM version 4 (GraphPad, San Diego, CA) and SPSS version 16 (Chicago, IL). Parametric and non-parametric data was presented as mean (standard error of the mean) and median (interquartile range). Log transformed data was presented as geometric mean (95% confidence interval) throughout. Exacerbation events were assumed to be independent of each other. No adjustments for multiple comparisons were made across biomarkers.

For comparison of clinical and biomarker changes between baseline and exacerbation visits the paired T-test or Wilcoxon matched pairs test was used. For comparison of exacerbations associated with or without bacteria, virus and sputum eosinophilia the T-test and Mann-Whitney test was used respectively. To determine suitable biomarkers, the ROC curves were plotted for i) exacerbation versus stable state, ii) bacteria versus non-bacteria associated exacerbations, iii) virus versus non-virus associated exacerbations and iv) sputum eosinophilia (>3% non-squamous cells) versus non sputum eosinophilia associated exacerbations, to determine sensitivity and specific biomarkers of sub-phenotypes of COPD exacerbations.

A p value of < 0.05 was taken as the threshold of statistical significance.

Results

One hundred and fifty six patients were enrolled; 145 (101 men, 44 women) completed the first visit and 115 completed 12 months (figure 5.3). At baseline 45%, 40% and 17% had GOLD II, III, and IV respectively. The majority of patients recruited were current or ex-smokers (142/145), with a mean (range) pack year history of 49 (10 to 153) with an absolute and percentage mean (SEM) reversibility to inhaled bronchodilator on study entry of 47mL (11) and 4% (1) respectively. Skin prick testing or serum specific IgE to a wide panel of aeroallergens confirmed that 20% were atopic. Bacterial colonisation, defined as the presence of *H. influenzae, M. catarrhalis, S. pneumoniae, S. aureus* or *P. aeruginosa* by standard culture techniques (Health Protection Agency 2009, 2009) was present in 28% of patients at baseline. Using qPCR a bacterial pathogen (*H. influenzae, M. catarrhalis, S. pneumoniae, or S. aureus)* was detected in 86% of patients at the baseline stable visit. A virus was detected in 5% of patients at study entry, whilst eosinophilic airway inflammation was present in 27% of patients.

The baseline characteristics of all patients entered into the study and completing the first study visit are presented in table 5.3.

Figure 5.3 Consort diagram for 12 month observation biomarker study

| Visit 0 | **170 subjects screened** |

3 co-morbidity; 11 did not fulfil inclusion

| Visit 1 | **156 subjects entered** |

4 died; 7 co-morbidity

| Visit 2 | **145 subjects completed baseline** |

4 died; 5 co-morbidity; 3 withdrew

| Visit 3 | **133 subjects completed 3 months** |

2 died; 4 co-morbidity; 3 withdrew

| Visit 4 | **124 subjects completed 6 months** |

2 died; 5 co-morbidity; 1 withdrew

| Visit 5 | **116 subjects completed 9 months** |

1 died

| Visit 6 | **115 subjects completed 12 months** |

Table 5.3; Baseline characteristics for patients entered into study

	Baseline characteristics
Male, n (%)	101 (70)
Age*	69 [43 to 88]
Age at diagnosis*	62 [30 to 83]
Current Smokers, n (%)	42 (29)
Ex Smokers, n (%)	100 (69)
Pack year history	49 [10 to 153]
Exacerbations rate in previous 12 months	3 [1 – 12]
Body mass index, (kg/m^2)	26.5 (0.4)
Maintenance prednisolone, %	6
Prednisolone dosage (mg)*	6 [4 to 10]
Inhaled corticosteroid usage, %	86
Inhaled corticosteroid dose (mcg) †	1540 (59)
Inhaled long acting beta agonist usage, %	76
FEV_1, (L)	1.33 (0.05)
FEV_1 % predicted	52 (2)
FEV_1/FVC ratio, %	50 (1)
Reversibility, (mL)	47 (11)
Residual volume, %	129 (4)
T_LCO % predicted	56 (2)
KCO % predicted	70 (3)
SGRQ $_{Total}$ (units)	60.4 (3.4)
CRQ $_{Total/4}$ (units)	4.1 (0.1)
VAS $_{Total}$ (mm)	142 (6)

FEV_1 Forced expiratory volume in 1 second; FVC Forced vital capacity; T_LCO diffusion capacity of the lung for carbon monoxide; KCO diffusion capacity of the lung for carbon monoxide corrected for alveolar volume; SGRQ St Georges Respiratory Questionnaire, scores ranging from 0 to 100 with higher score indicating worse health status (total score on domains of Impact, Symptoms and Activity); CRQ Chronic Respiratory Disease Questionnaire, scores range between 1 to 7 with higher score representing better health quality; VAS Visual Analogue Scale, performed on 100mm line from 'no symptoms' to 'worst symptoms', higher scores represent worse symptoms (total score addition of measured domains: cough, dyspnoea, sputum production and sputum purulence); † Beclomethasone dipropionate equivalent. Data presented as mean (SEM) and * mean [range]

Men compared to women smoked more (pack year mean difference (95%CI) 16 (6 to 27), p=0.002), had greater degree of airflow obstruction (mean FEV_1/FVC % difference (95%CI) -8 (-13 to -3), p=0.003) and reported better disease specific health status measured by the CRQ (CRQ mean difference (95%CI) 0.2 (0.1 to 0.9), p=0.02). There was no difference in inhaled corticosteroid dosage or gas transfer between genders. There was no difference in clinical characteristics between current smokers or ex-smokers. No differences in age, pack years smoked or CRQ and VAS symptom scores were detected according to severity of lung disease defined by GOLD. The exacerbation frequency prior to study entry, inhaled corticosteroid dose, lung function and degree of airflow obstruction and SGRQ total score was significantly different between GOLD severities (table 5.4).

Table 5.4: Clinical characteristics according to GOLD severity

	GOLD 2	GOLD 3	GOLD 4	p-value
Age	70 (67 to 72)	69 (67 to 72)	66 (61 to 71)	0.17
Pack year history	44 (37 to 51)	52 (43 to 61)	55 (45 to 65)	0.21
SGRQ $_{Total}$ (units)	**46.7 (41.8 to 51.6)**	**52.4 (47.9 to 56.9)**	**59.1 (52.3 to 65.8)**	**0.01**
CRQ $_{Total/4}$ (units)	4.2 (3.9 to 4.5)	4.0 (3.7 to 4.3)	4.0 (3.5 to 4.4)	0.63
VAS $_{Total}$ (mm)	140 (121 to 158)	151 (129 to 173)	126 (95 to 157)	0.39
Exacerbations in prev. yr	**2.8 (2.2 to 3.5)**	**3.8 (3.0 to 4.5)**	**4.5 (3.4 to 5.5)**	**0.02**
FEV_1, L [†]	**1.7 (1.6 to 1.8)**	**1.2 (1.1 to 1.3)**	**0.7 (0.6 to 0.8)**	**<0.01**
FEV_1/FVC ratio, % [†]	**61 (59 to 64)**	**48 (45 to 52)**	**38 (34 to 42)**	**<0.01**
FEV_1 % predicted [†]	**68 (65 to 70)**	**44 (41 to 47)**	**26 (22 to 30)**	**<0.01**
Inhaled corticosteroid dose, μg [‡]	**949 (741 to 1157)**	**1625 (1470 to 1781)**	**1660 (1335 to 1985)**	**<0.01**

SGRQ St Georges Respiratory Questionnaire, CRQ Chronic Respiratory Disease Questionnaire; VAS Visual Analogue Scale; FEV_1 Forced expiratory volume in 1 second; FVC Forced vital capacity; [†] post bronchodilator; [‡] Beclomethasone dipropionate equivalent, data presented as mean (95% CI)

At Exacerbation

A total of 182 exacerbation events were captured from 86 patients, 21 exacerbations warranted hospitalisation. Changes in lung function, health status and clinical characteristics form baseline to exacerbation are shown in table 5.5.

Table 5.5; Clinical characteristics for patients with captured exacerbations

	Study Entry	Exacerbation	p-Value
FEV_1 (L) [‡]	**1.33 (0.05)**	**1.10 (0.04)**	**<0.01**
FEV_1 % predicted [‡]	**52 (2)**	**42 (1)**	**<0.01**
Reversibility (mL)	47 (11)	37 (11)	0.50
FEV_1/FVC ratio [‡] %	52 (2)	50 (1)	0.65
$CRQ_{DYSPNOEA}$ (units)	**3.27 (0.10)**	**2.27 (0.08)**	**<0.01**
$CRQ_{EMOTION}$ (units)	**4.59 (0.11)**	**2.71 (0.15)**	**<0.01**
$CRQ_{FATIGUE}$ (units)	**3.65 (0.10)**	**2.71 (0.15)**	**<0.01**
$CRQ_{MASTERY}$ (units)	**4.81 (0.12)**	**3.80 (0.12)**	**<0.01**
CRQ_{TOTAL} (units)	**4.11 (0.10)**	**3.12 (0.08)**	**<0.01**
$VAS_{DYSPNOEA}$ (mm)	**47 (2)**	**71 (2)**	**<0.01**
VAS_{COUGH} (mm)	**35 (2)**	**61 (2)**	**<0.01**
$VAS_{SPUTUM\ PRODUCTION}$ (mm)	**33 (2)**	**58 (2)**	**<0.01**
$VAS_{SPUTUM\ PURULENCE}$ (mm)	**28 (2)**	**52 (2)**	**<0.01**
VAS_{TOTAL} (mm)	**142 (6)**	**239 (6)**	**<0.01**
Peripheral leukocyte count [#] ($x10^9$ cells/L)	**8.2 [7.9 to 8.6]**	**9.3 [8.9 to 9.8]**	**<0.01**
Peripheral neutrophil count [#] ($x10^9$ cells/L)	**5.3 [5.0 to 5.6]**	**6.3 [6.0 to 6.7]**	**<0.01**
Peripheral eosinophil count [#] ($x10^9$ cells/L)	0.21 [0.18 to 0.23]	0.19 [0.17 to 0.22]	0.84
Total sputum cell count [#] ($x10^6$ cells/g sputum)	**3.8 [3.1 to 4.7]**	**6.4 [5.2 to 7.8]**	**<0.01**
Sputum neutrophil count, %	**68 (2)**	**74 (2)**	**0.02**
Sputum eosinophil count, % [#]	1.2 [1.0 to 1.6]	1.1 [0.9 to 1.5]	0.58

FEV_1 Forced expiratory volume in 1 second; FVC Forced vital capacity; CRQ Chronic Respiratory Disease Questionnaire, scores range between 1 to 7 with higher score representing better health quality; VAS Visual Analogue Scale, performed on 100mm line from 'no symptoms' to 'worst symptoms', higher scores represent worse symptoms (total score addition of measured domains: cough, dyspnoea, sputum production and sputum purulence). Data presented as mean (SEM), unless stated. # Geometric mean [95% confidence interval]; [‡] post bronchodilator

There was a reduction in the FEV_1 and CRQ from baseline to exacerbation (FEV_1 (L) 1.33 vs. 1.10; mean difference 0.23, 95% confidence interval (CI) 0.12 to 0.36; p<0.001; CRQ (units) 4.11 vs. 3.12; mean difference 0.99, 95% CI 0.74 to 1.23; p<0.001). The magnitude of these changes was independent of smoking status, gender, GOLD severity (figure 5.4) or Anthonisen criteria (figure 5.5).

Hospitalised exacerbations were associated with a greater fall in lung function compared to exacerbations that were not hospitalised (ΔFEV_1 (mL) -355 vs. -131; mean difference 224, 95% confidence interval of difference -356 to -92, p<0.001), but not health status decline (ΔCRQ (units) -1.25 vs. -0.91; mean difference 0.34, 95% confidence interval of difference -0.83 to 0.15, p=0.18).

Figure 5.4; CRQ and FEV_1 for subjects at baseline and exacerbations categorised by GOLD severity. Data presented as mean (SEM). Red = baseline; Green = Exacerbation
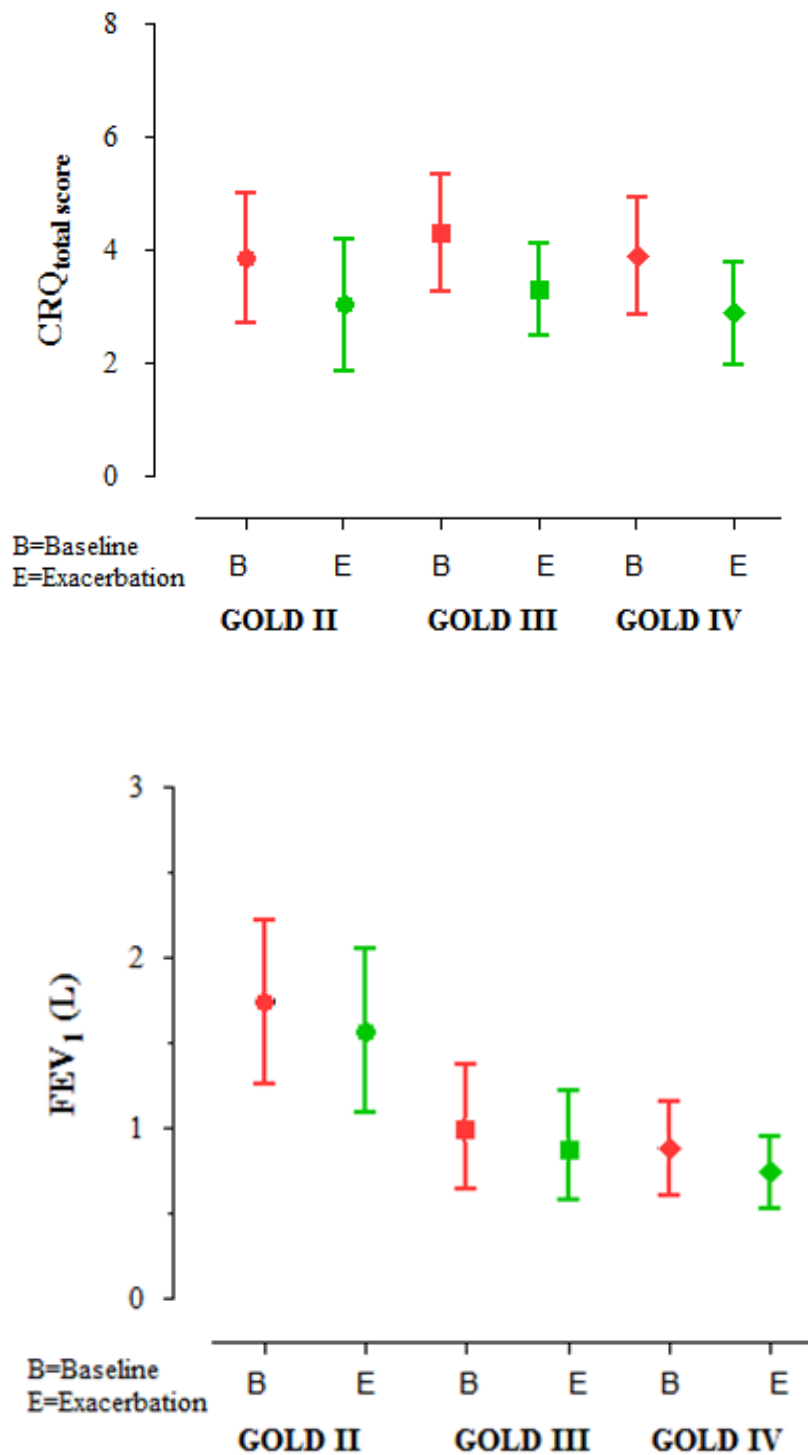
Figure 5.5; Change in CRQ and FEV$_1$ from baseline to exacerbation categorised by Anthonisen severity. Data presented as mean (standard error of the mean). Anthonisen I, II, III brown, blue and purple respectively
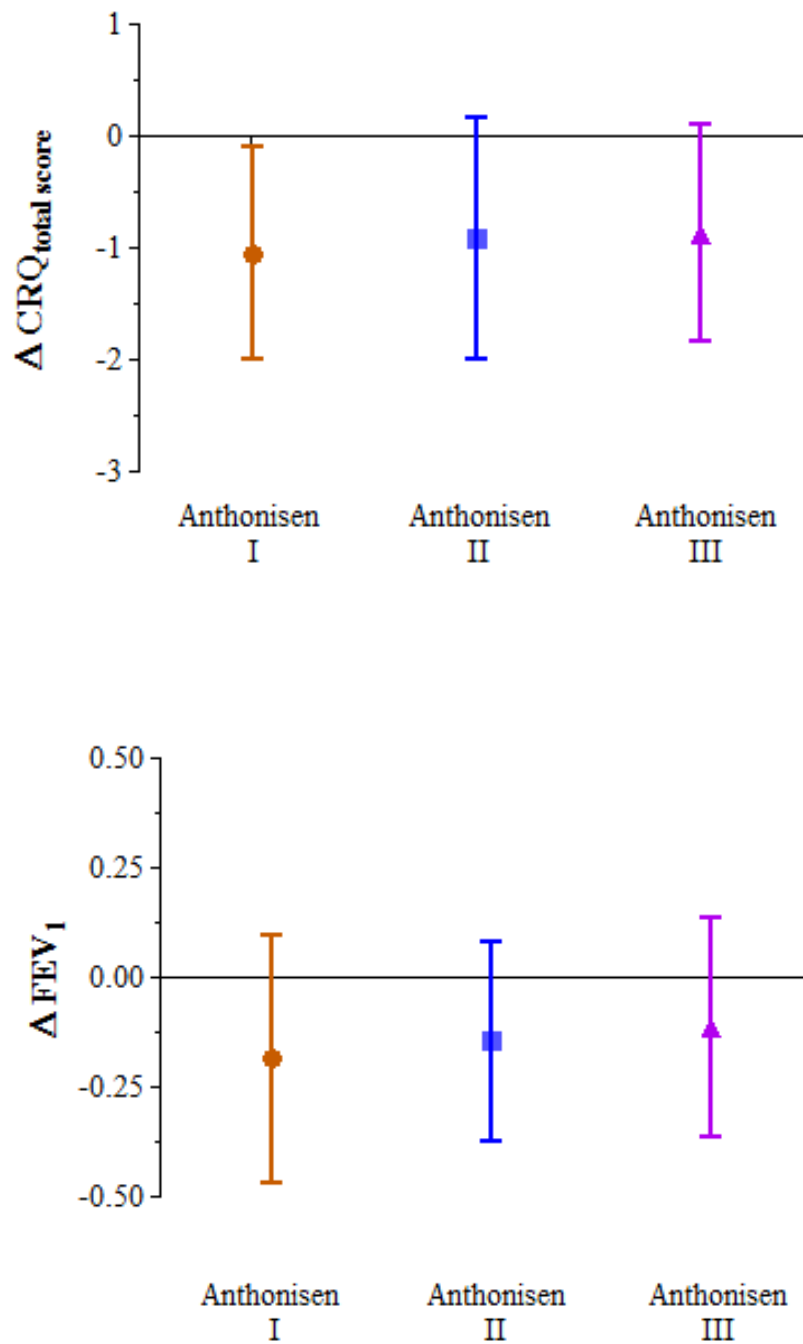
Sputum and serum biomarkers

Sputum and serum mediator data was available for 148 exacerbation events from 75 patients. Serum biomarkers that increased during an exacerbation were IL-6, TNF receptors I and II, sAA_1, CRP, PCT and ECP (table 5.6). Sputum biomarkers that increased were IL-1β, TNF-α, TNF-RI, TNF-RII, IL-6 and CCL5 (table 5.7).

Of all sputum and serum biomarkers measured there was a significantly increased level of serum CRP and TNF-α in subjects that were hospitalised (CRP median (IQR) 56 (102) vs. 8 (14), p=0.002; serum TNF-α geometric mean (95%CI) 4.3 (3.4 to 5.4) vs. 3.4 (3.2 to 3.6), p=0.02).

Table 5.6; Serum biomarkers at stable state and exacerbations

Serum	Baseline	Exacerbation	p-Value
IL1β (pg/mL)	6.9 [6.4 to 7.4]	7.2 [6.7 to 7.7]	0.35
IL-8 (pg/mL)	**6.7 [6.1 to 7.3]**	**5.9 [5.3 to 6.5]**	**0.04**
IL-6 (pg/mL)	**15.4 [11.6 to 20.4]**	**22.0 [18.8 to 25.8]**	**<0.01**
TNF-α (pg/mL)	3.5 [3.3 to 3.7]	3.5 [3.3 to 3.7]	0.71
TNF-RI (pg/mL)	**8104 [7567 to 8679]**	**8794 [8206 to 9426]**	**<0.01**
TNF-RII (pg/mL)	**5126 [4845 to 5423]**	**5333 [5036 to 5648]**	**0.03**
CCL17 (pg/mL)	**545 [469 to 633]**	**436 [375 to 507]**	**<0.01**
IL-5 (pg/mL)	2.2 [2.1 to 2.4]	2.4 [2.2 to 2.5]	0.11
IL-13 (pg/mL)	30.8 [27.7 to 34.3]	29.5 [27.2 to 32.1]	0.05
CCL2 (pg/mL)	655 [619 to 692]	623 [585 to 669]	0.07
CCL4 (pg/mL)	406 [379 to 435]	386 [361 to 413]	0.05
IL-10 (pg/mL)	1.5 [0.8 to 2.8]	1.0 [0.8 to 1.4]	0.26
IFN-γ (pg/mL)	0.2 [0.1 to 0.3]	0.2 [0.1 to 0.3]	0.85
CXCL10 (pg/mL)	58.7 [53.3 to 64.6]	61.6 [54.9 to 69.3]	0.36
CXCL11 (pg/mL)	63.9 [58.0 to 70.4]	65.4 [58.9 to 72.6]	0.63
CCL3 (pg/mL)	**20.0 [16.0 to 24.8]**	**18.4 [16.7 to 20.2]**	**<0.01**
CCL13 (pg/mL)	**692 [634 to 755]**	**603 [550 to 662]**	**<0.01**
sAA1 (ug/mL)	**4.8 [3.8 to 6.0]**	**11.1 [8.1 to 15.1]**	**<0.01**
GM-CSF (pg/mL)	0.3 [0.2 to 0.7]	0.2 [0.2 to 0.3]	0.14
CRP (mg/l)‡	**4 (9)**	**8 (26)**	**<0.01**
SPD (ng/mL)‡	**188 (141)**	**164 (144)**	**0.02**
PCT (ng/mL)‡	**0.05 (0.03)**	**0.06 (0.04)**	**0.01**
ECP (ug/L)	**59.3 [52.1 to 67.4]**	**78.8 [68.9 to 90.1]**	**<0.01**
Neopterin (nmol/L)	9.1 [8.4 to 9.9]	11.4 [10.5 to 12.4]	0.06

Data presented as geometric mean (95%CI); ‡ median (interquartile range)

Table 5.7; Sputum biomarkers at stable state and exacerbations

Sputum	Baseline	Exacerbation	p-Value
IL1β (pg/mL)	**112 [50 to 249]**	**214 [151 to 303]**	**<0.01**
IL-8 (pg/mL)	6043 [4088 to 8933]	5482 [4565 to 6582]	0.09
IL-6 (pg/mL)	**619 [407 to 940]**	**747 [590 to 947]**	**<0.01**
TNF-α (pg/mL)	**157 [136 to 181]**	**261 [217 to 315]**	**<0.01**
TNF-RI (pg/mL)	**9.8 [5.1 to 18.7]**	**27.4 [20.0 to 37.5]**	**<0.01**
TNF-RII (pg/mL)	**1240 [1069 to 1438]**	**1886 [1584 to 2246]**	**<0.01**
CCL5(pg/mL)	**398 [333 to 476]**	**730 [597 to 891]**	**<0.01**
CCL17 (pg/mL)	**3.8 [2.7 to 5.3]**	**6.6 [5.4 to 8.0]**	**<0.01**
IL-5 (pg/mL)	**24.6 [15.2 to 39.6]**	**13.3 [11.0 to 16.2]**	**<0.01**
CCL2 (pg/mL)	1.9 [1.2 to 3.2]	1.5 [1.2 to 1.8]	0.23
CCL4 (pg/mL)	626 [533 to 736]	523 [430 to 642]	0.16
CXCL10 (pg/mL)	**1007 [875 to 1159]**	**1463 [1204 to 1778]**	**<0.01**
CXCL11 (pg/mL)	215 [139 to 332]	389 [286 to 528]	0.11
CCL13 (pg/mL)	86.8 [58.2 to 129.4]	98.4 [78.3 to 123.5]	0.40
Neopterin (nmol/g)	**34.0 [22.2 to 52.0]**	**19.3 [16.1 to 23.1]**	**0.01**

Data presented as geometric mean (95%CI)

No single biomarker had an area under the ROC curve >0.70 in determining an exacerbation from stable state (figure 5.6).

Figure 5.6; Receiver operator characteristic curves for biomarkers that positively predict exacerbations from baseline stable state. Area under the ROC curve (95% confidence interval) is shown in the parentheses
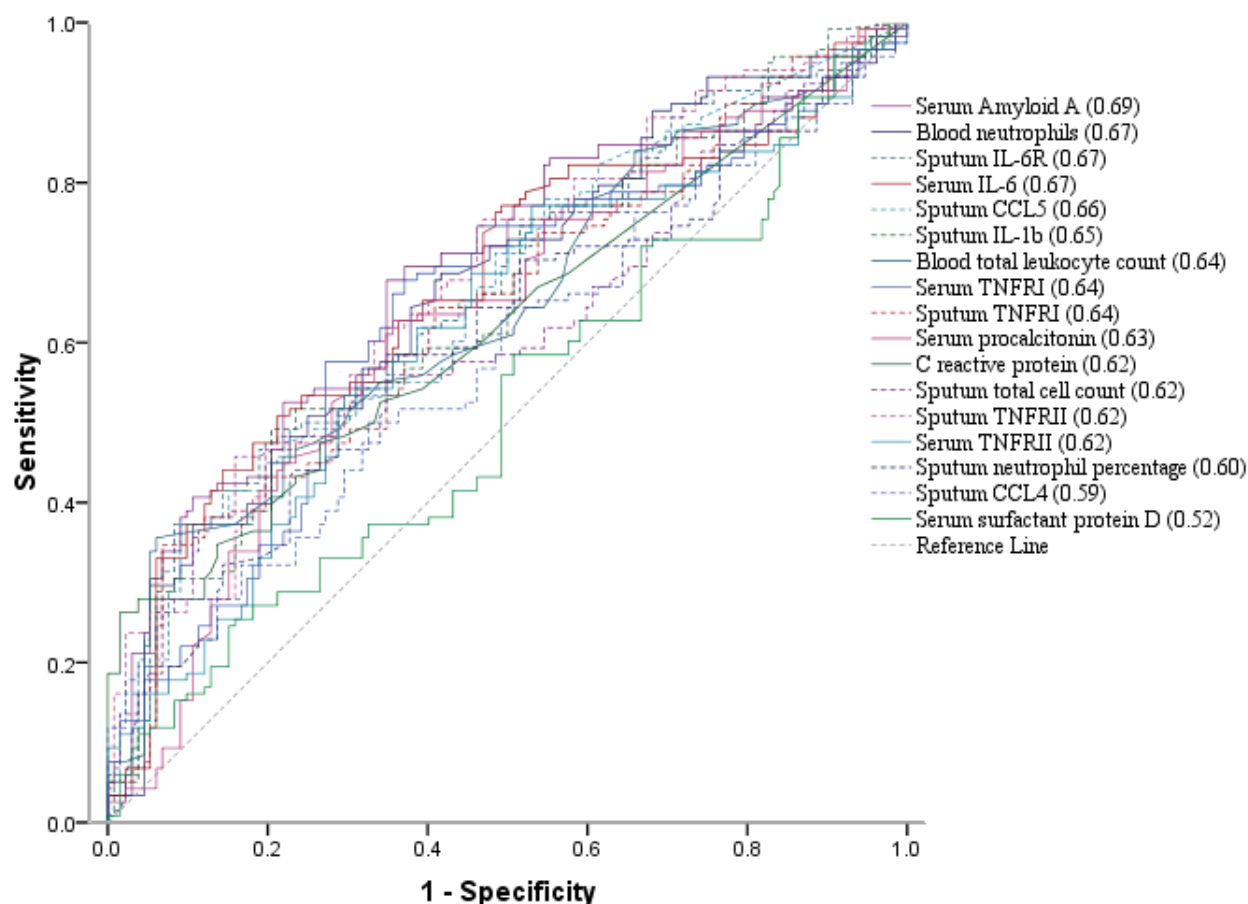
Exacerbations associated with bacteria

Bacteria associated exacerbations occurred in 55% of all exacerbations (positive bacterial pathogen on routine culture and/or CFU $\geq 10^7$). Standard microbiological culture techniques were positive for a PPM in 35% of exacerbation samples. 67 organisms were detected; 50, 7 and 1 exacerbations were associated with 1, 2 and 3 organisms respectively. The detection rates were as follows: *H. influenzae* 27/67 (40%),

M. catarrhalis 22/67 (33%), *S. pneumoniae* 10/67 (15%), *P. aeruginosa* 4/67 (6%) and *S. aureus* 4/67 (6%).

There were 104 paired baseline and exacerbation qPCR results. These showed either a gain, loss or no change from baseline of *H. influenzae* in 23 (22%), 23 (22%) and 58 (56%) events; *S. aureus* in 4 (4%), 7 (7%) and 93 (89%); *S. pneumoniae* in 14 (13%), 29 (28%) and 61 (59%); *M. catarrhalis* 24 (23%), 22 (21%) and 58 (56%). This showed a gain or acquisition of a qPCR detected pathogen in 15%, a loss in 20% and no change in 65% from baseline to exacerbations samples.

In bacteria associated exacerbations blood and sputum neutrophils were increased compared to baseline. There was a significant increase in CFU's from baseline to exacerbation (geometric mean (95% CI) 1.7^6 (1.2^6 to 2.4^6) vs. 2.9^6 (1.9^6 to 4.6^6), p=0.027) and the proportion of samples with a CFU bacterial load of $>10^7$ increased from 32% at baseline to 46% during exacerbations (χ^2 p=0.02). Total bacterial load (16S) was higher in subjects with a bacteria associated exacerbation than those without (geometric mean (95% CI) 7.6^7 (4.2^7 to 1.4^8) vs. 2.8^8 (1.7^8 to 4.7^8), p=0.001). There was no difference in the 16S signal across exacerbations of Anthonisen types (ANOVA p=0.64). Clinical assessments of change in FEV$_1$, symptoms of sputum production and sputum purulence had an area under the ROC curve (95%CI) of 0.45 (0.35 to 0.55), 0.50 (0.40 to 0.60) and 0.58 (0.48 to 0.68) respectively. The most suitable biomarker for determining bacteria associated exacerbations was sputum IL-1β with an area under the ROC curve (95% CI) of 0.89 (0.83 to 0.95). A cut off of 125pg/mL had a sensitivity of 90% and a specificity of 80%. The best serum biomarker was CRP with an area under the ROC curve (95% confidence interval) of 0.65 (0.57 to 0.74). A serum CRP cut off of 10 mg/L had a sensitivity of 60% and specificity of 70% (figure 5.7).

Figure 5.7; Receiver operator characteristic curves for biomarkers that positively predict bacteria associated exacerbations. Area under the ROC curve (95% confidence interval) is shown in the parentheses.
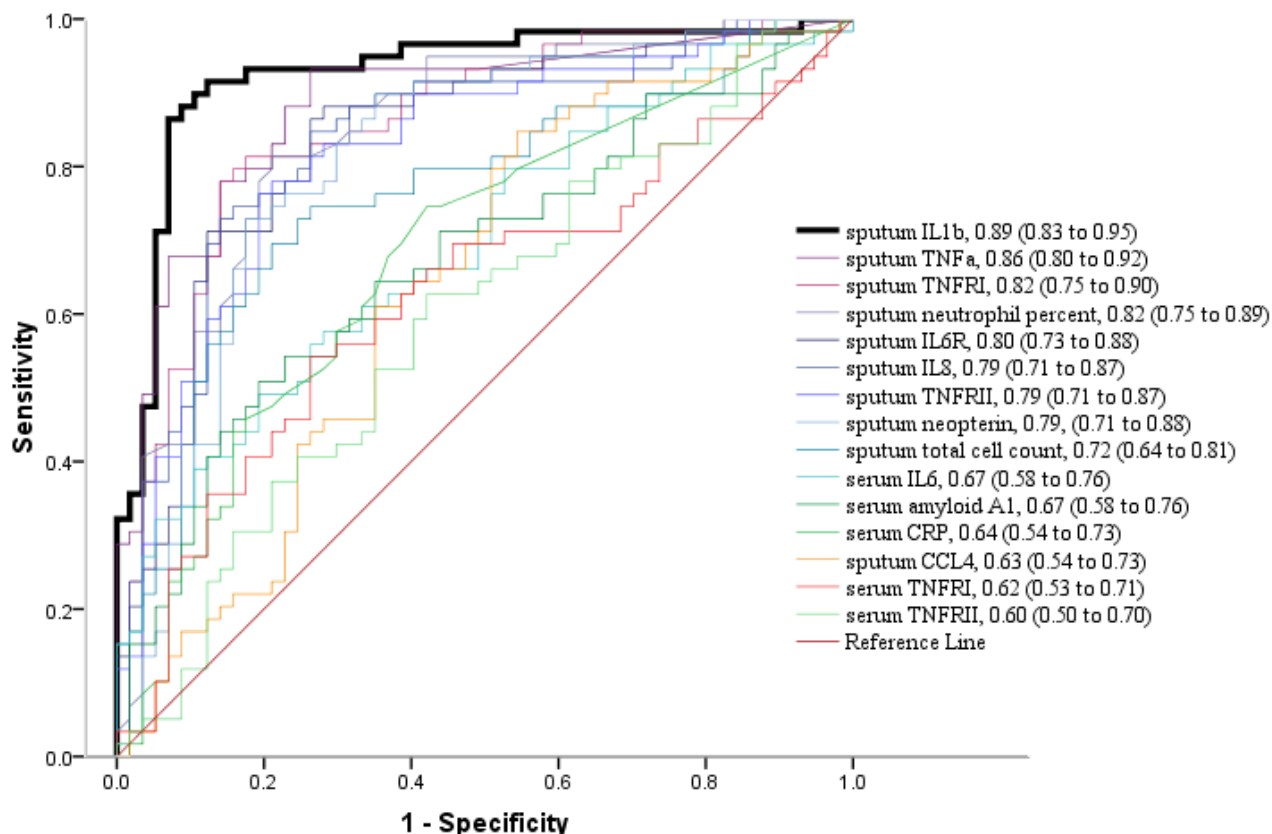
Exacerbations associated with virus

29% of exacerbations were associated with a virus, and RV was the most frequently detected. In total 47 exacerbations with a virus were identified. The detection rates for any of the identified viruses are as follows*: rhinovirus* 26/47 (45%), *respiratory syncytial virus* 14/47 (30%), *influenza* 4/47 (9%), *parainfluenza* 1/47 (2%), *coronavirus* 1/47 (2%) and *human bocavirus* 1/47 (2%). Virus associated exacerbations had a larger fall in % FEV_1 compared to non-virus associated exacerbations (-17% vs. -9%; mean difference -8%, 95% CI -16 to -1; p=0.04). Clinical assessments of change in FEV_1,

symptoms of cough and breathlessness had an area under the ROC curve (95%CI) of 0.43 (0.32 to 0.53), 0.62 (0.52 to 0.72) and 0.51 (0.41 to 0.62) respectively. The best marker for distinguishing the presence of a virus at exacerbation was serum CXCL10 (IP-10), with an area under the ROC curve (95% CI) of 0.76 (0.67 to 0.86). A serum CXCL10 cut off of 56pg/mL gave a sensitivity of 75% and specificity of 65% (figure 5.8). In exacerbations associated with virus alone the area under ROC curve (95% CI) for serum CXCL10 improved to 0.83 (0.70 to 0.96).

Figure 5.8; Receiver operator characteristic curves for biomarkers that positively predict virus associated exacerbations. Area under the ROC curve (95% confidence interval) is shown in the parentheses.
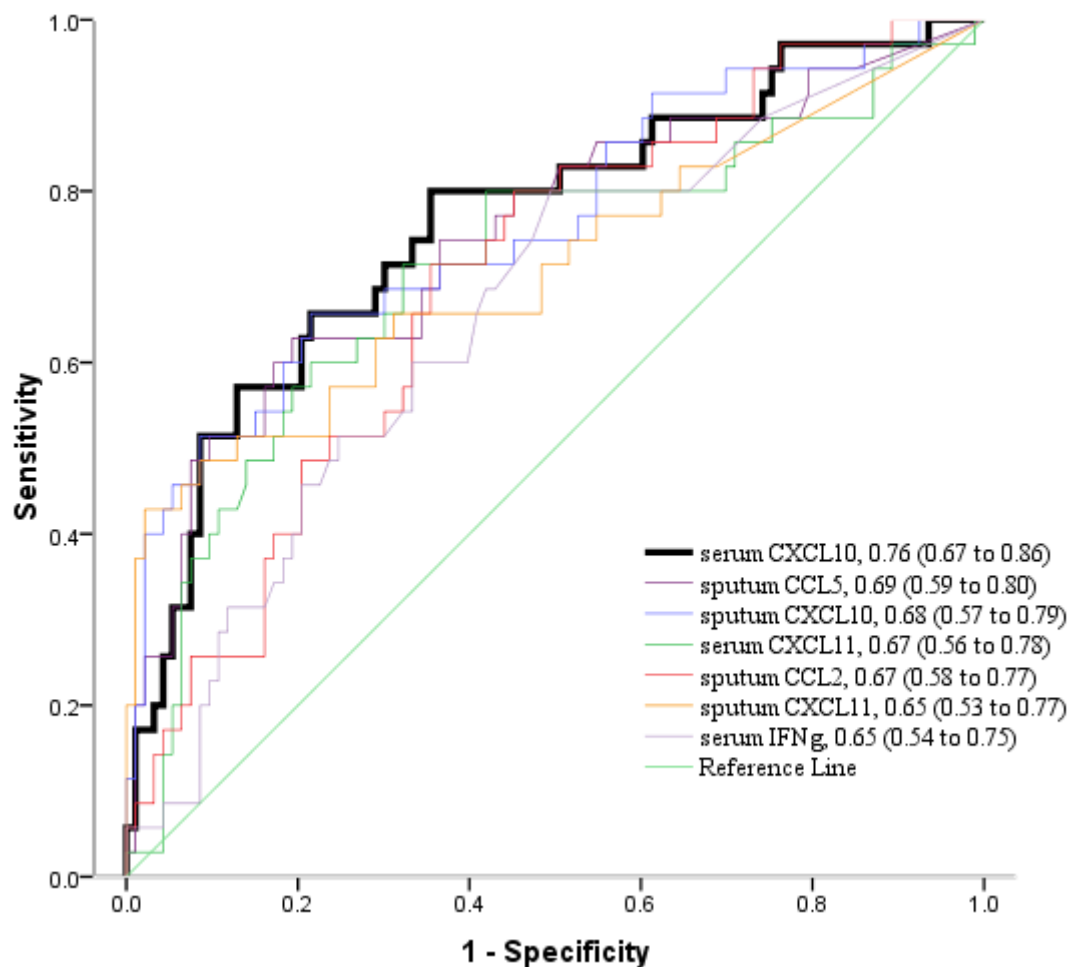
Exacerbations associated with sputum eosinophilia

A sputum eosinophilia was observed in 28% of exacerbations. The most sensitive and specific measure to determine a sputum eosinophilia at exacerbation was the percentage peripheral blood eosinophil count with an area under the ROC curve (95% CI) of 0.85 (0.78 to 0.93). A cut off of 2% peripheral blood eosinophils had a sensitivity of 90% and specificity of 60% for identifying a sputum eosinophilia of > 3% at exacerbation (figure 5.9).

Figure 5.9; Receiver operator characteristic curves for biomarkers that positively predict sputum eosinophil associated exacerbations. Area under the ROC curve (95% confidence interval) is shown in the parentheses.
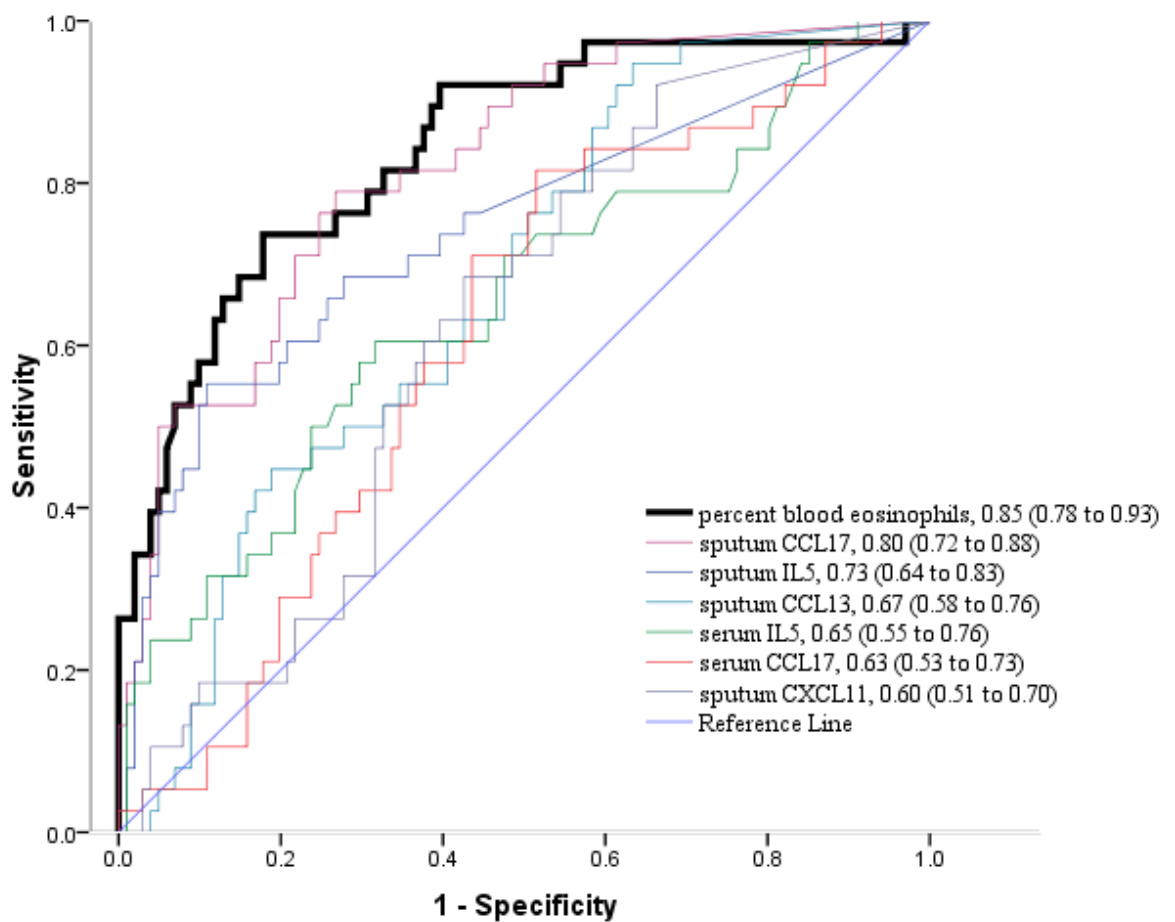
In summary, the associated pathogens and inflammatory hallmarks identified in association with an exacerbation were as follows: *bacteria alone* 37%; *virus alone* 10%; *sputum eosinophilia alone* 17%; *bacteria + virus* 12%; *bacteria + sputum eosinophilia* 6%; *virus + sputum eosinophilia* 3%; *bacteria + virus + sputum eosinophilia* 1%; none 14% (figure 5.10)

Figure 5.10; Non proportional Venn diagram for aetiological and inflammatory causes of exacerbation events
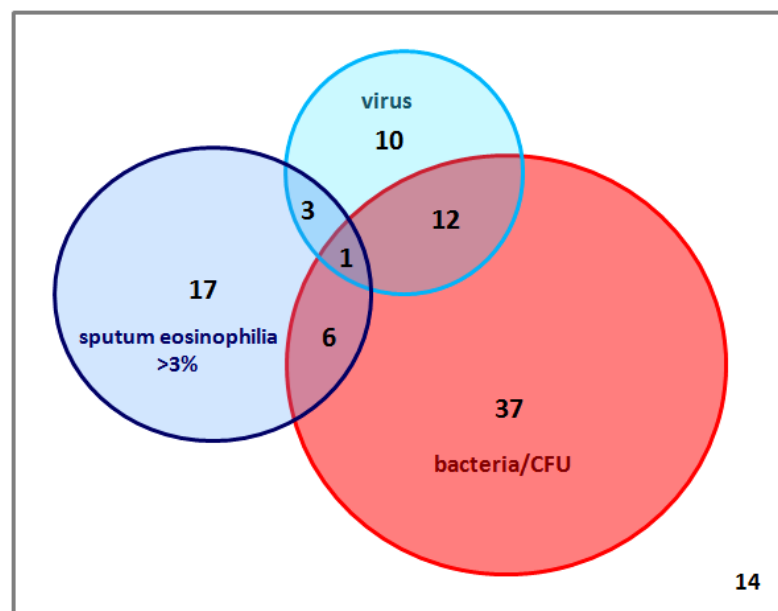
Multivariate modelling using combinations of 2 or 3 biomarkers for the detection of bacteria, virus and eosinophil associated exacerbations did not significantly improve on the use of a single mediator alone. Differential clinical and biomarker expression for exacerbations associated with bacteria, virus and sputum eosinophilia are shown in table 5.8 to 5.10.

Table 5.8; Clinical features of exacerbations associated with bacteria, virus or eosinophilic airway inflammation

	Bacterial	Non Bacterial	p value	Virus	No Virus	p value	Eosinophilic	Non Eosinophilic	p value
Δ FEV$_1$ (L) [‡]	-0.18 (-0.24 to -0.13)	-0.14 (-0.22 to -0.07)	0.35	**-0.24 (-0.32 to -0.16)**	**-0.14 (-0.19 to -0.09)**	**0.03**	-0.11 (-0.18 to -0.03)	-0.18 (-0.22 to -0.13)	0.13
FEV$_1$ % fall [‡]	-13 (-17 to -9)	-9 (-15 to -3)	0.24	**-17 (-22 to -12)**	**-9 (-13 to -5)**	**0.04**	-8 (-15 to 0)	-11 (-16 to -8)	0.27
Δ CRQ $_{Total}$ (units)	-1.11 (-1.31 to -0.91)	-0.84 (-1.09 to -0.59)	0.10	-1.01 (-1.35 to -0.68)	-0.98 (-1.16 to -0.81)	0.86	-0.99 (-1.28 to -0.70)	-0.95 (-1.14 to -0.77)	0.85
Δ VAS $_{Total}$ (mm)	84 (64 to 104)	96 (75 to 121)	0.55	100 (72 to 128)	80 (64 to 96)	0.22	80 (52 to 108)	88 (76 to 104)	0.50
Peripheral leukocytes (x10^9 cells/L) [#]	**9.9 [9.3 to 10.6]**	**8.5 [7.9 to 9.2]**	**<0.01**	9.0 [8.3 to 9.9]	9.4 [8.9 to 10.0]	0.46	**8.6 [7.9 to 9.3]**	**9.7 [9.2 to 10.3]**	**0.01**
Peripheral neutrophils (x10^9 cells/L) [#]	**6.9 [6.4 to 7.5]**	**5.6 [5.1 to 6.1]**	**<0.01**	5.9 [5.3 to 6.6]	6.4 [6.0 to 6.9]	0.25	**5.5 [5.0 to 6.2]**	**6.6 [6.2 to 7.2]**	**0.01**
Peripheral eosinophils (x10^9 cells/L) [#]	0.19 [0.16 to 0.22]	0.20 [0.16 to 0.25]	0.28	0.20 [0.15 to 0.25]	0.20 [0.17 to 0.24]	0.32	**0.34 [0.27 to 0.43]**	**0.15 [0.13 to 0.18]**	**<0.01**
Sputum total cell count (x10^6 cells/g) [#]	**10.5 [8.0 to 13.9]**	**3.7 [2.8 to 5.0]**	**<0.01**	7.4 [4.8 to 11.4]	7.1 [5.5 to 9.0]	0.56	**3.6 [2.5 to 5.0]**	**8.2 [6.5 to 10.4]**	**<0.01**
Sputum neutrophils, %	**85 (2)**	**63 (3)**	**<0.01**	71 (4)	76 (2)	0.51	**59 (3)**	**80 (2)**	**<0.01**
Sputum eosinophils, % [#]	**0.7 [0.5 to 0.9]**	**2.2 [1.4 to 3.3]**	**<0.01**	1.0 [0.6 to 1.5]	1.3 [0.9 to 1.8]	0.42	**12.1 [9.5 to 15.3]**	**0.5 [0.4 to 0.5]**	**<0.01**

Δ Change from baseline; FEV$_1$ Forced expiratory volume in 1 second; CRQ Chronic Respiratory Disease Questionnaire, scores range between 1 to 7 with higher score representing better health quality; VAS Visual Analogue Scale, performed on 100mm line from 'no symptoms' to 'worst symptoms', higher scores represent worse symptoms (total score addition of measured domains: cough, dyspnoea, sputum production and sputum purulence). Data presented as mean difference (95% confidence interval) unless stated; # geometric mean [95% confidence interval]. ‡post bronchodilator

Table 5.9; Serum biomarker levels at exacerbations associated with bacteria, virus or eosinophilic airway inflammation. Data presented as geometric mean (95%CI) ‡ median(IQR)

Serum	Bacterial	Non Bacterial	p-value	Viral	Non Viral	p-value	Eosinophilic	Non eosinophilic	p-value
IL1β (pg/mL)	6.9 [6.3 to 7.5]	7.1 [6.2 to 8.1]	0.63	7.4 [5.9 to 9.3]	7.0 [6.5 to 7.5]	0.49	7.7 [6.6 to 8.8]	7.1 [6.6 to 7.6]	0.19
IL-8 (pg/mL)	5.9 [5.1 to 6.8]	5.8 [4.9 to 6.8]	0.87	6.2 [4.9 to 7.9]	5.9 [5.2 to 6.7]	0.70	5.4 [4.5 to 6.5]	5.9 [5.3 to 6.5]	0.32
IL-6 (pg/mL)	**29.1 [22.5 to 37.8]**	**15.8 [13.3 to 18.3]**	**<0.01**	27.4 [19.4 to 38.7]	21.2 [17.4 to 25.9]	0.55	**15.1 [12.8 to 17.8]**	**25.6 [20.7 to 31.6]**	**0.01**
TNF-α (pg/mL)	3.6 [3.3 to 3.9]	3.3 [3.0 to 3.7]	0.37	3.6 [3.1 to 4.2]	3.4 [3.2 to 3.7]	0.52	3.6 (3.2 to 4.1)	3.5 [3.3 to 3.7]	0.58
TNF-RI (pg/mL)	**9496 [8562 to 10532]**	**8112 [7287 to 9030]**	**0.04**	8587 [7164 to 10293]	8873 [8175 to 9630]	0.71	**7353 (6684 to 8088)**	**8788 [8206 to 9411]**	**<0.01**
TNF-RII (pg/mL)	**5674 [5271 to 6108]**	**5022 [4549 to 5545]**	**0.05**	5492 [5164 to 6228]	5327 [4971 to 5709]	0.67	**4684 (4254 to 5157)**	**5333 [5040 to 5643]**	**0.01**
IL-5 (pg/mL)	2.3 [2.1 to 2.5]	2.3 [2.0 to 2.6]	0.92	2.4 [2.1 to 2.8]	2.3 [2.1 to 2.5]	0.50	**2.8 [2.5 to 3.1]**	**2.4 [2.2 to 2.5]**	**0.01**
CCL2 (pg/mL)	638 [583 to698]	601 [533 to 677]	0.41	646 [553 to 774]	627 [581 to 676]	0.60	608 [513 to 720]	623 [583 to 665]	0.70
CCL4 (pg/mL)	402 [367 to 441]	386 [346 to 431]	0.57	389 [336 to 450]	389 [358 to 422]	0.99	384 [338 to 435]	382 [357 to 409]	0.97
IFN-γ (pg/mL)	0.2 [0.1 to 0.3]	0.2 [0.1 to 0.3]	0.93	**0.4 [0.2 to 0.6]**	**0.2 [0.1 to 0.2]**	**0.02**	0.2 [0.1 to 0.3]	0.2 [0.2 to 0.3]	0.69
CXCL10 (pg/mL)	59.5 [51.9 to 68.3]	66.1 [52.6 to 83.0]	0.43	**90.9 [66.9 to 123.3]**	**55.6 [48.9 to 63.1]**	**<0.01**	55.9 [42.7 to 73.2]	62.2 [55.4 to 69.8]	0.24
CXCL11 (pg/mL)	60.9 [52.9 to 70.1]	71.0 [59.1 to 85.2]	0.18	**83.0 [62.7 to 109.9]**	**60.3 [53.5 to 67.9]**	**<0.01**	75.2 [58.8 to 96.3]	65.4 [59.0 to 72.5]	0.12
CCL3 (pg/mL)	19.1 [16.6 to 22.0]	18.1 [15.4 to 21.4]	0.62	19.1 [15.6 to 23.5]	18.5 [16.4 to 20.9]	0.06	17.6 [15.0 to 20.6]	18.9 [16.7 to 21.3]	0.32
CCL13 (pg/mL)	630 [545 to 728]	547 [479 to 624]	0.16	549 [450 to 670]	617 [551 to 691]	0.31	691 [596 to 801]	598 [546 to 656]	0.05
sAA1 (ng/mL)	**18.2 [11.9 to 28.0]**	**6.2 [4.0 to 9.7]**	**<0.01**	13.0 [6.7 to 25.3]	10.4 [7.2 to 15.1]	0.56	**4.6 [2.9 to 7.5]**	**11.4 [8.4 to 15.4]**	**<0.01**
CRP (mg/L)‡	0.2 [0.1 to 0.3]	0.3 [0.2 to 0.4]	0.73	0.3 [0.1 to 0.6]	0.2 [0.1 to 0.3]	0.84	0.2 [0.1 to 0.3]	0.2 [0.2 to 0.3]	0.69
SPD (ng/mL)‡	**13 (41)**	**5 (15)**	**<0.01**	12 (21)	9 (30)	0.92	**7 (13)**	**11 (32)**	**0.02**
PCT (ng/mL)‡	**133 (112)**	**174 (148)**	**0.02**	156 (168)	164 (154)	0.96	156 (105)	166 (150)	0.22

Table 5.10; Sputum biomarker levels at exacerbations associated with bacteria, virus or eosinophilic airway inflammation (data presented as geometric mean (95%CI)

Sputum	Bacterial	Non Bacterial	p-value	Viral	Non Viral	p-value	Eosinophilic	Non eosinophilic	p-value
IL1β (pg/mL)	857 [570 to 1289]	47 [32 to 69]	<0.01	278 [154 to 501]	245 [156 to 384]	0.51	44 [27 to 72]	417 [284 to 615]	<0.01
IL-8 (pg/mL)	8926 [7077 to 11259]	3221 [2490 to 4168]	<0.01	6294 [4575 to 8658]	6226 [5079 to 7633]	0.70	3204 [2351 to 4366]	6984 [5699 to 8559]	<0.01
IL-6 (pg/mL)	950 [683 to 1321]	604 [408 to 895]	0.08	1105 [674 to 1813]	761 [591 to 982]	0.18	148 [112 to 195]	339 [272 to 422]	0.16
IL-6R (pg/mL)	469 [366 to 600]	146 [114 to 188]	<0.01	313 [216 to 453]	277 [224 to 344]	0.59	148 [112 to 195]	261 [217 to 315]	<0.01
TNF-α (pg/mL)	89.7 [61.9 to 129.9]	7.5 [5.2 to 10.8]	<0.01	55.9 [30.6 to 102.1]	23.5 [15.9 to 34.8]	0.07	7.5 [4.8 to 11.7]	47.0 [32.5 to 67.8]	<0.01
TNF-RI (pg/mL)	3272 [2634 to 4065]	1084 [855 to 1375]	<0.01	1932 [1404 to 2661]	2110 [1706 to 2609]	0.69	938 [713 to 1233]	1886 [1584 to 2246]	<0.01
TNF-RII (pg/mL)	1250 [964 to 1620]	412 [314 to 542]	<0.01	864 [580 to 1286]	762 [608 to 955]	0.60	401 [274 to 587]	730 [597 to 891]	<0.01
CCL5 (pg/mL)	8.9 [6.9 to 11.4]	4.5 [3.2 to 6.2]	<0.01	14.9 [9.8 to 22.7]	5.0 [4.1 to 6.2]	<0.01	3.8 [2.5 to 5.7]	8.4 [6.7 to 10.4]	<0.01
CCL17 (pg/mL)	8.9 [7.1 to 11.2]	21.2 [15.3 to 29.4]	<0.01	9.9 [6.7 to 14.8]	15.3 [12.0 to 19.6]	0.31	34.8 [24.2 to 50.0]	9.2 [7.5 to 11.2]	<0.01
IL-5 (pg/mL)	1.1 [0.9 to 1.4]	1.8 [1.3 to 2.5]	0.02	1.6 [1.1 to 2.4]	1.5 [1.1 to 1.9]	0.90	3.3 [2.1 to 5.1]	1.1 [0.9 to 1.3]	<0.01
CCL2 (pg/mL)	427 [331 to 551]	620 [459 to 837]	0.06	924 [578 to 1477]	447 [356 to 562]	0.01	568 [381 to 847]	526 [430 to 642]	0.60
CCL4 (pg/mL)	1992 [1536 to 2585]	1184 [866 to 1619]	0.01	2212 [1275 to 3837]	1382 [1127 to 1695]	0.11	1665 [1149 to 2413]	1463 [1204 to 1778]	0.44
CXCL10 (pg/mL)	317 [214 to 469]	526 [312 to 885]	0.12	1513 [755 to 3032]	230 [169 to 313]	<0.01	405 [221 to 743]	391 [270 to 568]	0.79
CXCL11 (pg/mL)	11.4 [6.6 to 19.7]	42.7 [20.9 to 87.2]	<0.01	130 [41 to 412]	10 [7 to 15]	<0.01	31.6 [14.0 to 71.4]	18.4 [10.9 to 31.1]	0.09
CCL13 (pg/mL)	14.8 [11.8 to 18.4]	35.6 [19.0 to 34.5]	<0.01	17.9 [11.6 to 27.5]	20.4 [16.3 to 25.4]	0.90	29.0 [22.7 to 37.1]	16.6 [13.2 to 20.9]	<0.01
Neopterin (ng/mL)	55.0 [43.8 to 69.1]	25.0 [19.6 to 31.7]	<0.01	38.1 [26.3 to 55.0]	36.6 [29.4 to 45.6]	0.08	19.9 [15.0 to 26.4]	43.2 [35.0 to 53.2]	<0.01

Discussion

In this observational longitudinal biomarker investigation study, I have shown that a single biomarker for COPD exacerbations cannot be identified despite using a wide panel of clinical, physiological, serum and sputum indices. However, sensitive and specific biomarkers can be defined to correctly identify bacteria, virus and sputum eosinophil associated exacerbations of COPD. I have shown that there was little overlap in aetiological and inflammatory associations at exacerbation and demonstrated that a proportion of exacerbations are not associated with bacteria, virus or sputum eosinophils at exacerbation.

Exacerbations of COPD

In this study, patients with COPD were followed up at stable state and during exacerbations, with sampling performed in longitudinal visits and in treatment naïve exacerbations. This is the largest study to date that has used biomarker sampling in longitudinal follow-up and exacerbation visits. Recruitment of patients was performed after fulfilment of specific entry criteria with strict exclusion of patients with a current or previous history of asthma. There was deterioration in lung function, health status and symptom scores irrespective of the disease severity, underlying inflammation or aetiology during exacerbations. This decline in lung function and health status was significant and similar to other studies (Seemungal, 2000; Donaldson, 2002; Aaron, 2003; Wilkinson 2006). In my study, an exacerbation of COPD was defined according to Anthonisen criteria and diary cards were used to trigger contact to the research department. Consistent with previous exacerbation studies (Seemungal 1998, Aaron 2003, Donaldson 2005), all patients were asked at study entry to contact the research department if there was an increase in symptoms of breathlessness, sputum production

or sputum purulence in comparison to baseline stable state. Following review by a clinician and demonstration that there was not an alternative cause for symptom change (using clinical examination, electrocardiogram and CXR as necessary), exacerbation data was captured in those patients who required treatment with systemic corticosteroids and/or antibiotic therapy. These methods were used to capture exacerbation events in accordance with current definitions of COPD exacerbations (Anthonisen 1986; Rodriguez 2000, Rabe 2007). The diary card was provided as an exacerbation trigger for patients and not used to define an exacerbation. Designed and used extensively by Wedzicha *et al* in studies from the East London COPD cohort, the diary card can identify reported and unreported exacerbation events and has been designed using the original Anthonisen criteria of an increase in major and minor symptoms (Seemungal, 1998; Wedzicha, 2000; Donaldson, 2002; Hurst 2006). However, these diary cards have not been validated by other research groups and their close basis on Anthonisen criteria adds little to detection of exacerbations from the original Anthonisen criteria which were used in my study.

Biomarkers in COPD exacerbations

An extended panel of serum and sputum biomarkers were assessed at stable state and during exacerbations using the MSD platform and single ELISA. Serum biomarkers that were significantly raised at exacerbation were IL-8, IL-6, TNF-RI, TNF-RII, CRP, PCT, and sAA_1 from paired baseline and exacerbation samples. These findings are consistent to findings previously determined by Hurst *et al* (Hurst, 2006) and Bozinovski *et al* (Bozinovski, 2008). In the study by Hurst *et al* 36 plasma biomarkers were measured using a proteome array in 90 subjects at paired stable and exacerbation visits. The exacerbation was defined by symptom based criteria and healthcare utilisation and

showed elevated IL-6, CRP, IL-1, and TNF-RI levels similar to the findings observed in this chapter. Bozinovski *et al*, used a mixture of ELISA, immunolumunometric assays and surface enhanced laser desorption ionisation time of flight (SELDI-ToF) protein chip analysis (analogous to mass spectrometry) to measure the plasma biomarkers IL-6, CRP, PCT and sAA$_1$. In keeping with my findings and that by Hurst *et al*, Bozinovski found CRP and serum AA$_1$ to be differentially increased during exacerbations. Studies by Perera *et al* have also shown that during exacerbations of COPD there is elevation in IL-8, IL-6 and CRP, which is often predictive of the rate of recovery of the exacerbation (Perera 2007).

Elevated sputum biomarkers during exacerbations compared to baseline in my study were IL-1β, IL-6, TNF-α, TNF-RI, TNF-RII and RANTES. These biomarkers are responsible for the inflammatory cascade and the recruitment of neutrophils and macrophages to sites of tissue injury and inflammation. Elevated sputum IL-6 levels have been demonstrated during exacerbations in studies by Bhowmick and Perera *et al*, although IL-8 was not significantly elevated (Bhowmick, 2000; Perera, 2007). In findings comparable to my results in this chapter, it was observed by Kersul *et al* (Kersul, 2011) that sputum levels of IL-6, IL-8 and TNF- α were increased during exacerbations albeit non-significantly for TNF- α. These subtle differences between the published literature and my work are likely to reflect the differences in sputum collection techniques, sputum processing and methods used to measure the cytokines.

Studies by Hurst *et al* (Hurst 2006) and Bozinovski *et al* (Bozinovski 2008) have investigated biomarkers to correctly identify exacerbations of COPD using ROC analysis to determine biomarkers that are sensitive and specific with significant area

under the curve results. Similar to findings in this chapter, these authors could not demonstrate a single candidate biomarker to correctly identify an exacerbation with an AUC of > 0.8 (which is standardised as a cut off for a suitable test). Both Hurst and Bozinovski *et al* determined that CRP and/or sAA_1 respectively were the most useful plasma biomarker with an AUC of approximately 0.7. The ability of these markers to correctly identify an exacerbation was then only improved by the addition of major symptoms to the ROC analysis. In my study, sAA_1 had the highest ROC for determining an exacerbation (AUC 0.69); however, the addition of symptoms to improve on the biomarker sensitivities and specificities was not analysed in my study because there are minimal benefits in defining an objective biomarker for exacerbations using the addition of subjective symptoms. Sputum biomarkers to correctly identify exacerbations of COPD have never been investigated, whilst combination of biomarkers have also failed to provide further benefits.

Bacteria in COPD exacerbations

Bacteria are considered to play a role in up to 50% of exacerbations (Sethi, 2008) while current guidelines propose that sputum purulence is used to guide antibiotic therapy (Ram, 2006). Using standard bacterial culture techniques, my results showed that bacteria were detected in 35% of exacerbation samples. There was also a significant increase in CFU's during exacerbations and the proportion of subjects with a CFU bacterial load of $> 10^7$ during an exacerbation was 46%. The use of qPCR identified greater than 85% of subjects with pathogenic bacteria during stable state and exacerbations; in addition there was little difference in bacterial species using qPCR between stable state and exacerbations. In this chapter I have defined bacteria associated exacerbation of COPD as an exacerbation that had pathogenic bacteria using

standard culture techniques and/or an elevated CFU bacterial load greater than 10^7. There is currently no unified definition for a bacterial exacerbation of COPD. Studies investigating COPD exacerbations have often used the presence of bacteria from standard culture techniques (Soler 2007); however newer molecular detection methods have suggested a change in pathogen strain as a major cause of exacerbations of COPD (Sethi 2002). In a study by Sethi *et al* gel electrophoresis was used to type isolates of *H. influenzae, M. catarrhalis, S. pneumoniae and P. aeruginosa* from exacerbation samples and observed that acquisition of a new stain was associated with an exacerbation of COPD and proposed that this conclusively supported the causative role of bacteria during exacerbations of COPD (Sethi, 2002). In this chapter, qPCR was performed in all samples at stable state and exacerbations; the detection of bacteria using qPCR was common and change or acquisition of pathogen identified using molecular techniques was not helpful to define bacterial exacerbations. Furthermore, Hilty *et al* have shown that pathogens are ubiquitous in the COPD airway (Hilty 2010). Thus a clear definition is lacking and so the presence of a pathogenic bacteria on standard culture was used to define a bacteria associated exacerbation. My findings do not provide evidence of causality for the role of bacteria during exacerbations.

An increase in bacterial load has been shown to occur during exacerbations of COPD (Stockley 2000; Sethi 2008) and was similarly demonstrated in this chapter, by standard CFU techniques and by molecular total 16S bacterial load. The cut off of CFU $> 10^7$ was selected from previously published data by Stockley *et al* which has showed that COPD exacerbations with a CFU bacterial load of $\leq 10^6$ and macroscopic mucoid sputum were not associated with worse outcomes despite withholding antibiotic treatment (Stockley, 2000). Sputum purulence has been shown to be sensitive for

detecting bacterial culture or high bacterial loads at exacerbation (Stockley, 2000). However the use of sputum purulence is confounded by detection at stable state and chronic bacterial colonisation. The mechanism responsible for sputum colonisation has been proposed to be related to poor bacterial clearance and defective macrophage phagocytosis (Taylor, 2010). Furthermore the change in sputum purulence or sputum production measured using the VAS in my study was not sensitive or specific for identifying bacteria associated exacerbations with a ROC curve of 0.50 and 0.58 respectively.

The most sensitive and specific assay for determining bacteria associated exacerbations was found to be sputum IL-1β. This cytokine belongs to the IL-1 superfamily and is involved in the immune system as defence against infection. IL-1β is produced by macrophages, monocytes, fibroblasts, NK and dendritic cells in response to infection. IL-1β increases the expression of adhesion molecules in endothelial cells and allows migration and transmigration to sites of infection and inflammation. Sputum TNF-α and sputum neutrophils were also identified as suitable biomarkers for bacteria associated exacerbations. These findings are not unsurprising and relate to bacterial infection stimulating an inflammatory response. IL-1β in bronchoalveolar lavage has been shown to be a good biomarker in ventilator associated pneumonia (Conway, 2010) and suggests that this airway marker may suitably determine bacterial infections, above that of serum CRP or PCT whose utility could not be demonstrated in this study or in others (Hurst, 2006; Bozinovski, 2008).

Virus in COPD exacerbations

Viruses have been implicated as a major cause of COPD exacerbations and are detected in up to 80% of severe COPD exacerbations (Seemungal, 2001; Papi, 2006). Using PCR, a virus in this chapter was detected in approximately 30% of exacerbations. This low yield is likely a reflection of the moderate severity of exacerbations in this cohort and is similar to other out-patient based COPD exacerbation studies (Wilkinson, 2006). Exacerbations associated with a virus had the biggest drop in lung function (FEV$_1$ and FEV$_1$ % predicted) compared to non-virus associated exacerbations. In a study by Wilkinson *et al* 56 out-patient exacerbations with paired baseline data from the East London COPD cohort were analysed and showed that detection of RV was associated with the greatest decline in lung function and worsened symptom scores irrespective of bacterial co-culture (Wilkinson 2006); and were similar to findings presented in this chapter. The identification of a virus at the onset of an exacerbation was defined as a virus associated exacerbation in this chapter and in accordance with published literature. The detection of a virus using PCR is likely to reflect a measurable virus threshold and exacerbations with an earlier exposure to viruses may have been missed. Experimental models of RV induced COPD exacerbations have determined peak lower respiratory tract symptoms occur 9 days following RV exposure (Mallia 2011), although in this experimental work subjects did not have true exacerbations as defined by Anthonisen, GOLD or Rodriguez-Roisin. Despite results from this chapter and the studies by Wilkinson and Mallia *et al* (Wilkinson, 2006; Mallia, 2011) the role of viruses during exacerbations remains unclear.

The total sputum eosinophil count has been proposed as a potential biomarker of a viral exacerbation (Papi, 2006). In this chapter, the total absolute sputum eosinophil count

was increased in virus associated exacerbations, but not the differential sputum eosinophil count suggesting the association was a consequence of a change in the total cell count. The most sensitive and specific biomarker for a virus associated exacerbation was serum CXCL10. CXL10 is secreted by monocytes, endothelial cells and fibroblasts in response to interferon-gamma (IFN-γ) and itself can further propagate the inflammatory response via chemoattraction of monocytes, macrophages, NK cells and dendritic cells. IFN-γ is critical for immunity against viruses, by direct inhibition of virus replication, immunomodulatory effects and Th1 cell differentiation. The biomarkers identified in this chapter that were positively predictive of a virus associated exacerbation were related to IFN-γ, and the power of the biomarker was increased further in exacerbations that were solely associated with a virus. The application of clinical symptoms in combination with serum CXCL10 has been proposed as a possible biomarker for RV infection at exacerbation by Quint *et al* (Quint, 2010). However, my findings have confirmed that serum CXCL10 as a potential predictor of a virus associated exacerbation, independent of a requirement for symptom evaluation.

Eosinophilic inflammation in COPD exacerbations

The inflammatory profile of a COPD exacerbation is typically neutrophilic, but eosinophilic airway inflammation during stable disease and exacerbations has been demonstrated (Saetta, 1993; O'Donnell, 2004; O'Donnell, 2006). The presence of a sputum eosinophilia has also been repeatedly shown to be associated with a favourable response to corticosteroid therapy (Shim, 1978; Pizzichini, 1998; Brightling, 2000).

In this study, the peripheral percentage eosinophil count was the most sensitive and specific biomarker of a sputum eosinophilia. The presence of a peripheral blood

eosinophilia has been shown to be an independent predictor of persistent airflow obstruction in asthma (Bumbacea 2004), whilst Hospers *et al* showed that a peripheral eosinophilia was an independent risk factor for all-cause mortality in subjects with COPD (Hospers 2000). Investigation of peripheral blood eosinophils in airways disease has predominately focussed on asthma with little work published in COPD. In this study a cut off of 2% was selected as one that has the greatest sensitivity for a sputum eosinophilia without significant compromise in the specificity. Alterations in the threshold cut off will affect the sensitivities and specificities of the test to accurately predict the presence of a sputum eosinophilia during exacerbations.

In this study I have employed clinical exacerbation phenotypes of COPD to identify biomarkers related to potential aetiology and inflammation, namely exacerbations that are associated with bacteria, virus or a sputum eosinophilia. There were 14% of exacerbations that did not have an identified possible aetiological pathogen or eosinophilic associated airway inflammation. This may be reflective of the tools used to detect pathogens in this study, or that these are non-pathogen related events such as responses to allergen, irritants or pollution.

Limitations

A limitation in this study is that only moderate exacerbations were captured and the identified biomarkers IL-1β, CXCL10 and peripheral blood eosinophils need to be validated in a severe population. Severe exacerbations are associated with significant morbidity and mortality whilst presentation to hospital with an exacerbation is often associated with a new diagnosis of COPD. However, these biomarkers biologically relate to the clinical exacerbation phenotypes associated with bacteria, virus and sputum

eosinophils and have been previously demonstrated as markers of infection in COPD and ventilator associated pneumonia. The method to define bacteria associated exacerbation used standard culture and bacterial load and may also be a limitation; however there is no current gold standard for this definition and the causal links between bacteria and exacerbations have not fully been determined. The presence of co-infection in this study was lower than previously published and is likely to be reflective of the severity of the exacerbations. One statistical limitation is that corrections for repeated observations were not made. Exacerbations have been shown to be a good predictor of the risk of future exacerbations but there is no evidence to show that the presence of a pathogen during an exacerbation predicts the aetiology of a further exacerbation, and in practice, exacerbations are treated independently.

Conclusions

In this study I have determined that sensitive and specific biomarkers can be used to define clinical exacerbation phenotypes associated with bacteria, virus and sputum eosinophils. These biomarkers may be used to guide targeted treatment at the onset of an exacerbation.

6. Biological clusters analysis to define COPD exacerbation phenotypes

Abstract

Mathematical methods have been used to phenotype COPD and include factor and cluster analyses which are unbiased in their evaluation. Current descriptions of COPD clusters have focussed on clinical indices at stable state. There is no data however investigating the use of biomarkers to cluster and phenotype COPD exacerbations.

Factor and cluster analysis using sputum biomarkers during exacerbations of COPD were investigated. This study demonstrated that there were 4 biological exacerbation clusters. These clusters were related to a pro-inflammatory axis, Th1 axis and a Th2 axis and were found to be dependent on inflammation and possible aetiology. The fouth cluster had low levels of inflammatory mediators and no dominant associated pathogen. These biological exacerbation clusters were clinically indistinguishable at exacerbation or baseline state and related to clinical exacerbation phenotypes associated with bacteria, virus and sputum eosinophils. Biological exacerbations clusters were also repeatable for multiple exacerbations.

The identification of biological exacerbation clusters lends to the multi-dimensional approach to phenotyping COPD.

Introduction

COPD heterogeneity is demonstrated by pathological processes (Hogg, 2004), airway inflammation (Saetta, 1993; Brightling, 2001; O'Donnell, 2006), microbial characterisation (Sethi, 2002; Sethi, 2008; Hilty, 2010) and imaging (Coxson, 2008; Coxson, 2009) and are likely to reflect different disease processes and treatment responses (Naunheim, 2006; Calverley, 2007; Tashkin, 2008). A phenotype corresponds to any observed quality with traits and characteristics that are controlled genetically and environmentally (Agusti, 2011). Phenotype descriptions of COPD have looked at clinical, physiological and pathological processes (Dornhorst, 1955; Filley, 1968; Hogg, 2004) and entertained the use of statistical tools to identify clinical COPD phenotypes using clinical indices. These have included lung function, symptom scores and radiology and determined clinical COPD phenotypes at stable state (Pistolesi, 2008; Weatherall, 2009; Burgel, 2010). There is no data in the literature examining cluster analysis to examine exacerbations of COPD or the use of biomarkers to cluster exacerbations of COPD and inform the biological fingerprint. The use of biomarkers to phenotype COPD and in particular COPD exacerbations can provide a further understanding of the pathogenesis, treatment and disease modulation. In this chapter I have investigated whether during exacerbations of COPD there are definable biological phenotypes using factor and cluster analysis (Everitt, 2001; Fu, 2010) and whether these relate to clinical characteristics of COPD at exacerbations or stable state.

Methods

Patient selection, study design and data collection were performed as described in chapter 5. The baseline characteristics of all patients are presented in table 5.1. Factor analysis was used to identify patterns of relationships in large datasets from sputum mediators at exacerbations. Cluster analysis was used to classify groups on similar chosen characteristics alone. Both these methods are described in detail below.

Factor analysis

Factor analysis is a mathematical method used to identify patterns of variation in characteristics of variables in large datasets. Factor analysis can be used to define relationships within datasets, classify or reduce data, control variables or make inferences from datasets. The factor analysis method employs i) data reduction and ii) data optimisation techniques to deduce patterns of variation. In the data reduction step, vectors are drawn from the sum of the individual variable components. Each variable is plotted as a vector in space with the same point of origin (0, 0, and 0 for example). Variables that are similar can thus be found to lie close to each other in space; the cosine angle (cosϴ) between vectors is representative of the correlation coefficient between the variables. A factor is constructed as one that maximises the vectorial sum from each of the contribution of the variable components. Repeated iterations allow for further factor construction which accounts for variation that is not included in the first iteration and so forth. As a consequence, the calculated factors become representative of the data within that component and are independent of other calculated factors. This can be represented mathematically in geometric space; factors are perpendicular to each other. This is schematically illustrated in figure 6.1.

Figure 6.1; a) Measured variables are represented in space as vectors from a point of central origin. The direction of each vector depends on the relationship of that variable with the other variables in the analysis; b) Factor axis, delineated in the blue, orange and green direction of the vectors are constructed so that each factor accounts for the variance of the variables within the factor and are independent of each other.
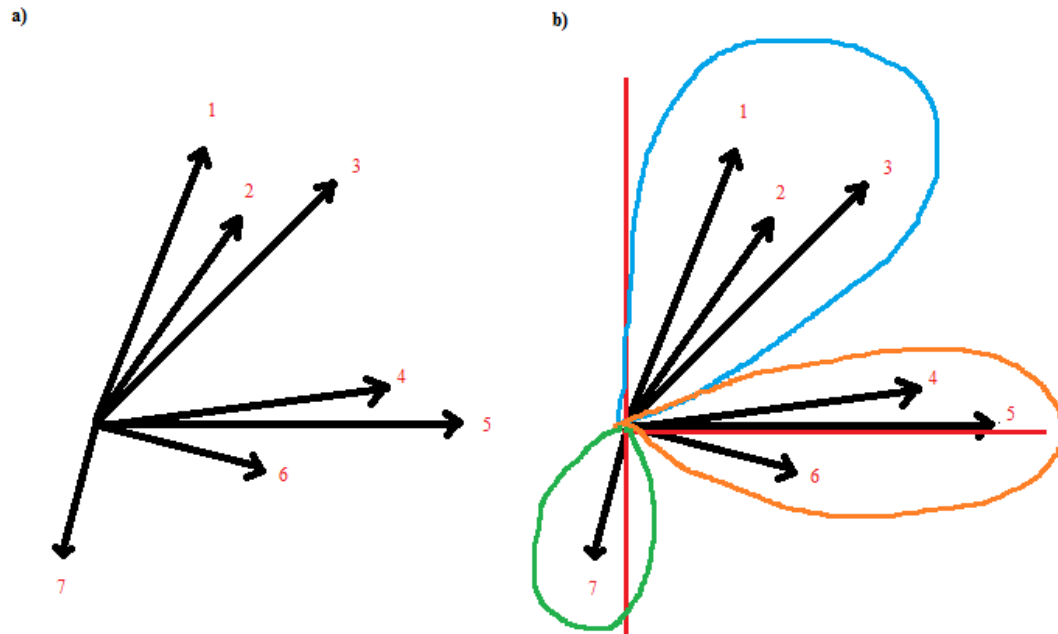
Two commonly used methods of data reduction are principal factor analysis (PFA) and principal components analysis (PCA). In PFA, the common variability of each item is used; in PCA the variance from each item is used. The algorithm for generating each method is different but these differences are minor when large datasets are used. In this thesis the PCA method was used. In the data optimisation step, maximisation of the factor representation for the variation in the variable dataset is performed by rotation in geometric space. Rotation defines a structural model of factors which delineate distinct groups of interrelated data; compared to un-rotated factors which delineate comprehensive data patterns alone. In this thesis, orthogonal rotation was used,

maintaining factor independence compared to oblique rotation, where factors are correlated to one another.

Once the factors are generated, it is critical to assess the validity of the factor matrix output. For a factor analysis model to be statistically valid, the model (inclusive of all generated or selected factors) should account for most of the variance of the dataset (> 60%) and is a measure of the pattern's completeness and strength. The factor matrix output represents the correlation matrix, an expression of the degree of the linear relationship or loadings between entered variables and designated factors. The loadings, α, is a measure of the degree of patterns between variable and the resulting factor. This is tantamount to the correlation coefficient. Similarly the relevance of the factor is the amount of variation accounted for by the determined pattern and is equal to the eigenvalue. In brief, the eigenvalue of a factor represents the amount of the total variance explained by that factor. A factor with an eigenvalue of less than 1 accounts for less variance than that contributed by 1 variable. Estimation of the number of factors relies on interrogation of the Cattell scree plot. This is a graphical display of the size of the eigenvalue associated with each factor. The scree plot can then be used to determine how many factors are retained in the analysis, namely by review of the number of factors above the 'elbow' of the plot (where a dramatic point change occurs) (Cattell, 1966). This is often associated with factors that have an eigenvalue of greater than 1. Only extracted factors with an eigenvalue of >1 were used for further analysis in the statistical methods.

In this thesis factor analysis was used to explore patterns in biomarker measurements during exacerbations of COPD and to reduce individual variables from the large sputum biomarker dataset into factors. The factors are representative of the dataset and are independent of each other. These relationships are mathematically calculated and are

not biological. The derivations of factors are not used in isolation and are applied to characterise the heterogeneity of the measures collected at the onset of the exacerbation in addition to cluster analysis which is discussed below.

Cluster analysis

Cluster analysis is a mathematical method designed to classify groups or clusters with similar characteristics (grouping of homogenous observations) within datasets that are heterogeneous. In a method akin to factor analysis, the difference is that cluster analysis explores patterns within the population based on similar characteristics; whereas patterns of variable expression are determined in factor analysis.

There are predominately two types of clustering techniques which use a two-step algorithm. These can be grouped into hierarchical and partitional (non-hierarchical) clustering. In partitional clustering, the groups (clusters) are divided without overlapping and the number of groups/clusters can be pre-selected. In hierarchical clustering, the grouping permits the clustering to have sub-clusters, which are organised into a branching tree by starting at a single point with repeated iterations. This is often displayed as a dendrogram. Other clustering techniques include exclusive (each case is assigned to a single cluster), overlapping (a case can belong simultaneously to more than one cluster) and fuzzy (a case belongs to every cluster where there is an appropriate membership weight). In this thesis only work involving hierarchical and partitional clustering is used; this is because of the limitations associated with the formation of clusters that are either attached to a single cluster (exclusive) or being in more than one (overlapping or fuzzy).

Once selected, these clustering techniques use comparable algorithms to find useful groups of clusters. In essence, the first step employs quantification of similarities between cases (a geometric distance in space and the Euclidean distance is often used) followed by the second step which involves placing cases into groups based on the measured similarity (linkage). Different types of clusters are classed according to how the degrees of similarities are formed. These include *well-separated* in which the cases within each cluster are closer to each other than to other cases not in the cluster; and *prototype-based* where the cases within the cluster are closer to the prototype that defines the cluster than to the prototype of any other cluster. The prototype is selected and can be either a *centroid* (average/mean), *medoid* (a representative point), *density-based* (the cluster is a dense region of cases surrounded by less dense regions) and *conceptual* (encompasses all the definitions of a cluster, i.e. *well-separated, prototype* and *dense*). Similarity or case within cluster relationship is often calculated as the distance between the cases in a geometric space. Linkage is used to determine distance between calculated clusters. In this thesis the Euclidean distance was used to calculate the distance in geometric space (similarity) and Ward's method was performed to calculate linkage between clusters. Ward's method calculates compact clusters. Estimation of cluster numbers was performed by review of the output from the linkage dendrogram. Although these are arbitrary, natural groupings are sought and defined by long stems and their level of similarity; a schematic example is illustrated in figure 6.2.

Figure 6.2; Schematic example of hierarchical tree diagram or dendrogram generated by Ward's method for determination of linkage and number of clusters. Top) Cases are numbered (10 in this example) and fused together where there is similarity. The 'height' is the equivalent of the linkage distance, and fusion of cases to generate clusters are formed with cases of similar or close linkage; Bottom) Dendrogram is reviewed for cluster estimation and two clusters (circled) are identified
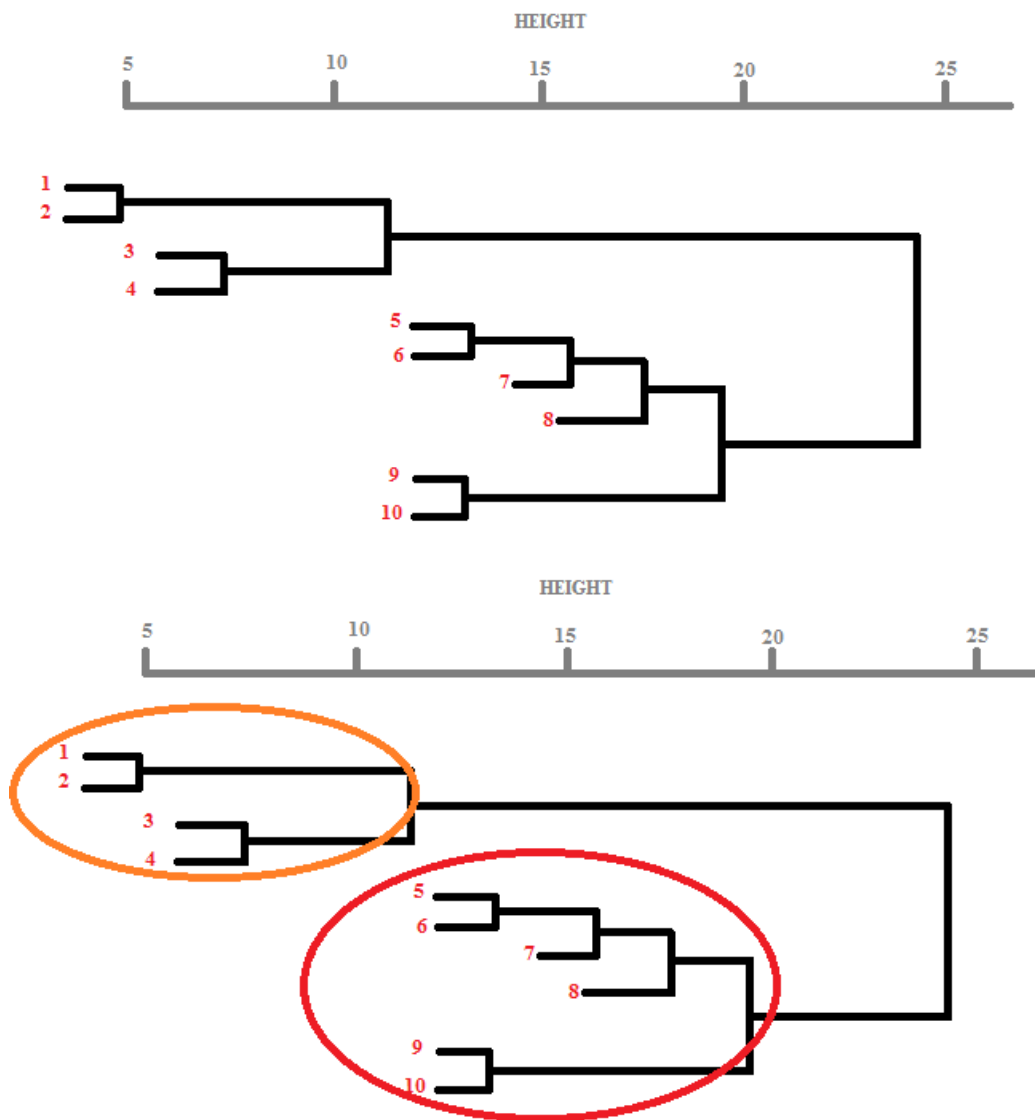
In this thesis, hierarchical cluster analysis was applied to determine biological clusters for exacerbations of COPD. Cluster estimation was performed using Ward's linkage method to generate a dendrogram within the exacerbation population based in both

events and cases independently. Subsequent to this, biomarkers measured during the exacerbation were entered into partitional prototype-based clustering (K-means) to determine biological clusters of exacerbations of COPD. There were 4 estimated clusters derived from the dendrogram and this was then pre-specified. Only the highest loading biomarker from the factor analysis was entered. The cluster analysis technique was used to investigate homogeneity within a heterogeneous population, but unlike work previously conducted by Pistolesi, Burgel and Weatherall *et al* (Pistolesi, 2008; Weatherall, 2009; Burgel, 2010), the investigation of homogeneity within COPD was analysed during exacerbations and using biological variables.

These statistical methods may demonstrate biological factors and biological clusters during exacerbations of COPD. Clinical characteristics for all exacerbation events were tabulated for each biological cluster. Individual patient baseline characteristics for each biological cluster was determined by i) the single or predominant cluster that the exacerbation event existed in or ii) the cluster that the first exacerbation event occurred for those without a single or predominant cluster event (15 subjects). Repeatability of biological clusters for subjects with multiple exacerbations was assessed using the intra-class correlation coefficient. Graphical depiction in three dimensions (3-D) for all exacerbation events and cases were plotted using Graphis (Kylebank Software Ltd, UK) in Cartesian coordinate (x, y, z) plots. Ellipsoid figures for the clusters in 3-D for exacerbation events were calculated from the equatorial radial components in the x, y, z directions and calculated from the variance of the factors with centres of the ellipsoid as the mean of the factors using the equation $r^2 = (1/v_{x1} - \mu_{x1})^2 \times (1/v_{y1} - \mu_{y1})^2 \times (1/v_{y1} - \mu_{y1})^2$.

Results

The patient baseline and exacerbation characteristics are identical to those in Chapter 5, presented in table 5.1 and table 5.2 respectively. Sputum biomarkers measured during exacerbations were available in 148 exacerbation events. Factor analysis of the sputum exacerbation biomarkers demonstrated 3 extracted biological factors with an eigenvalue greater than 1 (scree plot illustrating 'elbow notch' at 3 factors in figure 6.3).

Figure 6.3; Scree plot following factor analysis for sputum exacerbation biomarkers demonstrating 3 factors with an eigenvalue greater than 1
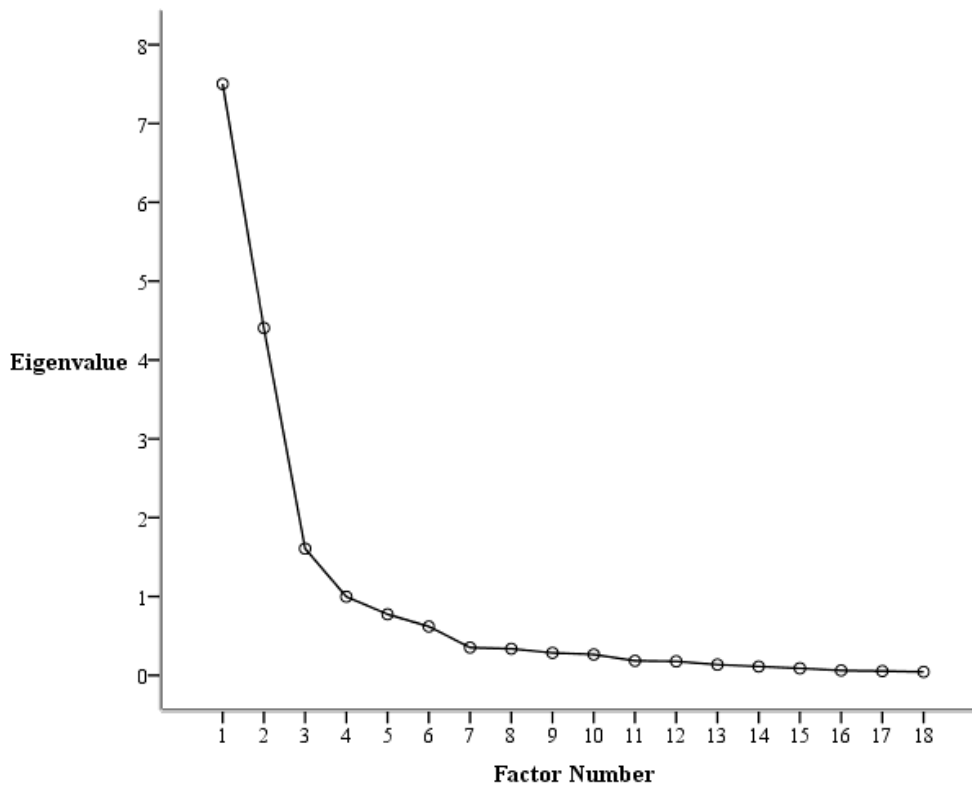
The factor analysis matrix output and corresponding α loading (correlation coefficients) of the sputum biomarkers for each of the three factors are tabulated in 6.1. The 3 factors identified accounted for 75% of the total variance of sputum mediators at exacerbation.

Table 6.1; Factor analysis output for all sputum biomarkers entered into factor analysis. The three factors show independent patterns of relationships between variables and the loading coefficients demonstrate the correlation between the variable and the factor axis. For simplicity only correlation coefficients of greater than 0.3 and biomarkers that are the highest loading are in bold

	Factor		
	1	**2**	**3**
	'pro-inflammatory'	**'Th1'**	**'Th2'**
TNF-α	0.80		-0.36
TNF-RI	0.88		
TNF-RII	**0.93**		
CCL2 (MCP-1)		0.44	0.57
CCL4 (MIP-1β)	0.62	0.44	0.39
CCL5 (RANTES)	0.58	0.64	
CCL13 (MCP-4)			0.82
CCL17 (TARC)			**0.90**
CXCL10 (IP-10)		0.92	
CXCL11 (I-TAC)		**0.93**	
IL-1β	0.89		-0.36
IL-5			0.76
IL-6	0.61	0.32	
IL-6R	0.89		
IL-8	0.83		

TNF Tumour necrosis factor; R receptor; CCL/CXC Chemokine ligand; MCP monocyte chemotactic protein; MIP macrophage inflammatory protein; RANTES Regulated upon activation Normal T cell expressed and secreted; TARC Thymus and activation regulated chemokine; IP Interferon gamma-induced protein; ITAC Interferon inducible T cell chemoattractant; IL interleukin

Factor 1 was termed a pro-inflammatory factor. In this factor the sputum biomarkers with the highest factor α loading (and thus correlation) were associated with inflammation and TNF-α, IL-1β, IL-6 and IL-8. Factor 2 was termed a Th1 factor with factor α loading of CXCL10 and CXCL11 and finally factor 3 was termed a Th2 factor as the highest factor α loading was with IL-5 and CCL17.

Hierarchical cluster analysis was then applied to explore the patterns within the population using the highest loading biomarker from each factor; TNF-RII, CXCL11 and CCL17 were chosen. The factor analysis revealed these biomarkers to have independence and minimal variance in the sputum biomarker dataset.

The cluster analysis revealed four distinct biological cluster populations for exacerbation events. Between the 4 clusters there were no differences in the change in lung function, health status or symptoms scores between baseline and exacerbation, but differential expression of biomarkers and in the proportions of bacteria, virus and sputum eosinophil associated exacerbations. These clusters were thus termed **bacteria-predominant**, **eosinophil-predominant**, and **virus-predominant** (according to the proposed aetiological cause). The final cluster showed low levels of the measured biomarkers and thus termed **pauci-inflammatory**.

The biomarker levels and clinical characteristics of these clusters are presented in table 6.2.

Table 6.2; Exacerbation characteristics of the biological clusters

	Cluster 1 bacteria-predominant	Cluster 2 eosinophil-predominant	Cluster 3 virus-predominant	Cluster 4 pauci-inflammatory	P Value ϕ
Number, (%)	52 (35)	44 (30)	36 (24)	16 (11)	-
Sputum TNF-α [#]	120 [84 to 173]	6 [4 to 10]	43 [24 to 76]	4 [3 to 7]	***
Sputum TNF-RII [#]	1722 [1402 to 2117]	353 [287 to 433]	1254 [969 to 1623]	77 [41 to 147]	***
Sputum CXCL10 [#]	130 [90 to 186]	230 [177 to 299]	5026 [3556 to 7103]	208 [80 to 539]	***
Sputum CXCL11 [#]	3.1 [2.2 to 4.3]	10.9 [7.7 to 15.5]	799 [415 to 1539]	17.3 [5.6 to 53.1]	***
Sputum IL-5 [#]	0.7 [0.6 to 0.9]	2.0 [1.4 to 3.0]	4.3 [3.1 to 6.2]	0.6 [0.5 to 0.7]	***
Sputum CCL17 [#]	5.5 [4.5 to 6.7]	34.8 [27.3 to 44.5]	23.5 [16.2 to 34.1]	4.7 [3.5 to 6.3]	***
% Bacterial (95%CI)	86 (73 to 92)	29 (18 to 45)	44 (28 to 61)	31 (12 to 58)	***
% Viral (95%CI)	22 (13 to 35)	10 (3 to 23)	57 (39 to 73)	30 (10 to 61)	***
% Eosinophilic (95%CI)	6 (1 to 16)	60 (45 to 74)	28 (16 to 44)	27 (10 to 52)	***
Δ FEV$_1$ (mL)[†]	-132 (-251 to -35)	-110 (-230 to -31)	-232 (-340 to -124)	-280 (-524 to -36)	0.32
Δ CRQ (units) [†]	-0.89 (-1.15 to -0.63)	-0.90 (-1.33 to -0.48)	-0.90 (-1.39 to -0.41)	-1.00 (-1.89 to -0.12)	0.99
Δ VAS$_{TOTAL}$ (mm)[†]	79 (42 to 116)	80 (41 to 119)	120 (86 to 154)	73 (38 to 108)	0.39

Δ Change from baseline; FEV$_1$ Forced expiratory volume in 1 second; CRQ Chronic Respiratory Disease Questionnaire; VAS Visual Analogue Scale. # Geometric mean [95% confidence interval]; † Mean difference (95% confidence interval); ϕ One-way ANOVA, ***p<0.001

Biological cluster ellipsoids were calculated and plotted for all exacerbation events to schematically represent biological clusters of COPD exacerbations in 3-D and presented in figure 6.4.

Figure 6.4; Graphical and proportional representation of biological COPD exacerbation clusters
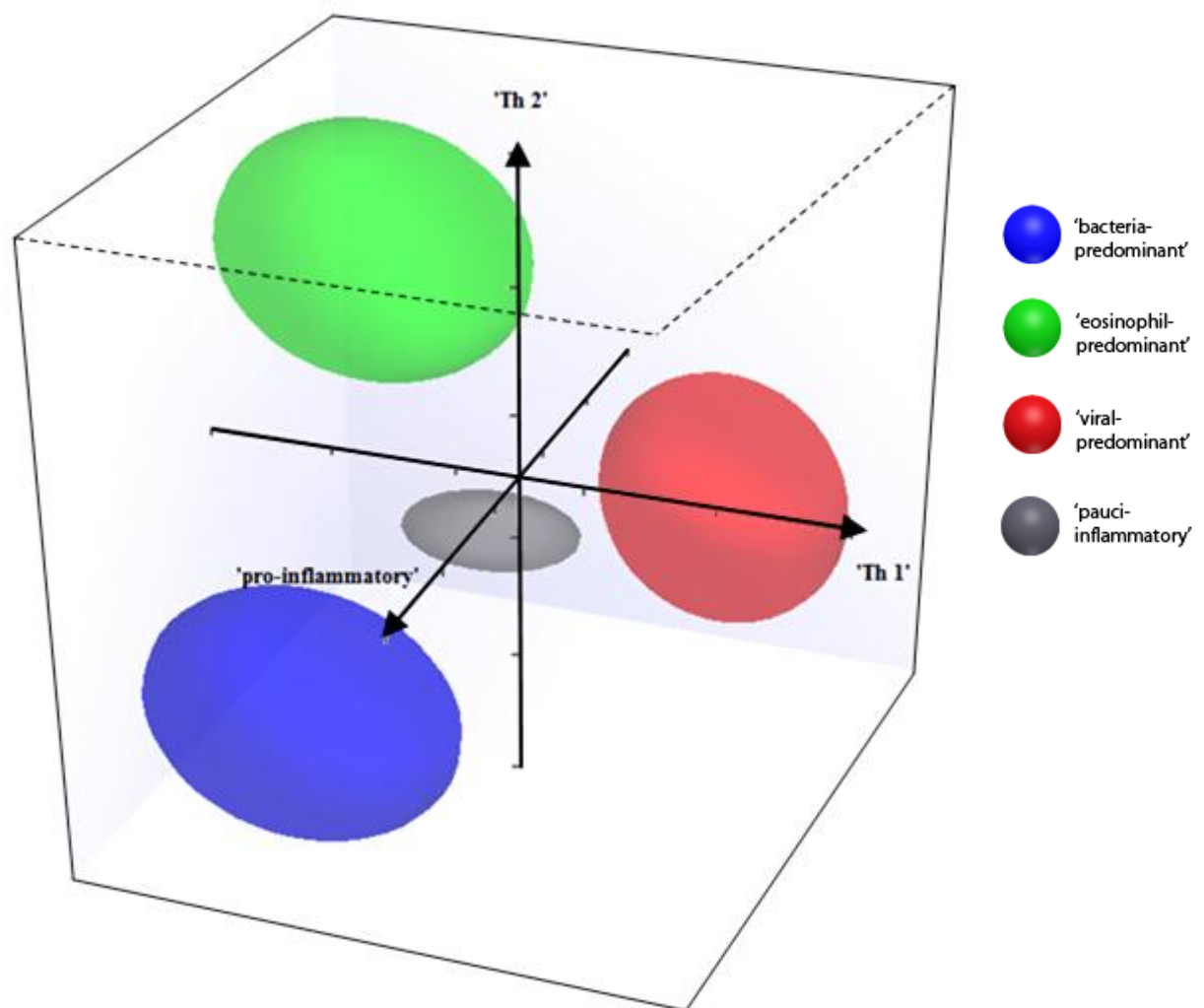
The baseline characteristics for each patient within each cluster are presented in table 6.3. This showed that the baseline clinical characteristics of the patients within each exacerbation cluster were indistinguishable, apart from the degree of airway inflammation during the stable state.

Table 6.3; Baseline clinical characteristics of the biological exacerbations clusters

	Cluster 1 bacteria-predominant	Cluster 2 eosinophil-predominant	Cluster 3 virus-predominant	Cluster 4 pauci-inflammatory	P Value ϕ
Number, (%)	28 (37)	19 (25)	20 (27)	8 (11)	-
Male, n (%)	18 (64)	14 (74)	14 (70)	7 (88)	0.63
Age, years*	69 (52 to 84)	68 (45 to 88)	70 (49 to 84)	69 (61 to 85)	0.62
Current smokers, n (%)	8 (29)	8 (42)	4 (20)	3 (38)	0.48
Pack years smoked*	44 (10 to 122)	50 (20 to 106)	47 (10 to 134)	72 (23 to 120)	0.11
Exacerbation rate in last yr	3.8 (0.5)	4.3 (0.5)	4.0 (0.7)	4.9 (1.2)	0.58
Exacerbation rate in study	3.8 (0.3)	3.6 (0.4)	3.2 (0.3)	3.1 (0.5)	0.64
Inhaled corticosteroid dose,‡	1507 (147)	1567 (133)	1470 (160)	1150 (188)	0.55
Body mass index, kg/m^2	26.0 (0.8)	25.2 (0.8)	27.8 (1.2)	25.3 (2.0)	0.24
Residual Volume, %	134 (8)	150 (9)	120 (8)	146 (23)	0.11
Total Lung Capacity, %	109 (3)	131 (15)	100 (4)	103 (9)	0.05
T_LCO % predicted	56 (5)	59 (5)	57 (6)	46 (7)	0.62
KCO % predicted	67 (6)	67 (5)	72 (6)	60 (11)	0.59
FEV$_1$ % predicted, baseline	53 (3)	51 (5)	53 (5)	40 (7)	0.34
Reversibility (%)	5 (2)	-2 (6)	11 (2)	2 (3)	0.09
FEV$_1$/FVC ratio (%)	51 (2)	47 (2)	50 (3)	47 (5)	0.67
CRQ$_{TOTAL}$ (units)	4.14 (0.20)	3.90 (0.22)	4.10 (0.26)	3.66 (0.50)	0.74
VAS$_{TOTAL}$ (mm)	178 (15)	142 (18)	124 (18)	147 (37)	0.14
VAS$_{COUGH}$ (mm)	49 (5)	38 (6)	33 (6)	48 (13)	0.24
VAS$_{DYSPNOEA}$ (mm)	51 (5)	43 (5)	37 (6)	46 (12)	0.37
VAS$_{SPUTUM\ VOLUME}$ (mm)	42 (5)	38 (6)	28 (6)	34 (14)	0.54
VAS$_{SPUTUM\ PURULENCE}$ (mm)	37 (5)	24 (5)	27 (6)	18 (9)	0.09
Total sputum cell count,# (x10^6 cells/g)	**8.3 [5.5 to 12.5]**	**2.3 [1.6 to 3.2]**	**2.5 [1.2 to 5.3]**	**3.5 [1.2 to 10.7]**	**<0.01**
Sputum neutrophil count, %	**75 (5)**	**53 (4)**	**68 (4)**	**81 (6)**	**<0.01**
Sputum eosinophil count, %#	**1.0 [0.6 to 1.6]**	**3.1 [1.4 to 6.6]**	**1.0 [0.5 to 1.9]**	**0.5 [0.2 to 1.0]**	**0.01**

FEV$_1$ Forced expiratory volume in 1 second; CRQ Chronic respiratory health score; VAS Visual analogue score; T_LCO Carbon monoxide diffusion capacity; KCO Carbon monoxide diffusion capacity corrected for alveolar volume; FVC Forced vital capacity; Data presented as mean (standard error of the mean), unless stated. # Geometric mean (95% confidence interval), †mean change (95% confidence interval) between exacerbation and baseline, * mean (range), ‡ Beclomethasone dipropionate equivalent); ϕ One-way ANOVA

The biomarker levels from sputum during the stable state are presented according to their biological exacerbation clusters in table 6.4. This demonstrated that there was a significant difference in stable state levels of IL-1β (one-way ANOVA, p=0.008), TNF-α (one-way ANOVA, p=0.003), TNF-RII (one-way ANOVA, p=0.038), IL-10 (one-way ANOVA, p=0.002) and IL-5 (one-way ANOVA, p=0.019) between the biological exacerbation clusters.

Table 6.4; Stable sputum biomarker levels in the biological exacerbation clusters. Asterisked (*) mediators were significantly different between the 4 biological cluster groups

	Cluster 1 bacteria-predominant	Cluster 2 eosinophil-predominant	Cluster 3 virus-predominant	Cluster 4 pauci-inflammatory
IL1β*	205 (127 to 329)	90 (58 to 140)	99 (66 to 148)	128 (66 to 245)
IL-6R	150 (118 to 190)	178 (135 to 235)	151 (117 to 194)	158 (113 to 222)
IL-8	3473 (2912 to 4143)	3165 (2567 to 3903)	3307 (2646 to 4133)	3396 (2457 to 4693)
IL-6	469 (318 to 692)	515 (348 to 762)	357 (240 to 531)	363 (205 to 641)
TNF-α*	15.6 (8.6 to 28.2)	3.6 (1.9 to 6.7)	5.0 (2.9 to 8.7)	6.2 (2.6 to 14.9)
TNF-RI	1361 (1040 to 1780)	1025 (824 to 1275)	986 (759 to 1280)	853 (585 to 1244)
TNF-RII*	607 (447 to 824)	469 (345 to 639)	416 (296 to 586)	354 (236 to 530)
IL-5*	0.8 (0.6 to 1.2)	0.7 (0.4 to 1.1)	1.3 (0.9 to 1.9)	0.8 (0.4 to 1.6)
CXCL10	317 (213 to 472)	308 (192 to 492)	442 (293 to 668)	602 (314 to 1153)
CXCL11	21.6 (13.8 to 33.9)	18.1 (10.5 to 31.3)	26.6 (16.7 to 42.2)	41.6 (21.0 to 82.2)
CCL2	810 (610 to 1075)	584 (441 to 773)	1014 (733 to 1403)	585 (354 to 967)
CCL4	1064 (723 to 1568)	930 (676 to 1280)	1310 (873 to 1965)	1095 (666 to 1801)
CCL5	7.2 (4.7 to 10.9)	5.3 (3.6 to 7.8)	9.4 (6.8 to 12.9)	7.2 (4.6 to 11.3)
CCL13	43 (30 to 62)	48 (37 to 63)	66 (47 to 94)	40 (28 to 56)
CCL17	43 (31 to 60)	58 (41 to 80)	65 (47 to 89)	25 (16 to 38)

IL interleukin; TNF Tumour necrosis factor; CXC/CCL Chemokine ligand

Data presented as geometric mean (95%CI)

Discussion

In this chapter I have shown that factor analysis of exacerbation sputum biomarkers demonstrated 3 biological factors. These factors were biologically independent of each other. Subsequent cluster analysis using the biomarkers which were most independent of each other, derived from the initial factor analysis, identified 4 COPD biological exacerbation clusters. These exacerbation biomarker based clusters were clinically indistinguishable at either stable state or at exacerbations but related to inflammation and pathogen identification.

Mathematical modelling and biological phenotyping of COPD

Biological taxonomy in simple terms is the science of identifying and naming species and arranging into classifications (from the Greek τάξις, taxis 'arrangement' and νομία, nomia 'method'). The process of defining the taxonomic unit derivative usually requires exploration and interrogation of large datasets. Taxonomy uses the principle that the greater the number of shared features or characteristics between organisms then the greater the likelihood of a biological relationship (Wardlaw, 2005). These methods to characterise biology and to construct the biological taxonomy, are not standardised and can range from simple qualitative or quantitative methods to comprehensive statistical and biological techniques. As evolution is part of biology, it is not surprising that biological taxonomy, phenotyping and classifications, evolve as newer information is sought and novel methods are used to interrogate data. Factor and cluster analysis are thus becoming widely used in investigating datasets for relationships between variables. These can aid defining the heterogeneity and taxonomy of biological systems and importantly are methods which can be free from investigator bias. Factor analysis and

cluster analysis have already been shown to determine phenotypes of COPD (Pistolesi 2008; Weatherall 2009; Burgel 2010) and clinical variables during stable COPD have been used as the basis of the mathematical analysis in these studies. However, in this chapter unlike the previous COPD cluster analysis phenotype studies, my data has demonstrated for the first time, the use of biomarkers to characterise exacerbations of COPD and I have determined biological clusters of COPD independent of symptoms and physiology. This unbiased statistical method derived phenotypes of exacerbations of COPD based on biological markers.

Results of the factor analysis

In this chapter, I have used factor analysis to investigate relationships of sputum biomarkers during an exacerbation of COPD. This mathematical method was used to identify patterns of variations within the measured sputum exacerbation biomarkers. The factor analysis reduced the exacerbation sputum biomarker dataset and demonstrated that 3 factors were able to explain over 75% of the variance of the biomarker dataset. Importantly this showed that the factor analysis model was representative of the dataset and satisfied the statistical rule required to interpret the factor analysis further. The 3 factors were independent of each other, by default from the mathematical calculation (PCA and orthogonal rotation). The factor analysis output contained the correlation matrix, which was equivalent to the correlation of the individual biomarker (or variable) to the factor. Correlation matrixes of less than 0.3 (equivalent to a weak relationship) were suppressed. The sputum biomarker with the highest factor α loading and non cross loading with the other factors (i.e. the correlation was high in one factor alone) was subsequently used in the cluster analysis.

The first factor was weighted predominately by TNF-α, IL-1β, IL-6 and IL-8 was termed 'pro-inflammatory'. These cytokines are key mediators in the acute and chronic inflammatory response and are the products of activated mononuclear phagocytes and macrophages. In particular IL-1β and TNF-α, are primary cytokines of septic shock and responsible for stimulating immune responses against bacterial LPS. These pro-inflammatory biomarkers have been found to be elevated in stable COPD and during exacerbations (Chung 2001; Hurst, 2006; Perera, 2007). The second factor was termed 'Th1' and was weighted by CXCL10, CXCL11 and CCL5. The receptor for CXCL10 and CXCL11 is CXCR3 and is preferentially expressed on Th1 cells. The synthesis of these cytokines is mediated by IFN-γ. IFN-γ is pivotal in innate and adaptive immunity acting as a macrophage activating factor. The major actions of IFN-γ are inhibiting virus replication and promotion of macrophage activity. Elevated levels of serum CXCL10 have been found in COPD patients compared to non-smoking controls in response to virus (Quint, 2010). Finally, the last factor was predominantly associated with CCL17 and IL-5 and was termed 'Th2'. Th2 cells predominately secrete IL-4, IL-5, IL-10 and IL-13 and are responsible for the activation of mast cells and eosinophils. CCL17 is induced by IL-4 and IL-13 and binds to the CCR4 receptor on Th2 cells. CCL17 has been associated with allergic disease in several studies (Sekiya, 2000, Fuke 2004). The relationships of the biomarkers to the factors yielded mathematically independent patterns, which were reassuringly also biologically linked.

Results of the cluster analysis

After determining the biological factors in COPD exacerbations, cluster analysis was performed and distinct biological clusters which related to underlying aetiology and inflammation during an exacerbation of COPD were found. These biological clusters could not be distinguished clinically or by Anthonisen criteria and the exacerbation severity was similar across the clusters with similar and significant falls in lung function at exacerbation compared to baseline. These clusters demonstrated mathematical independence but were computed on biology alone.

Biomarker profiling during COPD exacerbations has the potential to further our understanding of disease mechanisms. The identification of the pro-inflammatory, Th1 and Th2 clusters may be related to differences in gene expression, protein synthesis or cellular defects; and further focussed approaches to investigate mechanism of disease in these phenotypes is warranted. The biological exacerbation clusters were repeatable for multiple exacerbation events and suggest that these exacerbation phenotypes are consistent; and these novel phenotypic approaches could lend to alternative prognostic and therapeutic strategies. Previously determined COPD phenotypes by Pistolesi and Burgel *et al* (Pistolesi, 2008; Burgel, 2010) have focussed on clinical variables to characterise COPD subjects, but results have been similar to the original 'pink puffer' and 'blue bloater' phenotypes described by Dornhorst (Dornhorst, 1955). These statistical methods can derive further hypotheses; however their use in isolation cannot conclude causality.

The identified clusters were interrogated for their biological and clinical characteristics and defined according to their predominant findings. Cluster 1 had the highest concentrations of sputum TNF-α and the greatest proportion of bacteria-associated exacerbations; Cluster 2 had the highest level of sputum CCL17 and eosinophil-associated exacerbations; Cluster 3 had elevated concentrations of CXCL10 and IL-5 and the greatest proportions of virus-associated exacerbations. Cluster 4 had the lowest concentrations of TNF-α, CXCL10 and IL-5 with reduced proportions of bacterial and eosinophilic associated exacerbations of COPD. Previous attempts to phenotype COPD subjects have focussed on the use of clinical variables such as lung function and symptoms to identify phenotypes of COPD using cluster analysis (Pistolesi, 2008; Weatherall, 2009; Burgel, 2010). These have demonstrated subtle clinical differences within groups with similar degrees of airflow obstruction. There is currently no literature available which has examined biological markers to cluster and phenotype exacerbations of COPD.

Clusters 1, 2 and 3 were related to aetiology and inflammation and biologically validated the clinical exacerbation phenotypes associated with bacteria, virus and sputum eosinophils that were selected in chapter 5. It is unclear whether the identification of the 4th pauci-inflammatory biological cluster represented a proportion of patients without clear evidence of a cause, or merely reflects the insensitivity of the chosen defined clinical exacerbation phenotypes or the methods used to detect pathogens. Psychological distress measured by anxiety and depression has been shown to be associated with increased risk of exacerbations (Xu, 2008; Eisner, 2010). However a meta-analysis has failed to demonstrate a consistent association of psychological distress and exacerbations of COPD (Laurin, 2011). The pauci-inflammatory cluster had

a significant fall in lung function during the exacerbation in addition to reduction in health quality and symptom scores similar to the other three clusters. At exacerbation this pauci-inflammatory cluster had the lowest concentrations of pro-inflammatory and Th2 mediators, with a low proportion and mixed pathogen detection rate. This together with significant smoking history, evidence of gas trapping and impaired gas transfer suggests that the mechanism underlying this cluster may not simply relate to exacerbation symptoms associated with psychological distress. It is possible that this cluster reflects a sub-group of COPD patients that have defects in their immune response to a pathogen but have increases in symptoms and falls in lung function that may be attributable to bronchospasm as the other clusters.

These clusters were mathematically derived and clinically indistinguishable at baseline and during exacerbations. This is likely to reflect the limitations in current tools available in exacerbation phenotyping, but potentially derives methods to target specific treatment. The baseline characteristics of the clusters demonstrated similarities in demographics, exacerbation frequency and lung function. However the baseline cytokine and cellular airway inflammation was significantly different between the cluster groups. This suggests that the biological fingerprint of the exacerbation clusters might be predictive of the stable COPD phenotype and *vice versa*. In patients with multiple exacerbations the biological exacerbation clusters were repeatable. In Chapter 5, I have demonstrated that bacteria and sputum eosinophilic inflammation occurs at stable state (28% and 27% respectively). The repeatability of the biological exacerbation clusters would suggest that bacterial and eosinophilic exacerbations reflect instability within a complex and inherently stable system. However, as my data demonstrated in Chapter 5, virus detection was very infrequent at stable state (5%); the

repeatability of the virus-predominant exacerbation cluster is more likely to represent acquisition of a new virus pathogen in a group that may be susceptible. It is likely that both of these mechanisms drive exacerbations, but these biological phenotypes may respond to different management strategies but further studies are required.

Limitations

In this chapter, I have used multivariate modelling statistical analysis (factor and cluster analysis) to phenotype COPD exacerbations. A limitation of these methods is that they do not show causality. However, this method identified clusters of exacerbations in an unsupervised statistical approach. The use of clinical indices of symptoms and lung function to cluster COPD phenotypes is associated with bias. The methods I have used to cluster COPD exacerbations were free from investigator bias.

To limit statistical errors, stringent efforts were made to observe the rules that were required to provide optimal analysis. The factor analysis output accounted for 75% of the variance of the sputum biomarkers during exacerbations. Similarly the methods used to cluster the exacerbations have been extensively employed to explore phenotypes in COPD; in particular they are associated with the least bias and have been validated by other groups (Haldar, 2008; Weatherall 2009; Burgel, 2010). These analyses were performed in moderate exacerbations of COPD and therefore need to be validated in severe exacerbations and other COPD patient cohorts. Finally, only biological factors during exacerbations were determined. Assessment of biological factors during stable state and whether these stable COPD clusters predict exacerbation clusters or if there is movement between phenotypes with time (examining the stability of the phenotype) need to be explored further.

Conclusions

In summary biological phenotypes of exacerbations can be determined. Inflammation within exacerbations can be outlined into pro-inflammatory, Th1 and Th2 axes. These biological phenotypes are clinically indistinguishable at exacerbation but relate to inflammation and possible aetiology. Within exacerbation clusters there is repeatability. Identification of these biological exacerbation clusters lends to the multi-dimensional approach to phenotyping COPD.

7. The stability of airway inflammatory phenotypes of COPD and predictors of exacerbations

Abstract

The heterogeneity of COPD can be defined into phenotypes, but the stability of these phenotypes and how the phenotype predicts the exacerbation event is unclear. Using longitudinal stable state data from the COPD population I demonstrate for the first time that there are microbiological and airway eosinophilic inflammatory phenotypes which are repeatable during stable state and stay to type during exacerbations. Recognition of these microbiological and eosinophilic airway inflammatory phenotypes during stable state could identify sub-populations that may benefit from preventative exacerbation measures such as prophylactic antibiotics and corticosteroid therapy.

Introduction

The natural history of COPD is the pathological destruction of lung parenchyma (Hogg, 2004) and changes within the large and small airways (Jeffery, 1998). This has been shown to lead to progressive decline in lung function (Boushy, 1973; Burrows, 1969; Fletcher, 1977), exacerbations (Hurst, 2010) and a poor quality of life (Spencer, 2004). In particular exacerbations of COPD are associated with accelerated lung function decline (Donaldson, 2002) and a high risk of mortality noticeably in severe exacerbations requiring hospitalisation (Connors, 1996). It is has been demonstrated that there is a frequent exacerbation phenotype (Seemungal, 1998; Donaldson, 2002) and that the best predictor of this phenotype is a previous history of exacerbations (Burge, 2000; Wedzicha, 2002; Hurst, 2010). Furthermore, this frequent exacerbator phenotype is independent of disease severity (Hurst, 2010). However there is limited data investigating biomarkers to identify these clinical outcomes. Improvements in decoding these phenotypes are likely to be a key component in COPD management strategies. In this chapter I have investigated whether i) there are stable airway inflammatory COPD phenotypes; ii) if clinical exacerbations phenotypes can be predicted from the stable state; and iii) if there are biomarker predictors of frequent and infrequent exacerbation phenotypes.

Methods

Patient selection, study design, measurements and data collection were performed as described in chapter 5. The baseline characteristics of all patients are presented in table 5.1.

Phenotype stability was assessed using stable longitudinal visit data. Phenotype characteristics of interest associated with bacterial colonisation, airway inflammation and peripheral eosinophil counts were characterised into never, intermediate and always, which was dependant on the frequency over time. Only patients with at least two stable visits were included in this analysis, whilst the maximum number of stable visits per study design was 5 (observations were taken for the duration of 12 months with visits every 3 months as presented in figure 5.1).

The assigned longitudinal classification was as follows;

*Never***:** this was classified when none of the samples conferred to either i) sputum eosinophilia, ii) peripheral blood eosinophilia (defined as > 400 x10^6 cells/L), or iii) the presence of a pathogenic micro-organism on standard routine culture;

Intermediate: this was classified when >1 but not all of the samples confirmed to the phenotypes;

Always: this was classified when all 5 samples confirmed to the phenotypes.

Odds ratios (OR) and proportions of phenotype stability and their predictive capacity for exacerbation phenotypes were populated and analysed using contingency tables. Logistic and multiple regression statistical analysis was used to analyse the relationship and impact of independent variables (including sputum and serum biomarkers) on the frequent exacerbator phenotype in the COPD cohort. Put simply the analysis aims to answer the question 'what is the best predictor of exacerbations'. In logistic regression, analogous to simple linear regression (where a linear relationship is drawn between two quantitative variables) the relationship between independent predictor variables and a binary categorical outcome (the explanatory variable) of interest is explored. Logistic

regression analysis uses the natural log of the odds (the ratio of the proportions for two possible outcomes) and models these odds (of the independent variable of interest) as a linear function of the explanatory variable (binary outcome of interest) to determine relationships. In simple terms, using binary outcomes, the mean of the binary distribution (P) is equivalent to the proportion of 1's in the sample, whilst the mean or proportion of 0's in the distribution (Q) is denoted as 1-P. The variance and standard distribution is then denoted as PQ and √PQ respectively. Plotting this on a standard x and y axis would demonstrate that the regression line is non-linear with none of the points on the regression line. Log transformed data was used and maximised the goodness-of-fit of the line and the model. In this chapter, using logistic regression analysis the assigned explanatory variable of interest was the exacerbator phenotype. To draw this in a logistic regression format, the frequent exacerbation phenotype was defined as exacerbation frequency > 3, whilst the infrequent exacerbation phenotype was defined as exacerbations frequency ≤ 3 (thus the binary code used is frequent exacerbator phenotype yes =1, no=0).

Multiple regression analysis was also used and is similar to linear regression. However, multiple regression analysis explores the relationship between several independent variables and the explanatory variable using quantitative data. The simplest equation for multiple regression is $Y = \alpha + \beta \times X$, where Y is the explanatory variable, X is the independent variable, β is the regression coefficient (or slope of the line) and α, the intercept constant. The regression coefficient β represents the independent contribution of each independent variable. In the multiple regression analysis the explanatory variable was frequency of exacerbations (on a nominal scale). Independent predictor variables of interest (clinical and biomarker datasets) for both logistic and multiple

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regression analysis were chosen from initial univariate analysis and obeyed the function of association where p<0.10.

Results

Stable phenotypes of COPD

As shown in chapter 5 and figure 5.10, there was very little aetiological overlap in clinical exacerbation phenotypes associated with bacteria, virus and a sputum eosinophilia. This was a similar finding at the baseline stable visit and is shown in figure 7.1.

Figure 7.1; Non proportional Venn diagram for bacterial, eosinophilic and virus associated clinical COPD phenotypes at baseline stable state. Data presented as percentages and included only patients with full sputum dataset for detection of bacteria from standard culture, virus from polymerase chain reaction and sputum differential cell count
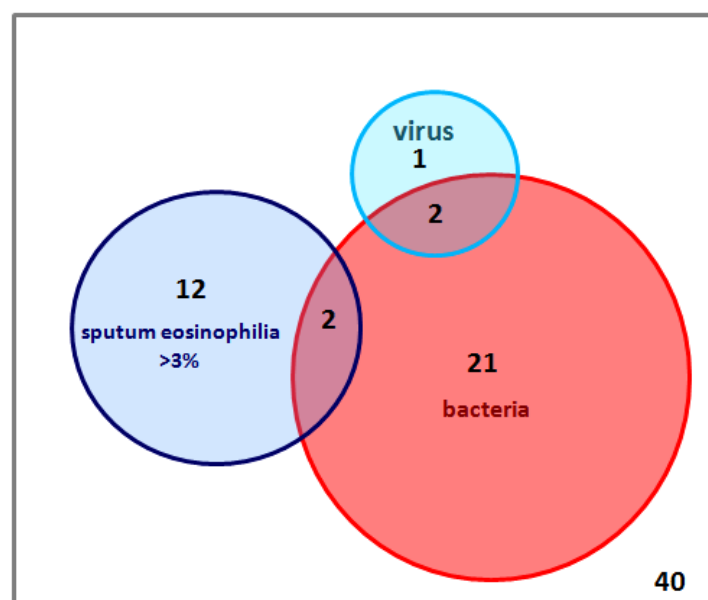
Microbiological COPD phenotypes

Firstly, microbiological phenotypes were examined during stable state in patients with a minimum and maximum of 2 and 5 available sputum cultures respectively (n=134) and analysed for the proportions of samples that were culture positive. The microbiological phenotype was classed as **always** culture positive, **intermediate** culture positive (proportion of positive samples ranged from 20 to 80%) and **never** culture positive.

The microbiological phenotype frequency in the COPD population over 1 year showed that 16% were always culture positive; 31% were intermediate; and 54% were never culture positive. This demonstrated that almost half of patients had a PPM on routine standard culture during 1 year. Always microbiological phenotype patients were more likely to have bacterial exacerbations than those that were never culture positive (89% vs. 34%, χ^2 p<0.0001).

Figure 7.2; Percentage of bacterial exacerbations that are associated with never, intermediate and always microbiological phenotypes in the stable state.
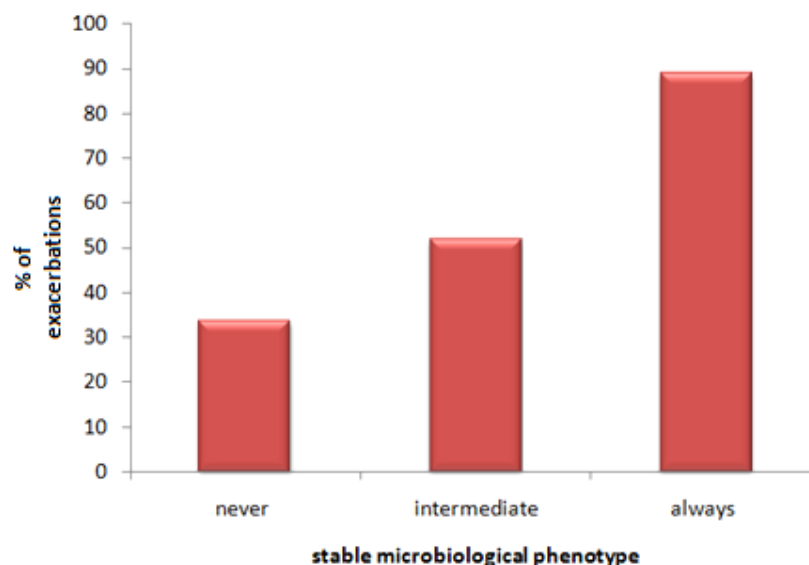
Patients in the always microbiological phenotype reported increased symptoms of cough, sputum production and purulence. The microbiology phenotype characteristics are presented in table 7.1.

Table 7.1; Clinical characteristics in COPD patients according to the microbiological phenotype

	Never **N=72**	**Intermediate** **N=41**	**Always** **N=21**	**p-** **value**
Age*	68 (43 to 85)	68 (49 to 88)	73 (53 to 84)	0.08
Pack years smoked*	45 (10 to 120)	55 (10 to 134)	53 (10 to 153)	0.17
Exacerbation frequency	3.1 (0.3)	3.9 (0.5)	4.4 (0.5)	0.11
FEV_1, L	1.38 (0.07)	1.39 (0.09)	1.26 (0.10)	0.62
FEV_1 % predicted	54 (2)	50 (3)	50 (4)	0.49
FEV_1/FVC, %	55 (2)	50 (2)	50 (3)	0.17
BMI, kg/m^2	27 (1)	26 (1)	27 (1)	0.41
T_LCO, %	60 (3)	55 (4)	52 (5)	0.38
SGRQ $_{total}$ units	50.1 (2.2)	50.8 (3.0)	55.1 (4.3)	0.60
CRQ $_{total}$ units	4.2 (0.)	4.2 (0.2)	3.8 (0.3)	0.28
VAS $_{total}$ mm	**126 (9)**	**167 (11)**	**168 (18)**	**0.01**
VAS $_{cough}$ mm	**30 (3)**	**45 (4)**	**39 (7)**	**0.02**
VAS $_{dyspnoea}$ mm	45 (3)	44 (4)	59 (6)	0.08
VAS $_{sputum\ volume}$ mm	**28 (3)**	**42 (4)**	**43 (6)**	**0.01**
VAS $_{sputum\ purulence}$ mm	**24 (3)**	**36 (4)**	**29 (6)**	**0.05**

FEV_1 Forced expiratory volume in 1 second; FVC Forced vital capacity; BMI Body mass index; T_LCO Diffusing capacity of the lung for carbon monoxide; SGRQ St Georges Respiratory Questionnaire, scores ranging from 0 to 100 with higher score indicating worse health status (total score on domains of Impact, Symptoms and Activity); CRQ Chronic Respiratory Disease Questionnaire, scores range between 1 to 7 with higher score representing better health quality; VAS Visual Analogue Scale, performed on 100mm line from 'no symptoms' to 'worst symptoms', higher scores represent worse symptoms (total score addition of measured domains: cough, dyspnoea, sputum production and sputum purulence). Data presented as mean (SEM) or *mean (range)

An elevated CRP and sputum neutrophil count was detected in subjects with the always microbiological phenotype compared to the intermediate and never microbiological phenotype (median (IQR) CRP 16 (17) vs. 5 (9) vs. 3 (6) respectively, p=0.004; % sputum neutrophil counts 81 (4) vs. 66 (3) vs. 66 (3) respectively, p=0.02).

Sputum eosinophil COPD phenotypes

In stable sputum samples the airway eosinophilic phenotype was also interrogated (n=126). This displayed that at stable state, 10% of patients were always eosinophilic, 45% were intermediate and 45% were never eosinophilic. Patients in the always sputum eosinophilic phenotype during stable state were more likely to have eosinophilic exacerbations of COPD compared to those that were of the never sputum eosinophilic phenotype (80% vs. 13%, χ^2 P<0.0001; figure 7.3).

Figure 7.3; Percentage of sputum eosinophilic exacerbations that are associated with never, intermediate and always airway eosinophilic phenotypes
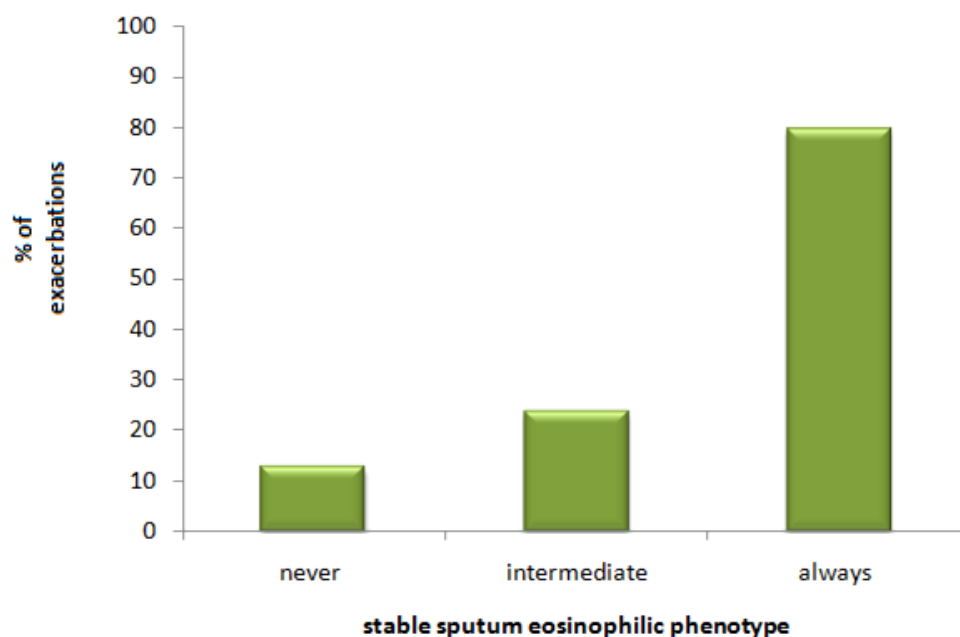
Only the SGRQ impact domain was found to be significantly different between patients in the 3 sputum eosinophilic phenotypes (see table 7.2).

Table 7.2; Clinical characteristics in COPD patients according to the sputum eosinophil phenotype

	Never N=57	Intermediate N=56	Always N=13	p-value
Age*	69 (47 to 88)	68 (43 to 88)	68 (45 to 83)	0.73
Pack years smoked*	51 (10 to 130)	50 (10 to 134)	38 (10 to 72)	0.32
Exacerbation frequency	3.4 (0.3)	3.5 (0.4)	4.3 (0.8)	0.56
FEV_1, L	1.38 (0.07)	1.42 (0.08)	1.12 (0.15)	0.21
FEV_1 % predicted	53 (2)	53 (3)	48 (6)	0.63
FEV_1/FVC, %	54 (2)	53 (2)	50 (5)	0.65
BMI, kg/m^2	27.0 (0.6)	26.4 (0.6)	25.7 (1.5)	0.67
RV, %	132 (6)	125 (6)	144 (13)	0.22
T_LCO, %	58 (3)	59 (3)	47 (5)	0.23
SGRQ $_{total}$ units	**53.0 (2.5)**	**46.6 2.6)**	**61.6 (4.9)**	**0.03**
SGRQ $_{symptoms}$ units	61.4 (2.7)	56.4 (3.1)	71.5 (6.5)	0.10
SGRQ $_{activity}$ units	67.4 (2.8)	63.2 (3.2)	75.4 (5.9)	0.23
SGRQ $_{impacts}$ units	**42.6 (2.9)**	**34.8 (2.6)**	**50.8 (5.5)**	**0.02**
CRQ $_{total}$ units	4.0 (0.1)	4.3 (0.2)	4.0 (0.4)	0.57
VAS $_{total}$ mm	152 (10)	134 (11)	159 (25)	0.38

FEV_1 Forced expiratory volume in 1 second; FVC Forced vital capacity; BMI Body mass index; T_LCO Diffusing capacity of the lung for carbon monoxide; SGRQ St Georges Respiratory Questionnaire, scores ranging from 0 to 100 with higher score indicating worse health status (total score on domains of Impact, Symptoms and Activity); CRQ Chronic Respiratory Disease Questionnaire, scores range between 1 to 7 with higher score representing better health quality; VAS Visual Analogue Scale, performed on 100mm line from 'no symptoms' to 'worst symptoms', higher scores represent worse symptoms (total score addition of measured domains: cough, dyspnoea, sputum production and sputum purulence). Data presented as mean (SEM) or *mean (range)

Peripheral blood eosinophil COPD phenotypes

Peripheral blood eosinophil counts have already been shown to be a sensitive and specific marker of a sputum eosinophilia during exacerbations in chapter 5. The proportion of COPD patients with a peripheral blood eosinophilia ($>0.4 \times 10^9$ cells/L) at baseline (single time point) was 20%. The proportion of COPD patients that always and never had a peripheral blood eosinophilia during stable state was 12% and 65% respectively. Patients that always had a peripheral blood eosinophilia were 3 times more likely to have a sputum eosinophilia during exacerbations (figure 7.4).

Figure 7.4; Percentage of sputum eosinophilic exacerbations that are associated with never, intermediate and always peripheral eosinophilic phenotypes
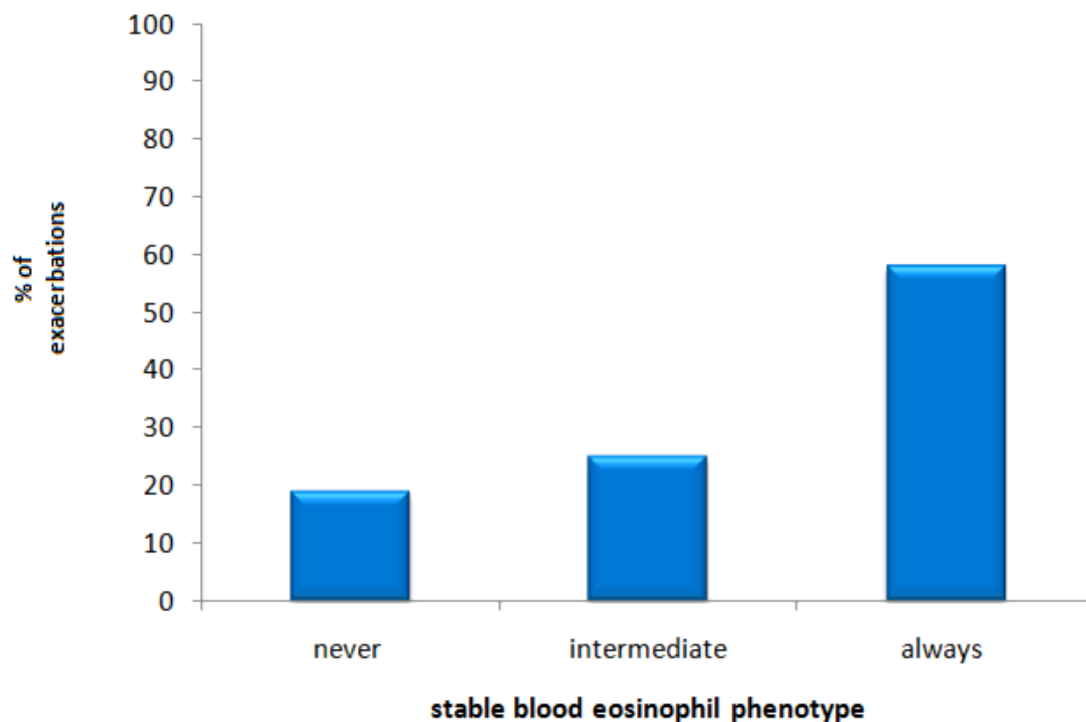
There was no clinical differences between patients in the never, intermediate and always peripheral blood eosinophil phenotypes (table 7.3).

Table 7.3; Clinical characteristics in COPD patients according to the peripheral blood eosinophil phenotype

	Never N=96	Intermediate N=29	Always N=20	p-value
Age*	68 (45 to 88)	70 (43 to 84)	70 (53 to 84)	0.56
Pack years smoked*	51 (10 to 153)	53 (12 to 134)	37 (10 to 95)	0.13
Exacerbation frequency	3.3 (0.3)	4.0 (0.6)	3.7 (0.6)	0.38
FEV_1, L	1.34 (0.06)	1.28 (0.11)	1.34 (0.12)	0.83
FEV_1 % predicted	52 (2)	52 (4)	51 (4)	0.98
FEV_1/FVC, %	53 (1)	54 (3)	49 (4)	0.51
BMI, kg/m^2	27.0 (0.5)	25.8 (1.0)	24.9 (0.8)	0.14
RV, %	134 (5)	125 (8)	120 (9)	0.34
T_LCO, %	58 (3)	56 (5)	54 (4)	0.80
SGRQ $_{total}$ units	51.1 (1.8)	50.6 (3.8)	52.6 (4.8)	0.93
CRQ $_{total}$ units	4.1 (0.1)	3.9 (0.3)	4.3 (0.3)	0.50
VAS $_{total}$ mm	143 (8)	135 (16)	144 (18)	0.87

FEV_1 Forced expiratory volume in 1 second; FVC Forced vital capacity; BMI Body mass index; T_LCO Diffusing capacity of the lung for carbon monoxide; SGRQ St Georges Respiratory Questionnaire, scores ranging from 0 to 100 with higher score indicating worse health status (total score on domains of Impact, Symptoms and Activity); CRQ Chronic Respiratory Disease Questionnaire, scores range between 1 to 7 with higher score representing better health quality; VAS Visual Analogue Scale, performed on 100mm line from 'no symptoms' to 'worst symptoms', higher scores represent worse symptoms (total score addition of measured domains: cough, dyspnoea, sputum production and sputum purulence). Data presented as mean (SEM) or *mean (range)

Predicting COPD clinical exacerbation phenotypes from stable state

The differentiation into microbiological, airway eosinophil and peripheral eosinophil phenotypes and the identification of exacerbation clinical phenotypes requires detailed

knowledge of phenotypic description over a series of visits for the duration of 1 year (minimum of 2 visits, maximum of 5 visits). An alternative approach is to determine the likelihood of defined clinical exacerbation phenotypes based on at least 1 event of microbiological culture, sputum eosinophilia or peripheral blood eosinophilia.

The OR (95% CI) for a bacteria associated exacerbation was 4.9 (2.4 to 9.9; p<0.001) if the patient had a PPM on diagnostic routine culture on ≥1 occasion at stable state. The OR (95% CI) for an eosinophil associated exacerbation was 2.7 (1.3 to 5.7; p=0.01) if the patient had a sputum eosinophilia on ≥1 occasion at stable state. The OR (95% CI) for an eosinophil associated exacerbation was 2.9 (1.4 to 6.0, p=0.004) if the patient had a peripheral eosinophil count >0.4 $x10^9$ cells/L on ≥1 occasion at stable state.

Virus detection during stable state was rare (5 patients at study entry). When studied, the presence of virus during stable state did not affect the occurrence of a virus associated exacerbation. The OR (95% CI) for a virus associated exacerbation if the patient had a virus at stable state was 0.5 (0.1 to 3.9, p=0.67).

Frequent exacerbator phenotypes

Exacerbations that were captured and non-captured were examined for all patients entered into the study. Factors associated with exacerbation frequency were assessed for frequent exacerbators. 50% of the COPD cohort had > 3 exacerbations in 1 year and classified as frequent exacerbators. Number of exacerbations correlated significantly with baseline lung function, markers of systemic inflammation and quality of life (table 7.4 to 7.6).

Table 7.4; Correlation coefficients of exacerbation frequency with lung physiology at baseline state

	Correlation coefficient r	p-value
FEV_1	-0.26	**<0.01**
FEV_1 % predicted	-0.33	**<0.01**
FEV_1 /FVC	-0.26	**<0.01**
BMI	-0.22	**0.01**
RV %	0.30	**<0.01**
T_LCO %	-0.41	**<0.01**

FEV_1 Forced expiratory volume in 1 second; FVC forced vital capacity; BMI Body mass index, RV Residual volume; T_LCO Diffusion capacity of the lung for carbon monoxide

Table 7.5; Correlation coefficients of exacerbation frequency with systemic inflammatory markers at baseline state

	Correlation coefficient r	p-value
Total leukocyte count	0.15	0.09
Neutrophil count	0.12	0.17
Eosinophil count	0.05	0.53
CRP	0.19	**0.03**
PCT	0.19	**0.03**
SPD	-0.03	0.75

CRP C reactive protein; PCT procalcitonin; SPD Surfactant protein D

Table 7.6; Correlation coefficients of exacerbation frequency with health status and symptom scores at baseline state

	Correlation coefficient r	p-value
SGRQ symptoms	0.15	0.08
SGRQ activity	0.34	**<0.01**
SGRQ impacts	0.31	**<0.01**
CRQ emotion	-0.09	0.30
CRQ fatigue	-0.14	0.11
CRQ dyspnoea	-0.24	**0.01**
CRQ Mastery	-0.19	**0.03**
VAS cough	0.13	0.14
VAS dyspnoea	0.15	0.09
VAS sputum volume	0.23	**0.01**
VAS sputum purulence	0.05	0.60

SGRQ St Georges Respiratory Questionnaire, scores ranging from 0 to 100 with higher score indicating worse health status; CRQ Chronic Respiratory Disease Questionnaire, scores range between 1 to 7 with higher score representing better health quality; VAS Visual Analogue Scale, performed on 100mm line from 'no symptoms' to 'worst symptoms', higher scores represent worse symptoms

There was no correlation detected for exacerbation frequency and sputum total cell counts, sputum neutrophil counts, sputum eosinophil counts or colony forming units at baseline. For all biomarkers measured at baseline, only sputum IL-6 correlated with exacerbation frequency (r=0.22, p=0.011). Frequent exacerbators significantly differed in GOLD severity (χ^2, p<0.001), but not with respect to the sputum eosinophil or microbiological phenotypes (see figures 7.5).

Figure 7.5; Bar charts representing proportions of frequent and non frequent exacerbators with COPD severity, sputum eosinophil and microbiological phenotypes
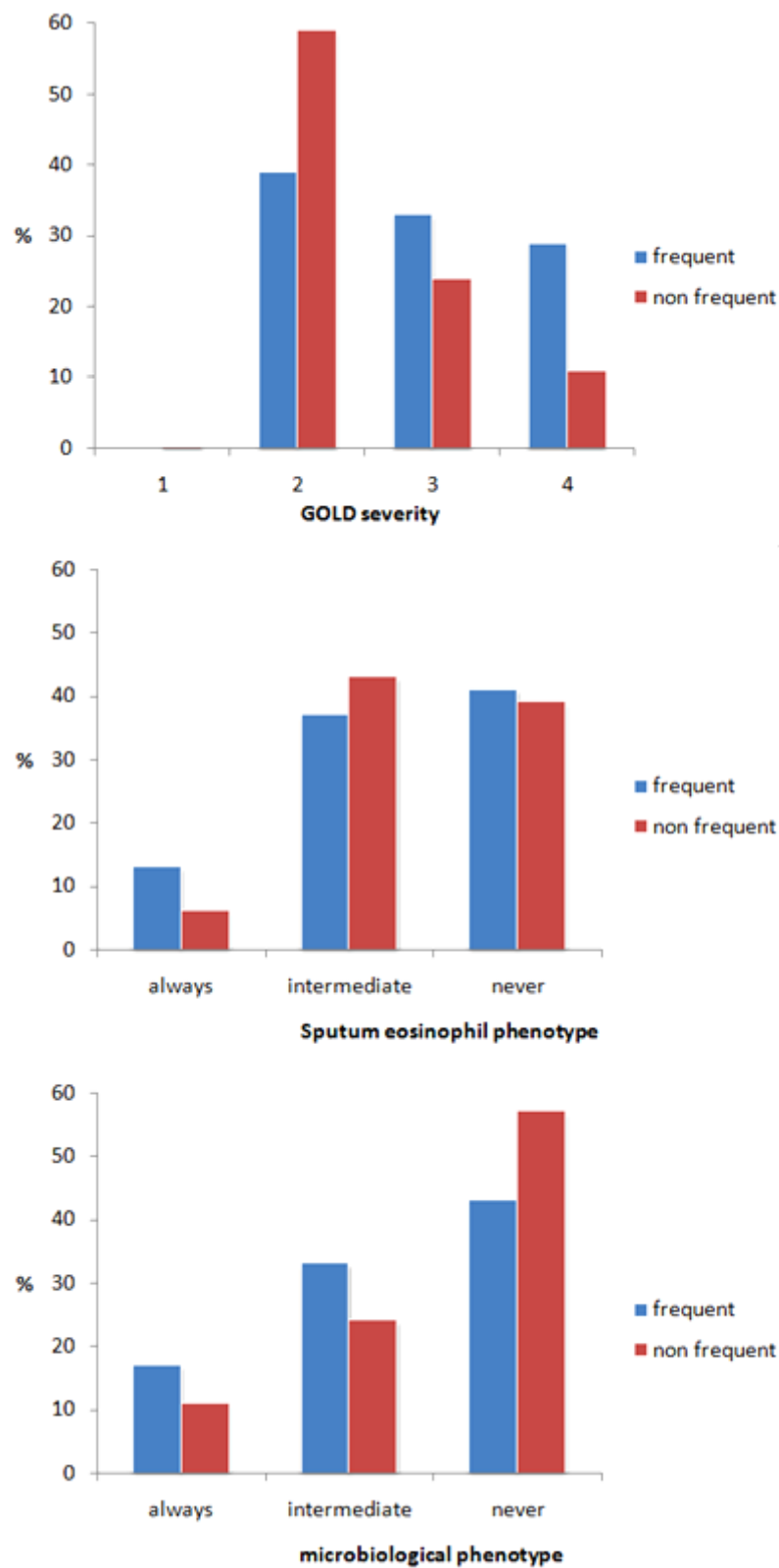
Univariate analysis determined that lung function, gas transfer and BMI were significantly lower in the frequent exacerbation phenotype, whilst CRP, sputum IL-10 and sputum IL-6 were significantly higher compared to non-frequent exacerbators.

Multivariate modelling using logistic regression analysis was applied to determine independent predictors of the frequent exacerbator phenotype using FEV_1 %, FEV_1 /FVC, T_LCO, BMI, CRP and sputum IL-6. The modelling also included sputum eosinophil differential cell count as univariate analysis demonstrated a $p<0.10$ ($p=0.08$ with trend to higher in frequent exacerbators). Model inspection showed no multicollinearity and observed the Hosmer-Lemeshow χ_2 goodness-to-fit test (an indicator of the regression line of best fit). The R^2 was 0.36 and indicated that 36% of the variance was explained by the model. Only T_LCO remained to be an independent predictor of frequent exacerbators using this model (OR (95%CI) 0.96 (0.93 to 0.99), $p=0.015$).

Repeatability of measures

The repeatability over two stable visits 3 months apart for lung function, sputum total and differential cell counts and blood eosinophil cell counts were examined using the intra-class coefficient for all patients in the study. This is presented in table 7.5.

Table 7.7; Measurements of repeatability at 2 visits, 3 months apart

	Visit 2	Visit 3	Intra Class Coefficient
FEV$_1$ *	1.3 (0.5)	1.4 (0.6)	0.94
Sputum TCC ^	3.8 [3.1 to 4.7]	2.8 [2.1 to 3.6]	0.68
Sputum Neutrophil % *	69 (22)	70 (23)	0.75
Sputum Eosinophil %^	1.2 [1.0 to 1.6]	1.0 [0.8 to 1.3]	0.77
Blood Eosinophils^	0.2 [0.18 to 0.23]	0.2 [0.17 to 0.23]	0.79
Blood Eosinophils %^	2.5 [2.2 to 2.8]	2.5 [2.2 to 2.9]	0.79

FEV$_1$ Forced expiratory volume in 1 second; TCC Total sputum cell count

Data presented as *mean (standard error of the mean) and ^ geometric mean (95% confidence interval of the geometric mean).

The inter-intra observer agreement was calculated over 2 consecutive visits for microbial culture and determined that the κ statistic was 0.6, suggesting good agreement and thus good repeatability of detection of a pathogenic bacteria over time.

Discussion

In this chapter using longitudinal data, I have demonstrated that airway inflammatory and peripheral blood COPD phenotypes exist and are predictive of the clinical exacerbation phenotype. These stable state phenotypes are clinically indistinguishable. I have examined clinical, physiological and biological associations with exacerbations in this cohort and determined that systemic and pulmonary inflammation is correlated to exacerbation frequency, whilst multivariate regression analysis showed that the most independent predictor of frequent exacerbations was the $T_L CO$.

Longitudinal stable disease phenotypes

A phenotype has been traditionally defined as the observable characteristics of an organism determined by its genotype and modulated by its environment (Rice, 2001). Clinical, physiological and radiological characteristics have been commonly used to describe phenotypes of COPD but do not provide information regarding prognostic or therapeutic characteristics (Friedlander, 2007; Weatherall 2009; Burgel, 2010). This has recently led to a variation in the definition of a COPD phenotype proposed by Han *et al* as 'a single or combination of disease attributes that describe differences between individuals with COPD as they relate to clinically meaningful outcomes' (Han, 2010). A major limitation with these definitions is the omission of a statement on the need for repeatability or stability in observations and no indication of a suitable time point at which to define the phenotype.

In this study I have investigated the presence of microbiological and eosinophilic phenotypes of COPD and demonstrated that they relate to clinical exacerbation outcomes and are stable over time. Importantly these phenotypes were clinically

indistinguishable using the parameters of lung function and disease health status. At baseline there were differences in symptoms of cough, sputum production and purulence in the microbiological phenotype. The microbiological phenotype was also found to predict bacteria associated exacerbations of COPD. The presence of bacteria during stable COPD is common (Monso, 1995; Soler, 1999) and is considered to reflect colonisation of the airways. However, the current definition of colonisation must fulfil the criterion of both the absence of infective symptoms and lack of a host immune response (Sethi, 2008). Despite its simplicity and frequent use, this definition is weak for several reasons. Firstly, COPD is a chronic inflammatory disease which is commonly associated with variation in symptoms. Studies have consistently demonstrated increased systemic and pulmonary inflammation during stable state (Bhowmick 2000; Donaldson 2005; Barnes 2006; O'Donnell 2006). I have also replicated these findings in chapter 5 and it is unlikely that microbial colonisation in COPD would occur without inflammation. Secondly, the current definition of colonisation does not explore the frequency of pathogen detection over time during stable state. In studies that have examined bacterial colonisation, this has been defined at a single time point (Soler 1999; Sethi 2008). This is unlikely to be representative of phenotype characteristics or phenotype stability. In an aim to eliminate these factors, I have categorised the phenotypes based on the proportion of time a patient demonstrated a particular type of bacterial or eosinophilic phenotype. I have used longitudinal data and investigated the associations of microbiological and eosinophilic phenotypes of COPD with exacerbations and demonstrated that the microbiological and eosinophilic phenotypes were stable and predictive of the clinical exacerbation phenotype associated with bacteria and sputum eosinophils. This is the first time COPD phenotype stability has been demonstrated.

Microbiological phenotypes of COPD

In this study I have shown that approximately 50% of COPD patients have bacteria isolated from the airway on greater than 2 occasions in a 12 month time period. Using current definitions of colonisation, it has been recognised that up to 30% of COPD subjects are 'colonised' with microbes and the detection of pathogens at stable state has been related to disease severity (Patel, 2002; Donaldson, 2005; Hurst, 2005). However the current methods used to define 'colonisation' are likely to under-estimate the frequency. In this study I have used the presence of pathogenic bacteria on standard culture to determine the microbiological phenotype. This is in accordance with currently used methods of detection, albeit at a single time point, for defining colonisation (Monso 1995; Sethi 2008). Development of newer molecular methods to identify bacteria may re-define colonisation. In work presented in chapter 5, qPCR analysis demonstrated that bacteria detection at stable state at a single time point was found in over 85% of samples. DNA sequencing methods by Hilty *et al* have demonstrated that the bronchial tree in healthy controls, asthmatics and COPD subjects are not sterile and the upper and lower airways of asthmatic and COPD subjects demonstrate similar distributions of the top seven bacterial phyla (Hilty 2010). Moreover, recent intricate molecular bacterial sequencing by Erb-Downward *et al* have shown that the bacterial community is diverse between smokers and COPD subjects, within the right and left lung of the same COPD subject and within the upper and lower lobes of the same lung (Erb-Downward 2011). These newer detection methods indicate that the lung microbiota is altered before there is spirometric evidence of lung disease and may suggest important insights into the pathogenesis of COPD. Perpetual microbial changes in the COPD lung (or within the same lobe) may thus be the stimulus for inflammation in COPD. Spatial diversity of the bacterial community in the COPD lung may give rise

to pathogenic and non-pathogenic anatomic damage. However, there is little information that exists using these techniques longitudinally and less data to show that exacerbation phenotypes can be predicted from a baseline stable state either at a single time point or over several time points. Investigation of pulmonary inflammation with corresponding bacterial molecular typing in different anatomic regions will improve further the complex interactions occurring in the lung and further studies are required.

Although not explored in this chapter there may be a role for prophylactic antibiotics in COPD patients with the microbiological phenotype. There has been a recent revival of interest in the role of prophylactic antibiotics in COPD. Previous studies have used erythromycin (Seemungal 2008), moxifloxacin (Sethi 2010) and azithromycin (Albert, 2011) to assess improvement in exacerbation frequency. Seemungal *et al* have demonstrated that time to first exacerbation, exacerbation frequency and recovery was superior in the low dose twice daily erythromycin group compared to placebo with the largest benefit in subjects that were frequent exacerbators. However the study did not achieve statistical power for the primary outcome and time to first exacerbation is a very difficult measurement to capture (Seemungal, 2008). Sethi *et al* demonstrated that pulsed moxifloxacin (5 day treatment duration) every 2 months in COPD subjects was associated with a reduction the exacerbation rates. This was only statistically significant in an intention to treat analysis but the strength of the association was favourable in a sub-group analysis of subjects reporting mucopurulent/purulent sputum at study entry (Sethi, 2010). More recent work by Albert *et al* showed that low dose once daily azithromycin had similar improvements to the erythromycin study by Seemungal *et al*, but surprisingly this efficacy occurred in milder GOLD stage 2 COPD; azithromycin was associated with significantly more side effects and no benefits in smokers with

severe disease were detected (Albert, 2011). The identification of microbiological phenotypes of COPD lends to the hypothesis that these patients may have a positive treatment response to antibiotics. A targeted study of prophylactic antibiotics in the microbiological phenotype investigating clinically important outcomes such as reduction in exacerbation frequency and improvement in health status are thus warranted.

Eosinophilic phenotypes of COPD

This chapter also identified an airway and peripheral blood eosinophilic phenotype, which were clinically indistinguishable. This inflammatory phenotype was predictive of sputum eosinophilic exacerbations of COPD. Importantly the eosinophil has been determined to be a feature of COPD exacerbations and in particular confers corticosteroid responsiveness at stable state (Pizzichini, 1997; Pizzichini, 1998; Brightling, 2000). As demonstrated in chapter 5 the peripheral blood eosinophil count is sensitive and specific to detect an eosinophilic exacerbation of COPD. The predictive value of identifying an eosinophilic phenotype at stable state introduces the concept of specific targeted eosinophil reduction strategies in COPD (Siva, 2007) which has been previously demonstrated in asthma (Green, 2002; Haldar, 2009).

Frequent exacerbator phenotype of COPD

In this study I also investigated predictors of exacerbations. I have shown that the frequent exacerbator phenotype, defined as > 3 exacerbations per year was common. Exacerbation frequency in this chapter was associated with poor quality of life, lung function and markers of systemic and pulmonary inflammation and is similar to previously published data from the East London Cohort (Seemungal, 1998; Donaldson,

2002). Interestingly however, multivariate regression analysis in this chapter revealed that the most independent predictor of the frequent exacerbator phenotype was the T_LCO.

The ECLIPSE work in COPD subjects has identified that a history of gastro-oesophageal reflux, worsened quality of life measures and exacerbations are predictive of the risk of future exacerbations (Hurst, 2010). My data, although smaller, had very similar findings using univariate analysis. A history of previous exacerbations was the most independent predictor of future exacerbations in the ECLIPSE cohort. However, Hurst *et al* (Hurst, 2010) did not analyse predictors of the frequent exacerbator phenotype which may address the subtle differences between my findings and ECLIPSE study. The most comprehensive work thus far on the frequent exacerbator COPD phenotype has been published from the East London COPD cohort. The frequent exacerbator phenotype was initially defined in a study by Seemungal *et al* according to the median value of exacerbations from 70 COPD patients followed up for 1 year (Seemungal 1998). Approximately half of these exacerbations were captured, whilst the remainder were counted as exacerbations from diary card records. Thus the frequent exacerbator phenotype was categorised as subjects with a median number of exacerbations of greater than or equal to 3. The frequent exacerbator phenotype had worsened health quality and symptoms measured by the SGRQ (for all the domains of activity, symptoms and impact) and factors predictive of this phenotype were a previous history of exacerbations and symptoms associated with either a cough, wheeze or sputum production. Furthermore, in a smaller study by Patel *et al*, in a sub-group of patients the presence of a pathogen was found to predict the frequent exacerbator phenotype in cross sectional analysis (Patel, 2002).

In this chapter, I found that the T_LCO in multivariate regression analysis was an independent predictor of frequent exacerbators. Additional data analysis on a larger number of variables similar to that studied by the ECLIPSE cohort may have violated the integrity of the statistical modelling in this chapter and was thus not performed. There is little published work by colleagues in the ECLIPSE cohort and the East London COPD cohort exploring comprehensive physiological testing and the frequent exacerbator phenotype. No measurements of diffusion capacity of the lung (uncorrected or corrected for alveolar volume) are presented in their published work. In a small study, Groenewegen *et al* has showed that a lower T_LCO, in addition to lower FEV_1 and higher levels of fibrinogen were independent predictors of a severe exacerbation (Groenewegen, 2008). The T_LCO is a physiologic measure reflecting i) parenchymal abnormalities, ii) pulmonary vasculature abnormalities and iii) impaired cardiac output. It could be construed that subjects with severe disease, demonstrated by increased parenchymal destruction and thus lower T_LCO have more frequent exacerbations. Several authors have shown that exacerbation frequency is related to disease severity measured by simple spirometry (Connors 1996; Donaldson 2002; Hurst 2010). A further possibility is that there is impaired diffusion across damaged lung due to the physical barrier of increased sputum and inflammatory exudates. The findings by Patel *et al* would corroborate this (Patel 2002) and relate to the 'British Hypothesis' and the original description of the 'blue bloater' by Dornhorst (Dornhorst, 1955). Pathological studies (Hogg 2011) have demonstrated vascular re-modelling in COPD. This may be a primary event of the systemic features of COPD or as a consequence of chronic hypoxia with secondary pulmonary hypertension. Finally, reduced cardiac output as a consequence of impaired left ventricular function and associated cardiovascular co-morbidities may also be a driver of the severity of COPD and thus frequency of

exacerbations. Although likely to be a multi-factorial driver in frequency of exacerbations, my findings suggest that assessments in the severity of lung disease in a clinical setting should include diffusion capacity and may infer that parenchymal destruction, inflammation and pulmonary vascular remodelling in COPD play an important role in the frequent exacerbation phenotype.

Limitations

In this chapter, I could not determine biomarkers measured at stable state, to predict exacerbation phenotypes of COPD. This is a similar finding to that observed by work performed by Hurst *et al* (Hurst, 2006). My study did not look at longitudinal biomarker levels throughout stable state. This limitation means that particular patterns of biomarkers were not investigated and may conceal biomarker signatures for exacerbator phenotypes. Repeatability of serum and sputum biomarkers needs to be assessed and assessments of biomarker stability over time is required. In this study airway phenotypes were classified arbitrarily into never, intermediate and always. No data or definition exists to characterise a stable phenotype and current definitions of colonisation have significant limitations. However my proposed method to define stable phenotypes of COPD will need validation in other COPD cohorts, but importantly were found to be associated with exacerbation phenotypes.

Conclusions

To conclude, there are important microbiological and eosinophilic phenotypes of COPD that can be stratified according to longitudinal data. These phenotypes relate to exacerbations of COPD and are associated with powerful predictive capacities.

8. The use of peripheral blood eosinophils to direct prednisolone treatment in exacerbations of chronic obstructive pulmonary disease: a randomised placebo controlled trial

Abstract

Exacerbations of COPD and responses to treatment are heterogeneous. I sought to investigate the utility of blood eosinophil counts to direct corticosteroid therapy during exacerbations, by performing a one-year double-blind randomised study of standard versus biomarker-directed corticosteroid therapy during COPD exacerbations. Prednisolone use in the biomarker-directed group was reduced without worsening symptoms or an increase in treatment failures. Exacerbations which were biomarker negative (peripheral blood eosinophil count $\leq 2\%$) and given prednisolone had more treatment failures and worse symptom scores after treatment compared to the biomarker negative group which received placebo. The peripheral blood eosinophil count is a promising biomarker to direct corticosteroid therapy during COPD exacerbations.

Introduction

Acute exacerbations of COPD have been shown to be heterogeneous with respect to inflammation (Saetta, 1994; Bhowmik, 2000), aetiology (Seemungal, 2001; Sethi, 2002; Papi, 2006) and treatment responses (Davies, 1999; Niewoehner, 1999; Aaron, 2003). The biomarker PCT has been used to target antibiotic therapy successfully in lower respiratory infections (Christ-Crain, 2004; Christ-Crain, 2006; Schuetz, 2009), whilst titrating inhaled and oral corticosteroid treatment according to the sputum eosinophil count was associated with reduction in exacerbation frequency in stable asthma (Green, 2002) and stable COPD (Siva, 2007). Current guidelines advocate the use of systemic corticosteroids during COPD exacerbations with improvements in the rate of recovery (Davies, 1999) despite being associated with significant side effects (Niewoehner, 1999) and with limited benefits in reducing mortality (Walters, 2009). Increased eosinophilic airway inflammation has been shown to occur during exacerbations of COPD (Saetta, 1994; Saetta, 1996; Bathoorn, 2009). It is not known if a sputum eosinophilia during exacerbations of COPD confers corticosteroid responsiveness or whether using a biomarker-targeted strategy – analogous to PCT to direct antibiotic therapy – can be used to target corticosteroid therapy.

In chapter 5, I have demonstrated that the peripheral blood eosinophil count is an accurate biomarker to predict a sputum eosinophilia during COPD exacerbations. I thus hypothesise that the peripheral blood eosinophil count can be used to direct systemic corticosteroid treatment during an exacerbation of COPD and can result in reduced total exposure to systemic corticosteroids without adversely affecting treatment outcomes. To test this hypothesis I undertook a randomised biomarker-directed prednisolone placebo controlled trial in patients presenting with an exacerbation of COPD.

Methods

In order to test the hypothesis, the blood eosinophil cut-off of 2% derived from chapter 5, was used as the biomarker to direct corticosteroid therapy in a prospective randomised placebo controlled interventional study. This biomarker was associated with the highest area under the ROC curve for correctly predicting a sputum eosinophilia (>3% non-squamous cells) at the onset of COPD exacerbation. The 2% cut-off was chosen as this had the highest sensitivity without a deficit in specificity. This ensures that true positives are treated with prednisolone.

Participants and study design

In this study, COPD patients were recruited either *de-novo* or following completion of the observational biomarker study presented in chapter 5 to enter the randomised biomarker-directed double-blind corticosteroid therapy versus standard care study. The peripheral blood eosinophil count at the exacerbation event was used to guide corticosteroid treatment in the biomarker-directed arm. There were 95 patients that were entered from the biomarker observational study (chapter 5 patient cohort) and a further 70 patients that were recruited *de-novo*. Patients with a physician diagnosis of COPD, defined according to GOLD were recruited consecutively from the general respiratory clinics at the Glenfield hospital, Leicester and from local advertising. The inclusion and exclusion criteria for entry into the study were similar to those presented in chapter 5. At exacerbation patients were randomised by minimisation for baseline lung function, exacerbation frequency and sputum eosinophil count and followed up at 2 (post therapy) and 6 (recovery) weeks after exacerbation. Figure 8.1 illustrates the study design.

Figure 8.1 Study design for biomarker-directed randomised placebo control trial

At Exacerbation

Randomisation by minimisation

FEV_1% predicted

Exacerbation frequency

Sputum eosinophil count

Biomarker-directed therapy Arm

Peripheral blood eosinophil count measured **And** treatment directed	
> 2%	≤ 2%

Standard therapy

Peripheral blood eosinophil count measured **But** treatment **not** directed	
> 2%	≤ 2%

Prednisolone + Antibiotic   Placebo + Antibiotic   Prednisolone + Antibiotic

Seen at 2 & 6 weeks for assessment of health status, symptom scores and lung function

Randomisation and minimisation were performed by an independent clinical team. Patients and study personnel involved in data collection and treatment failure assessment were blinded to randomisation, biomarker results and treatment allocation. Patients in the biomarker-directed group received a 30mg of prednisolone capsule once daily or an identical appearing placebo for 14 days when the peripheral blood eosinophil count was > and ≤ 2% respectively. Patients in the standard group received a 30mg prednisolone capsule once daily irrespective of the blood eosinophil biomarker results. All patients received open labelled broad spectrum oral antibiotic therapy (amoxicillin or doxycycline if amoxicillin allergic) for 7 days. Study medications were prepared by a single pharmacy (Royal Free Pharmacy Manufacturing Unit, London, UK) and were identical in taste, appearance and dispensing packaging.

Blood eosinophil counts were measured at exacerbation to define blood eosinophil biomarker-positive and -negative patients in both study groups (peripheral blood eosinophil levels ≤ 2% termed biomarker-negative; peripheral blood eosinophil levels > 2% termed biomarker-positive) but these results were not disclosed. Data sampling and randomisation were only obtained in patients with captured and treatment naïve COPD exacerbations. At all study visits the following measurements were undertaken; pre and post bronchodilator spirometry; health quality assessments using the CRQ (McMaster University, Hamilton, Canada); symptom assessment of cough, breathlessness, sputum production and sputum purulence using the VAS; blood for measurement of cell differential and CRP and finally sputum for analysis of bacteria, CFU, virus and sputum cell differential.

All patients were given daily diary cards to complete throughout the study and asked to contact the research team, if symptoms of cough, breathlessness, sputum volume production or purulence changed or increased over two consecutive days (diary card prompts), or if they felt they needed medical attention. Patients were then medically assessed at the exacerbation visit, where a full clinical history and examination was conducted. If clinically indicated, a CXR, electrocardiogram and blood gas analysis were performed to exclude other causes of increased symptoms and breathlessness. An exacerbation was defined as an increase in breathlessness, sputum purulence or sputum production, compared to the patients' day-to-day symptom variation and one that necessitated a change in medication (Anthonisen, 1987; Rodriguez-Roisin, 2000). After the exacerbation/randomisation visits, patients were then invited to attend a 2 week (post therapy) visit and a 6 week (recovery) visit, for further sampling and data analysis. Patients were asked to complete daily VAS diary cards for 14 days after treatment allocation. All patients gave informed written consent and the study was approved by the local ethics committee and the MHRA.

Validation of biomarkers in bacteria, virus and sputum eosinophil associated exacerbations of COPD

Validation of the biomarkers identified in chapter 5 was performed in all exacerbations captured from de-novo subjects in this interventional study.

Interleukin-1β (IL-1β)

Sputum IL-1β was measured for validation of determined biomarkers using a commercial ELISA assay (R&D systems, UK). In this experiment, 200 μL of standard, control and sputum supernatant was added to each well in duplicate, incubated for 2

hours and then washed. An equivalent volume of IL-1β conjugate was then added and incubated for 1 hour, followed by a further 3 wash steps. 200 μL of substrate solution was added and after 20 minutes 50 μL of stop solution was added. The plate was immediately read at 450nm. The intra- and inter- assay CV was <10% for both respectively.

Interferon gamma induced protein 10 (IP-10/CXCL10)

Serum IP-10/CXCL10 was measured for validation using a commercial ELISA (R&D systems, UK). In brief, 75μL of assay diluents was added to each well, followed by the addition of 75μL prepared control, standard and sample (in duplicate). This was covered and incubated for 2 hours at room temperature. After 4 wash steps, 200 μL of CXCL10 conjugate was added to each well and incubated for a further 2 hours. Another 4 wash steps were performed and 200 μL of substrate solution was added, with an additional incubation period of 30 minutes. Finally 50 μL of stop solution was added and the plate optical density was read at 450nm. The intra- and inter- assay CV was <5% and <9% respectively.

Statistical Analysis

Statistical analysis was performed using PRISM version 4 (GraphPad Software, San Diego, CA) and SPSS version 16 (SPSS, Inc. Chicago, IL). Parametric and non-parametric data is presented as mean (standard error of the mean) and median (interquartile range), unless stated otherwise. Log transformed data is presented as geometric mean (95% confidence interval). The primary objective of the study was to assess whether the blood eosinophil count can be used as a biomarker to direct

corticosteroid therapy at the onset of an exacerbation. The primary outcome was to show i) non-inferiority in the health status score following treatment between the standard therapy and biomarker-directed therapy study groups, ii) equivalence in the proportions of exacerbations associated with a treatment failure defined as the need to start or repeat treatment within 30 days of randomisation, hospitalisation for any cause or death, between the standard therapy and biomarker-directed therapy study groups, and iii) demonstration of a reduction in corticosteroid therapy prescription in the biomarker-directed therapy study group. To demonstrate non-inferiority in health reported outcomes following 14 days treatment, using the minimally clinical important CRQ mean change of 0.5 (SD 0.91, CI 0.3 to 0.6), 53 subjects were required in each arm to have 80% power at the 5% level. This also provided 95% power at the 5% level to show a 50% reduction in exacerbations requiring corticosteroid therapy, using an exacerbation frequency (SD) of 2.8 (1.7) per year. To exclude a change in the proportion of treatment failure of 20%, from 10% to 30%, between treatment arms, 60 exacerbations in each arm would have a power of 90% at the 5% level.

Secondary analysis of health status, symptom scores, lung function and treatment failures was performed in A) blood eosinophil biomarker-positive and biomarker-negative exacerbations, B) blood eosinophil biomarker-negative exacerbations prescribed prednisolone and placebo, and C) blood eosinophil biomarker-positive and -negative exacerbations prescribed prednisolone. Further exploratory analysis was performed to assess corticosteroid responsiveness and sputum eosinophilia during exacerbations of COPD.

Subjects could only be randomised into the study once, but multiple captured exacerbations were treated as independent events. A p-value < 0.05 was taken as the threshold of significance.

Results

One hundred and sixty four patients were recruited to enter the study (107 men, 57 women). 109 patients with 166 exacerbation events were captured during the study period; 55 and 54 patients with 86 and 80 exacerbations events respectively were randomised to the biomarker-directed and standard arm. The patients not randomised were those that had exacerbations but did not present to the research investigators. There were 66, 32, 8 and 3 patients that subsequently had 1, 2, 3, and 4 captured exacerbations. There were no differences in the clinical characteristics between patients that were randomised or not (table 8.1) or between patients in the biomarker-directed and standard therapy arm (table 8.2).

Table 8.1; Clinical characteristics of all patients recruited and patients that were randomised to enter the clinical study

	All patients N=164	Randomised patients N=109
Age*	70 (45 – 87)	69 (47 – 87)
Pack years smoked*	55 (10 – 207)	54 (10 – 207)
Exacerbation frequency	3 (1 - 12)	4 (1 – 12)
FEV_1, L‡	1.23 (0.04)	1.19 (0.05)
FEV_1 % predicted‡	49 (1)	48 (2)
FEV_1/FVC, %	48 (1)	46 (1)
BMI, kg/m^2	26.8 (0.5)	27.4 (0.6)
SGRQ $_{total}$ units	53.7 (1.3)	55.0 (1.6)
CRQ $_{total}$ units	4.0 (0.1)	4.0 (0.1)
VAS $_{total}$ mm	154 (7)	150 (8)

Data presented as mean (SEM) unless stated otherwise. *mean (range), ‡ post bronchodilator. FEV_1 Forced expiratory volume in 1 second; FVC Forced vital capacity; BMI Body mass index, SGRQ St Georges Respiratory Questionnaire, scores ranging from 0 to 100 with higher score indicating worse health status (total score on domains of Impact, Symptoms and Activity), CRQ Chronic Respiratory Disease Questionnaire, scores range between 1 to 7 with higher score representing better health quality; VAS Visual Analogue Scale, performed on 100mm line from 'no symptoms' to 'worst symptoms', higher scores represent worse symptoms (total score addition of measured domains: cough, dyspnoea, sputum production and sputum purulence).

Table 8.2 Clinical characteristics of patients in randomised placebo controlled trial

	Biomarker Arm N=55	Standard Arm N=54	P-value‖
Male, n (%)	30 (55)	39 (72)	0.07
Age*	70 (49 – 87)	68 (47 – 86)	0.27
Current smoker, n (%)	22 (40)	21 (39)	0.91
Ex smoker, n (%)	32 (58)	32 (59)	0.91
Pack year history*	52 (10 - 156)	57 (10 – 207)	0.47
Exacerbation frequency in previous yr*	3 (1 - 10)	4 (1 - 12)	0.12
Body mass index, kg/m^2	27.5 (6.7)	27.3 (5.3)	0.87
Inhaled corticosteroid usage, n (%)	48 (87)	47 (87)	0.97
Inhaled corticosteroid dose, µg[†]	1496 (595)	1489 (613)	0.96
Atopy, n (%)	13 (24)	7 (14)	0.21
Total IgE, kU/L[¥]	59 (166)	76 (141)	0.66
GOLD I, n, (%)	3 (5.5)	3 (5.6)	0.98
GOLD II, n (%)	23 (41.8)	16 (29.6)	0.18
GOLD III, n (%)	15 (27.3)	15 (27.8)	0.97
GOLD IV, n (%)	14 (25.5)	20 (37.0)	0.38
FEV_1, L[‡]	1.21 (0.53)	1.18 (0.47)	0.75
FEV_1, %[‡]	49 (19)	46 (18)	0.29
FEV_1/FVC Ratio, %[‡]	47 (12)	45 (12)	0.35
Reversibility, mL	27 (14)	26 (15)	0.96
Reversibility, %	3.7 (1.2)	3.8 (1.7)	0.95
Sputum total cell count, $x10^6/g$[#]	2.8 (1.7 to 4.4)	2.8 (1.9 to 4.2)	0.93
Sputum neutrophils, %	72 (26)	76 (21)	0.37
Sputum eosinophils, %[#]	0.9 (0.6 to 1.2)	0.8 (0.6 to 1.2)	0.88
CRQ total, units	3.86 (1.12)	4.14 (1.19)	0.21
VAS total, mm	149 (76)	150 (84)	0.96
Sputum eosinophil associated exacerbation, %	15	19	0.58
Virus associated exacerbation, %	32	31	0.95
Bacteria associated exacerbation, %	44	41	0.22

Data presented as mean (standard deviation), unless stated. *mean (range), [¥]median (interquartile range), †beclomethasone dipropronate equivalent, ‡ post bronchodilator, # geometric mean (95% CI); ‖ T-test or Mann Whitney for continuous variables or χ^2 for proportions. FEV_1 Forced expiratory volume in 1 second; FVC Forced vital capacity; CRQ Chronic Respiratory Disease Questionnaire, scores range between 1 to 7 with higher score representing better health quality; VAS Visual Analogue Scale, performed on 100mm line from 'no symptoms' to 'worst symptoms', higher scores represent worse symptoms (total score addition of measured domains: cough, dyspnoea, sputum production and sputum purulence)

There were ten severe exacerbations requiring hospitalisation. A sputum eosinophil, virus and bacteria culture positive -associated exacerbation was identified in 17%, 32% and 42% of all exacerbations respectively. There was no difference in the proportions of sputum eosinophil-associated, virus-associated and bacteria culture positive-associated exacerbations in the biomarker-directed and standard therapy arm at randomisation.

The consort diagram for all patients entered and randomised into the study is presented in figure 8.2. There were 24/55 patients in the biomarker-directed and 19/54 patients in the standard therapy arm with repeatedly captured exacerbation events. Only 10 patients (6 in biomarker-directed and 4 in standard therapy arm) had repeated exacerbation events that were associated with a change in the exacerbation biomarker status.

Figure 8.2; Clinical study consort diagram
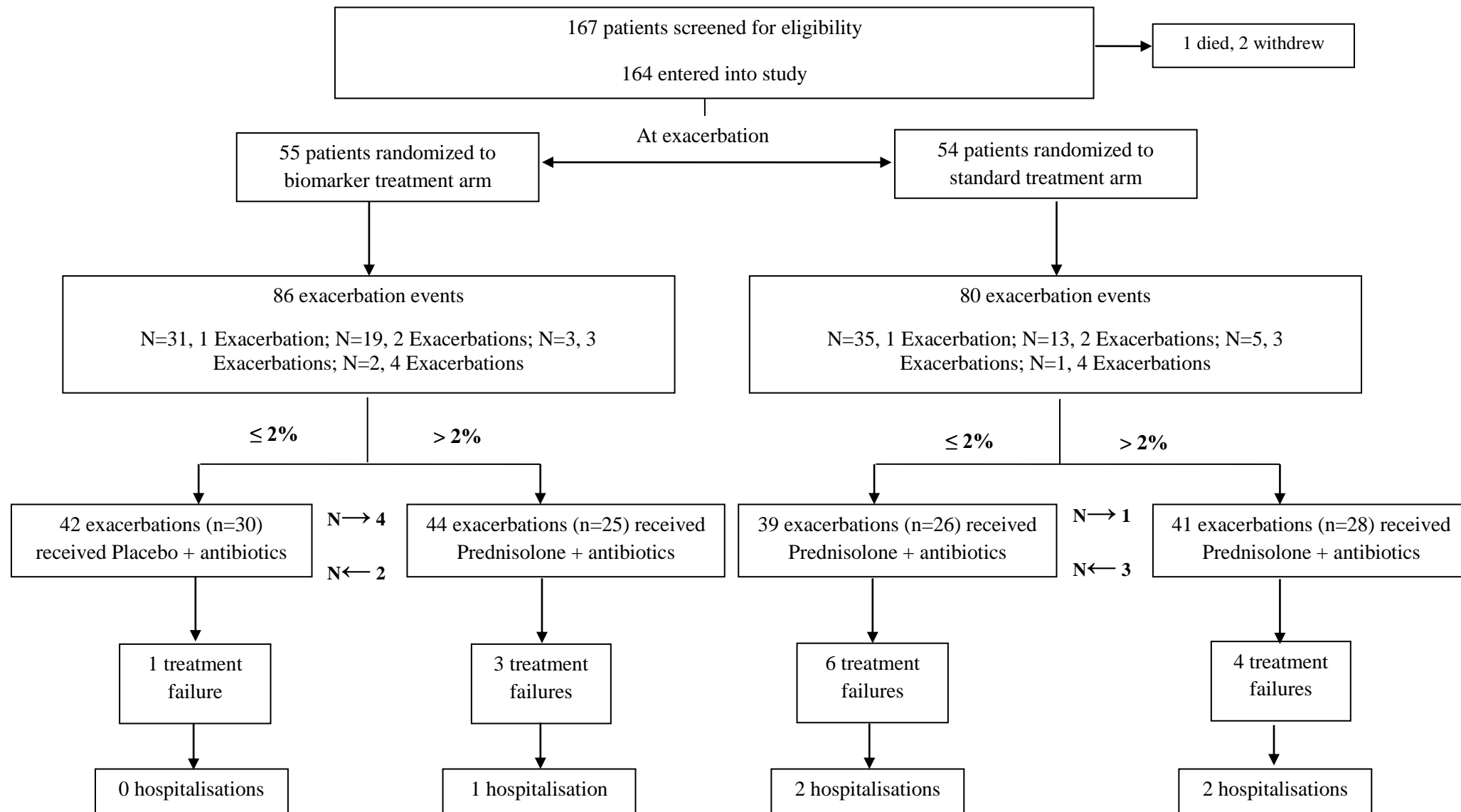
The primary outcome of non-inferiority of health status in the standard therapy and biomarker-directed groups following 2 weeks treatment was achieved (CRQ mean score change 0.8 vs. 1.1, mean difference 0.3, 95% CI 0.0 to 0.6, p=0.05, figure 8.3). There was a similar reduction in the CRQ score from baseline to exacerbation in the biomarker-directed and standard therapy arms (0.9 vs. 0.9, mean difference 0.0, 95% CI -0.3 to 0.3, p=0.97).

Figure 8.3; Disease specific health status (CRQ) in the biomarker (■) and standard (●) arm at study entry, exacerbation, 2 weeks after treatment and 6 weeks after treatment. No differences were detected after 2 weeks of treatment between biomarker-directed and standard therapy arms. Data presented as mean (standard error of the mean)
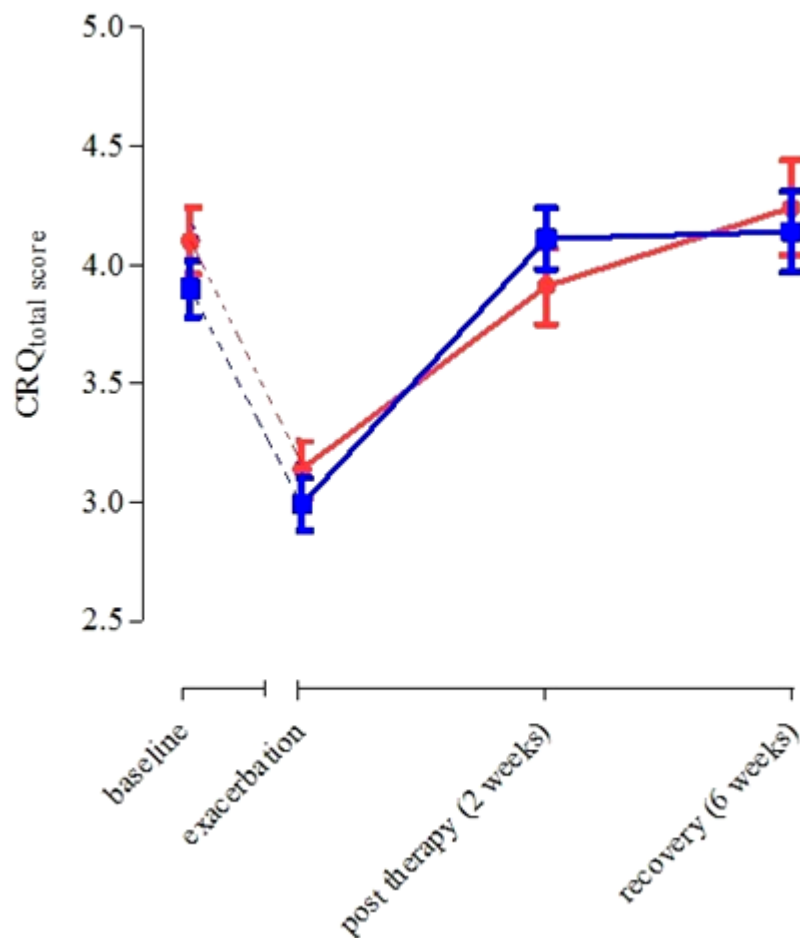
There were no differences in FEV_1 between biomarker-directed and standard therapy arm following treatment allocation (figure 8.4).

Figure 8.4; Lung function in the biomarker (■) and standard (●) arm at study entry, exacerbation, 2 weeks after treatment and 6 weeks after treatment. No differences were detected after 2 weeks of treatment between biomarker-directed and standard therapy arms. Data presented as mean (standard error of the mean)
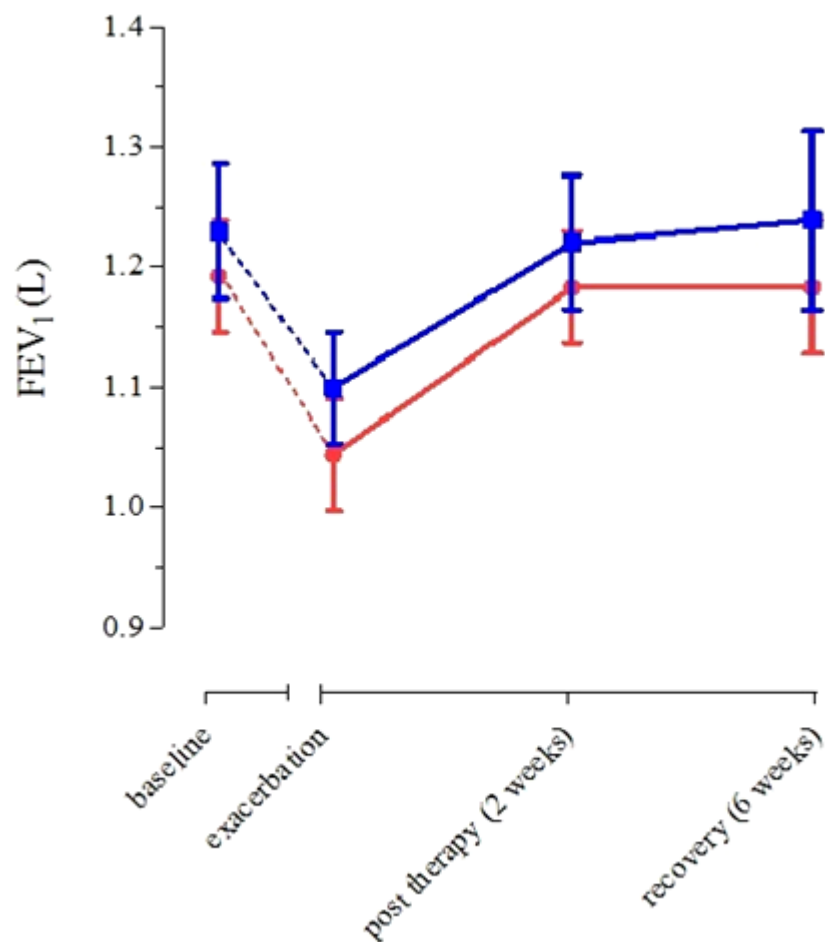
There was no difference in symptom improvement between the biomarker-directed arm and the standard arm, as measures by the % VAS improvement following treatment (figure 8.5).

Figure 8.5; The percentage improvement in visual analogue scale symptom total score (VAS) from exacerbation and for duration of treatment period in exacerbations in standard arm (●) and biomarker arm (■). No differences were detected in daily percentage improvement of symptoms (breathlessness, cough, sputum purulence and sputum production). Data points presented as mean (standard error of the mean)
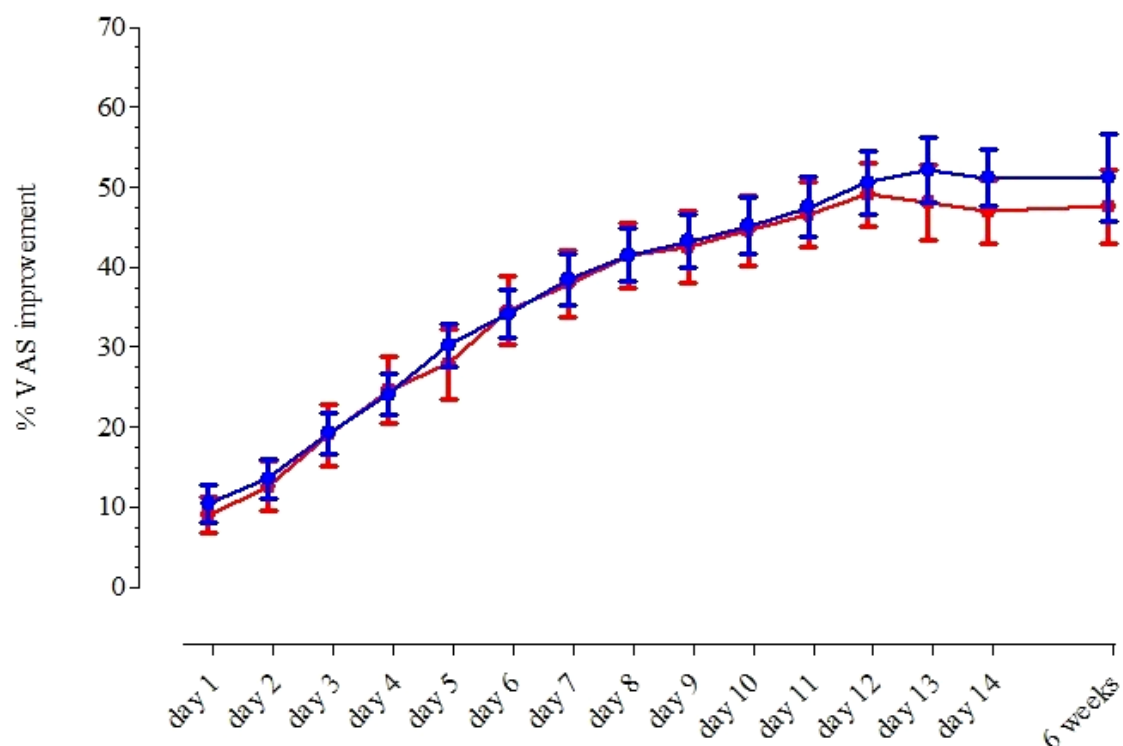
Sputum eosinophil differential cell count in the biomarker arm was higher in patients prescribed prednisolone compared to those prescribed placebo (7.8% vs. 0.7%; mean difference (95% confidence interval) 7.1 (2.1 to 12.1); $p<0.001$). Only 1 patient in the biomarker directed arm with a sputum eosinophil count of > 3% received placebo therapy (figure 8.6).

Figure 8.6; Sputum eosinophil differential cell counts in the biomarker-directed patients given prednisolone or placebo. Data presented as geometric mean (95% CI) and horizontal dashed line set at 3% sputum eosinophil count.
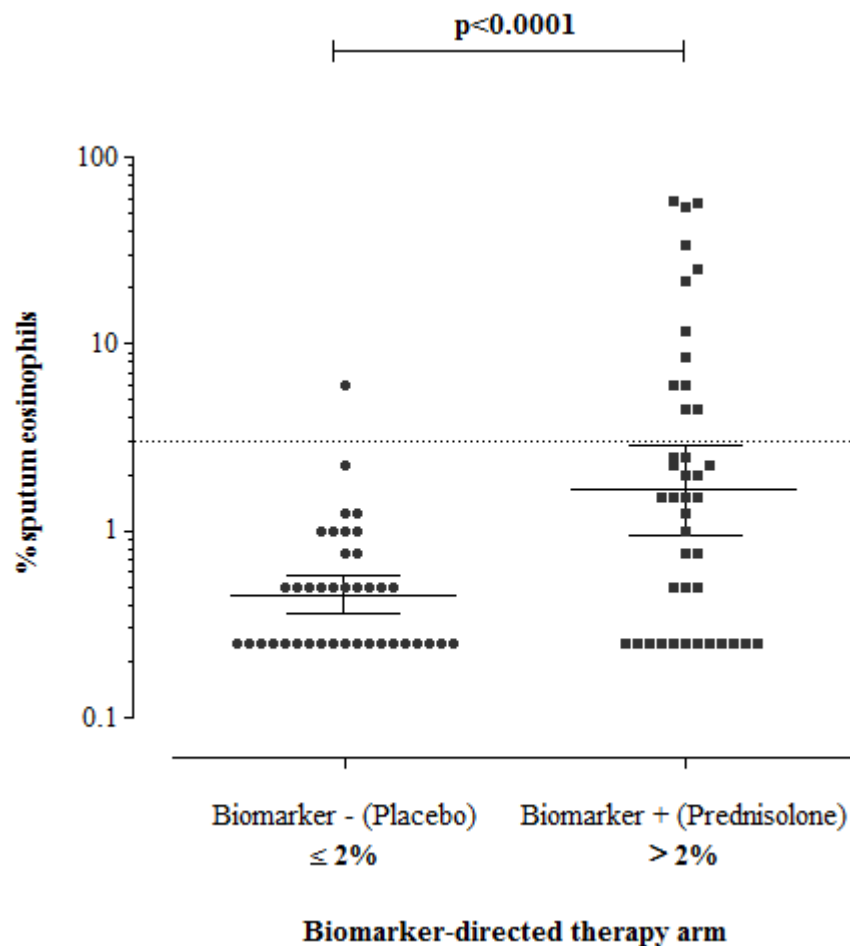
There were 14 treatment failures associated with worsening symptoms of COPD following treatment during the study; 10 occurred in the standard arm and 4 in the biomarker-directed arm. This demonstrated equivalence in treatment failure between the biomarker-directed and standard therapy arms. However there was a trend favouring the biomarker-directed arm with fewer treatment failures in this group (13% vs. 5%, 95% CI -1 to 16; p=0.07). In the biomarker-directed group 49% of the exacerbations were not treated with prednisolone. There were similar proportions of patients in the standard

therapy group and the biomarker-directed therapy group that had 1 exacerbation (35 vs. 31), 2 exacerbations (13 vs. 19), 3 exacerbations (5 vs. 3) and 4 exacerbations (1 vs. 2).

Blood eosinophil biomarker positive and negative exacerbations

There were 85 exacerbations that were blood eosinophil biomarker-positive given prednisolone, 39 exacerbations that were blood eosinophil biomarker-negative given prednisolone and 42 exacerbations that were blood eosinophil biomarker-negative given placebo. Changes in clinical characteristics for biomarker-positive and -negative exacerbations in the biomarker-directed and standard treatment arms at stable, exacerbation, post therapy and recovery visits are presented in table 8.3. Baseline and exacerbation health status, lung function and airway inflammation characteristics in blood eosinophil biomarker-positive and biomarker-negative exacerbations are presented in table 8.4.

The mean reduction in CRQ from baseline to exacerbation was similar between biomarker-positive and -negative exacerbations (1.0 vs. 0.9; mean difference 0.1; 95% CI -0.2 to 0.3; p=0.54). At exacerbation, blood eosinophil biomarker-negative exacerbations had higher sputum neutrophil counts, sputum total cell counts, serum CRP and FEV_1 % predicted compared to blood eosinophil biomarker-positive exacerbations (mean (SEM) sputum neutrophil count 86 (2) vs. 78% (3), p=0.03; geometric mean (95% CI) sputum total cell counts 9.2 (6.5 to 13.0) vs. 5.4 (3.9 to 7.5), p=0.03; median (IQR) CRP 20 (49) vs. 9 (22), p<0.01; mean (SEM) FEV_1 % predicted 46 (2) vs. 39 (2), p=0.03).

Table 8.3 Lung function and inflammation at baseline, exacerbation, 2 weeks post exacerbation (post therapy) and 6 weeks post exacerbation (recovery), categorised into exacerbations that were randomised to the standard arm or biomarker arm and were found to be biomarker negative and biomarker positive at exacerbation onset.

	Standard Arm Biomarker-Negative (n=26, n_E=39)				Standard Arm Biomarker-Positive (n=28, n_E=41)				Biomarker Arm Biomarker-Negative (n=30, n_E=42)				Biomarker Arm Biomarker-Positive (n=25, n_E=44)			
	Baseline	Exacerbation	2 weeks	6 weeks	Baseline	Exacerbation	2 weeks	6 weeks	Baseline	Exacerbation	2 weeks	6 weeks	Baseline	Exacerbation	2 weeks	6 weeks
FEV_1 (L)†	1.24 (0.49)	1.15 (0.48)	1.22 (0.45)	1.22 (0.43)	1.16 (0.42)	0.94 (0.41)	1.12 (0.43)	1.22 (0.39)	1.26 (0.61)	1.10 (0.58)	1.23 (0.58)	1.20 (0.54)	1.17 (0.45)	1.05 (0.40)	1.22 (0.46)	1.28 (0.42)
FEV_1, % predicted†	48 (20)	44 (19)	48 (19)	46 (17)	43 (17)	35 (16)	42 (16)	40 (14)	53 (20)	47 (19)	53 (19)	50 (19)	48 (20)	44 (19)	49 (20)	54 (19)
Sputum total cell count ($x10^6$/g)‡	2.4 (1.7 to 3.4)	10.6 (7.0 to 16.1)	3.8 (2.3 to 6.3)	2.0 (1.1 to 3.5)	3.2 (2.0 to 5.2)	6.3 (3.9 to 10.1)	2.8 (1.7 to 4.5)	2.3 (1.5 to 3.4)	3.5 (2.2 to 5.6)	8.1 (4.8 to 13.9)	2.3 (1.4 to 3.9)	1.7 (0.9 to 3.1)	2.5 (1.3 to 4.6)	4.7 (3.0 to 7.5)	2.1 (1.1 to 3.9)	2.7 (1.4 to 5.2)
Sputum neutrophils, %	73 (18)	82 (21)	80 (21)	77 (18)	77 (23)	83 (20)	73 (24)	69 (20)	72 (25)	88 (17)	78 (18)	77 (19)	75 (26)	74 (24)	75 (18)	74 (23)
Sputum eosinophils, %‡	0.6 (0.5 to 0.9)	0.5 (0.4 to 0.6)	0.4 (0.3 to 0.6)	0.5 (0.3 to 0.7)	1.1 (0.7 to 1.8)	1.5 (0.9 to 2.5)	0.6 (0.4 to 0.8)	2.1 (1.1 to 4.2)	0.7 (0.5 to 1.1)	0.4 (0.4 to 0.6)	0.7 (0.5 to 1.0)	0.8 (0.4 to 1.4)	0.9 (0.7 to 1.3)	1.7 (1.0 to 2.9)	0.8 (0.5 to 1.3)	0.9 (0.4 to 2.1)
Blood total cell count, $x10^9$ cells/L‡	9.0 (8.1 to 10.1)	10.8 (9.8 to 11.9)	11.9 (10.3 to 13.7)	8.4 (7.4 to 9.7)	9.4 (8.7 to 10.2)	9.1 (8.4 to 10.0)	12.0 (10.9 to 13.1)	9.1 (8.0 to 10.4)	7.8 (7.3 to 8.4)	9.7 (8.7 to 10.8)	8.2 (7.6 to 8.8)	7.7 (7.0 to 8.4)	8.8 (8.3 to 9.4)	8.5 (8.0 to 9.1)	11.2 (10.3 to 12.3)	8.8 (7.6 to 10.3)
Blood neutrophil count, $x10^9$ cells/L‡	5.7 (4.9 to 6.6)	7.7 (6.8 to 8.8)	8.2 (7.0 to 9.7)	5.2 (4.4 to 6.1)	6.0 (5.3 to 6.7)	5.8 (5.1 to 6.5)	8.3 (7.2 to 9.4)	5.6 (4.7 to 6.8)	5.0 (4.6 to 5.5)	6.9 (6.0 to 7.9)	5.4 (4.9 to 6.0)	5.0 (4.5 to 5.6)	5.5 (5.1 to 6.0)	5.5 (5.0 to 5.9)	8.0 (7.1 to 9.0)	5.7 (4.7 to 6.9)
Blood eosinophil count, $x10^9$ cells/L‡	0.15 (0.12 to 0.18)	0.10 (0.09 to 0.12)	0.11 (0.09 to 0.14)	0.12 (0.09 to 0.15)	0.32 (0.26 to 0.38)	0.38 (0.33 to 0.44)	0.19 (0.15 to 0.25)	0.31 (0.22 to 0.44)	0.15 (0.12 to 0.18)	0.12 (0.10 to 0.14)	0.14 (0.11 to 0.17)	0.17 (0.13 to 0.22)	0.28 (0.23 to 3.3)	0.31 (0.27 to 0.36)	0.18 (0.14 to 0.25)	0.20 (0.13 to 0.32)
Blood eosinophil %	2.0 (1.4)	1.1 (0.5)	1.1 (0.8)	1.7 (1.5)	4.0 (2.4)	4.8 (2.8)	2.4 (2.1)	4.8 (5)	2.3 (1.5)	1.3 (0.5)	2.0 (1.1)	2.5 (1.5)	3.7 (2.6)	4.1 (2.6)	2.3 (1.7)	2.9 (1.7)
C reactive protein, mg/L¶	5 (9)	18 (42)	10 (21)	6 (11)	7 (15)	10 (23)	3 (5)	3 (5)	3 (3)	24 (67)	3 (8)	3 (6)	3 (8)	9 (22)	3 (16)	6 (7)

FEV_1 Forced expiratory volume in 1 second; †post bronchodilator. Data presented as mean (standard deviation) unless stated. ‡Geometric mean (95% confidence interval); ¶ median (interquartile range)

Table 8.4 Lung function and inflammation at baseline and exacerbation in all exacerbations captured categorised as blood eosinophil biomarker-positive and biomarker-negative. Statistical analysis performed using a Paired T-test analysis or Wilcoxon Signed Ranks test. Differences between exacerbation and baseline presented as mean difference (95% confidence interval of difference); fold difference (95% confidence interval of fold difference); and median (interquartile range) of differences as appropriate. n= number of patients; n_E= number of exacerbation events

	Biomarker-negative n=56, n_E=81				Biomarker-positive n=53, n_E=85			
	Baseline	Exacerbation	mean difference (95%CI) ¶	p-value	Baseline	Exacerbation	mean difference (95%CI) ¶	p-value
FEV$_1$, L†	1.26 (0.56)	1.13 (0.53)	-0.13 (-0.19 to -0.07)	<0.01	1.16 (0.42)	0.99 (0.41)	-0.17 (-0.22 to -0.12)	<0.01
FEV$_1$, % predicted†	51 (20)	46 (19)	-5 (-7 to -3)	<0.01	46 (18)	39 (18)	-7 (-9 to -5)	<0.01
CRQ score, units	4.00 (1.13)	3.11 (1.05)	-0.88 (-1.06 to -0.70)	<0.01	3.99 (1.20)	3.03 (0.99)	-1.0 (-1.2 to -0.8)	<0.01
Sputum total cell count, x10^6/g‡	3.0 (2.2 to 4.0)	8.8 (6.1 to 12.6)	3.0 (2.0 to 4.3)	<0.01	2.9 (1.9 to 4.4)	5.6 (3.9 to 7.9)	2.0 (1.2 to 3.1)	<0.01
Sputum neutrophil counts, %	72 (22)	85 (20)	12 (6 to19)	<0.01	80 (20)	80 (22)	0.5 (-7 to 8)	0.90
Sputum eosinophil counts, %‡	0.7 (0.5 to 0.9)	0.5 (0.4 to 0.5)	0.7 (0.5 to 0.9)	<0.01	1.1 (0.8 to 1.6)	1.7 (1.1 to 2.6)	1.5 (0.9 to 2.3)	0.09
Blood total cell count, x10^9 cells/L‡	8.4 (7.8 to 8.9)	10.3 (9.5 to 11.1)	1.2 (1.2 to 1.5)	<0.01	9.1 (8.6 to 9.6)	8.8 (8.3 to 9.3)	1.0 (0.9 to 1.0)	0.19
Blood neutrophil count, x10^9 cells/L‡	5.3 (4.9 to 5.8)	7.3 (6.6 to 8.1)	1.4 (1.3 to 1.5)	<0.01	5.7 (5.3 to 6.2)	5.6 (5.2 to 6.0)	1.0 (0.9 to 1.1)	0.50
Blood eosinophil count, x10^9 cells/L‡	0.15 (0.13 to 0.17)	0.11 (0.10 to 0.13)	0.8 (0.7 to 0.9)	<0.01	0.30 (0.26 to 0.34)	0.34 (0.31 to 0.38)	1.2 (1.1 to 1.3)	<0.01
Blood eosinophil %	2.1 (1.4)	1.2 (0.5)	-0.9 (-1.1 to -0.7)	<0.01	3.9 (2.5)	4.4 (2.6)	0.6 (0.0 to 1.1)	0.05
CRP, mg/L	3 (5)	20 (49)	12 (29)	<0.01	5 (10)	9 (22)	0 (13)	0.04

Data presented as mean (standard deviation) unless stated. ‡Geometric mean (95% confidence interval); ¶mean, median or fold difference as appropriate. FEV$_1$ Forced expiratory volume in 1 second; CRQ Chronic respiratory disease questionnaire, scores range between 1 to 7 with higher score representing better health quality; CRP C reactive protein; †post bronchodilator.

There was a significant difference in absolute and percentage blood eosinophil counts at baseline, exacerbation, post therapy and recovery between biomarker-positive and -negative exacerbations (for each visit between groups, p<0.01, table 8.5). There were similar proportions of bacteria-associated biomarker-positive and biomarker-negative exacerbations (38% vs. 46%, p=0.31) and virus-associated biomarker-positive and -negative exacerbations (26% vs. 37%, p=0.16). The CFU count at exacerbation was significantly higher in biomarker negative exacerbations compared to biomarker positive exacerbations (CFU geometric mean (95%CI) 1.1^7 (6.2^6 to 1.9^7) vs. 2.9^6 (1.6^6 to 5.3^6), p=0.002). A sputum-eosinophil associated exacerbation was found in more biomarker-positive than biomarker-negative exacerbations (31% vs. 2%, p<0.001). For all exacerbation events captured, the 2% blood eosinophil count cut off had a positive predictive value of 91% for identifying a sputum eosinophilia of ≥3%.

Table 8.5 Lung function and inflammation (absolute data) at baseline, exacerbation, 2 weeks post exacerbation and 6 weeks post exacerbation, for all exacerbations categorised into biomarker-positive given prednisolone, biomarker-negative given prednisolone and biomarker-negative given placebo

	Biomarker-positive given prednisolone				Biomarker-negative given prednisolone				Biomarker-negative given placebo			
	Baseline	**Exacerbation**	**2 weeks**	**6 weeks**	**Baseline**	**Exacerbation**	**2 weeks**	**6 weeks**	**Baseline**	**Exacerbation**	**2 weeks**	**6 weeks**
	n=53	n_E=85	n_E =85	n_E =41	n=26	n_E =39	n_E =39	n_E =23	n=30	n_E =42	n_E =42	n_E =24
FEV_1 (L)†	1.16 (0.42)	0.99 (0.41)	1.17 (0.45)	1.19 (0.41)	1.24 (0.49)	1.15 (0.48)	1.22 (0.45)	1.22 (0.43)	1.26 (0.61)	1.10 (0.58)	1.23 (0.58)	1.20 (0.54)
FEV_1, % predicted†	46 (18)	39 (18)	46 (19)	46 (8)	48 (20)	44 (19)	48 (19)	46 (17)	53 (20)	47 (19)	53 (19)	50 (19)
Sputum total cell count ($x10^6$/g)‡	2.8 (1.9 to 4.2)	5.4 (3.9 to 7.5)	2.4 (1.6 to 3.6)	2.4 (1.7 to 3.4)	2.4 (1.7 to 3.4)	10.6 (7.0 to 16.1)	3.8 (2.3 to 6.3)	2.0 (1.1 to 3.5)	3.5 (2.2 to 4.4)	8.1 (4.5 to 10.7)	2.3 (1.4 to 2.7)	1.7 (0.9 to 2.0)
Sputum neutrophils, %	76 (24)	78 (23)	74 (21)	71 (21)	73 (18)	82 (21)	80 (21)	77 (18)	72 (25)	88 (17)	78 (18)	77 (19)
Sputum eosinophils, %‡	1.0 (0.8 to 1.4)	1.6 (1.1 to 2.3)	0.7 (0.5 to 0.9)	1.5 (0.9 to 2.6)	0.6 (0.5 to 0.9)	0.5 (0.4 o 0.6)	0.4 (0.3 to 0.6)	0.5 (0.3 to 0.7)	0.7 (0.5 to 0.9)	0.5 (0.4 to 0.5)	0.7 (0.5 to 0.8)	0.8 (0.4 to 0.9)
Blood total cell count, $x10^9$ cells/L‡	9.1 (8.6 to 9.6)	8.8 (8.3 to 9.3)	11.6 (10.9 to 12.4)	9.0 (8.1 to 9.9)	9.1 (8.1 to 10.1)	10.8 (9.8 to 12.0)	11.9 (10.4 to 13.7)	8.4 (7.4 to 9.7)	7.8 (7.3 to 8.1)	9.7 (8.7 to 10.2)	8.2 (7.6 to 8.4)	7.7 (7.0 to 7.9)
Blood neutrophil count, $x10^9$ cells/L‡	5.7 (5.3 to 6.2)	5.6 (5.2 to 6.0)	8.1 (7.4 to 8.9)	5.7 (5.0 to 6.5)	5.7 (5.0 to 6.6)	7.7 (6.8 to 8.8)	8.2 (7.0 to 9.7)	5.2 (4.4 to 6.1)	5.1 (4.6 to 5.3)	6.9 (6.0 to 7.4)	5.4 (4.9 to 5.6)	5.0 (4.5 to 5.2)
Blood eosinophil count, $x10^9$ cells/L‡	0.30 (0.26 to 0.34)	0.34 (0.31 to 0.38)	0.19 (0.15 to 0.23)	0.26 (0.19 to 0.34)	0.15 (0.12 to 0.18)	0.10 (0.09 to 0.12)	0.11 (0.09 to 0.14)	0.12 (0.09 to 0.15)	0.15 (0.12 to 0.17)	0.12 (0.10 to 0.13)	0.14 (0.11 to 0.15)	0.17 (0.13 to 0.18)
Blood eosinophil %	3.9 (2.5)	4.5 (2.7)	2.3 (1.9)	3.9 (3.9)	2.0 (1.4)	1.1 (0.5)	1.1 (0.8)	1.7 (1.5)	2.2 (1.5)	1.3 (0.5)	2.0 (1.1)	2.5 (1.5)
C reactive protein, mg/L¶	5 (10)	9 (22)	3 (9)	3 (6)	5 (8)	18 (42)	10 (20)	6 (10)	3 (2)	24 (67)	3 (7)	3 (5)

Data presented as mean (standard deviation) unless stated. ‡Geometric mean (95% confidence interval); ¶ median (interquartile range). n= number of patients; n_E= number of exacerbation events; FEV_1 Forced expiratory volume in 1 second; †post bronchodilator.

Blood eosinophil biomarker-negative exacerbations prescribed prednisolone and placebo

Biomarker-negative exacerbations given placebo compared to those given prednisolone had significant improvements in CRQ score after 14 days treatment (mean change 1.01 vs. 0.56; mean difference 0.45; 95% CI 0.01 to 0.90; p=0.045, figure 8.7).

Figure 8.7; Chronic Respiratory Disease Questionnaire total score (CRQ) at baseline, exacerbation, following 14 days treatment (2 weeks post exacerbation) and recovery (6 weeks post exacerbation) in exacerbations that were biomarker positive treated with prednisolone (●); biomarker negative treated with prednisolone (■); and biomarker negative treated with placebo (▼). Data points presented as mean (standard error of the mean)
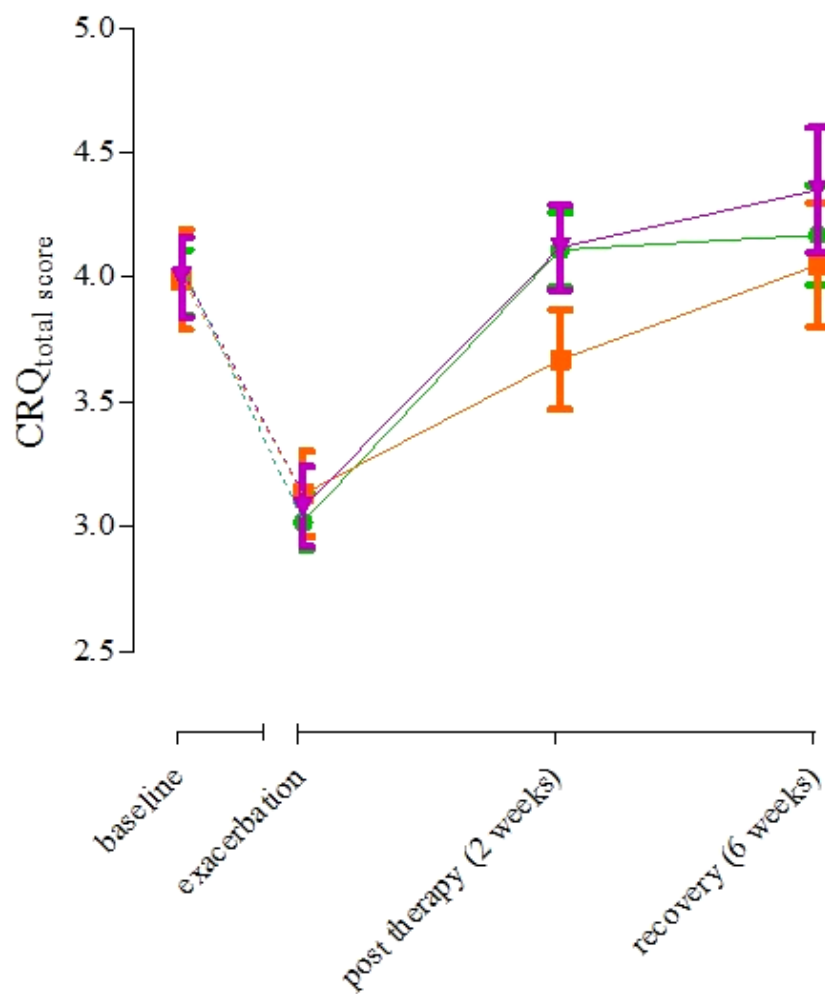
There was significantly more treatment failures in patients with biomarker-negative exacerbations given prednisolone than placebo (15% vs. 2% (95% CI 1 to 25), p=0.04). There was no difference in FEV_1 improvements after two weeks treatment for any of these groups (figure 8.8).

Figure 8.8; Forced expiratory volume in 1 second at baseline, exacerbation, following 14 days treatment (2 weeks post exacerbation) and recovery (6 weeks post exacerbation) in exacerbations that were biomarker positive treated with prednisolone (●); biomarker negative treated with prednisolone (■); and biomarker negative treated with placebo (▼). Data points presented as mean (standard error of the mean)
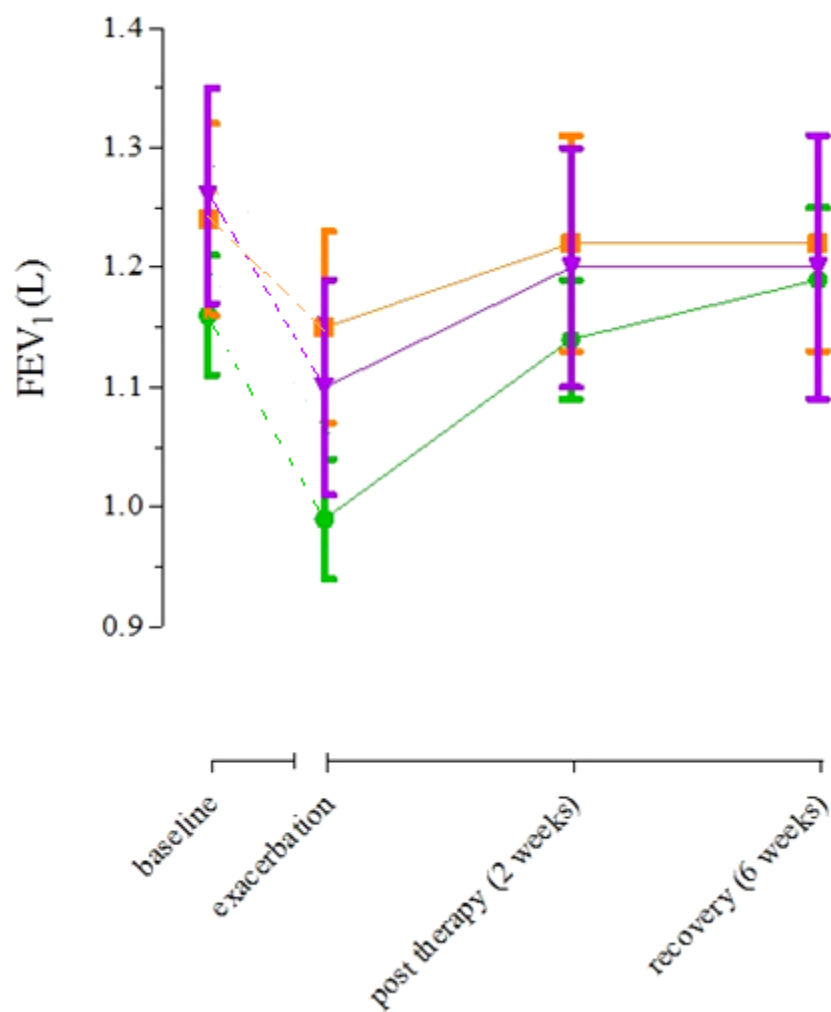
The proportion of exacerbations with no improvement in symptoms after 7 days treatment was higher in biomarker-negative treated with prednisolone compared to biomarker-negative treated with placebo (21% vs. 4% (95% CI 0 to 31), p=0.03). In biomarker-negative exacerbations treated with prednisolone or placebo, there were no differences in the proportions of those associated with bacteria (44% vs. 49%, p=0.70) or virus (36% vs. 38%, p=0.87).

Blood eosinophil biomarker-positive and -negative exacerbations prescribed prednisolone

There was a statistical and clinically significant difference in the CRQ improvement following prednisolone therapy in blood eosinophil biomarker-positive compared to biomarker-negative exacerbations (mean improvement in CRQ (units) 1.11 vs. 0.56; mean difference 0.56; 95% CI 0.15 to 0.96; p<0.01). There was no difference in treatment failure rates between the biomarker-positive and -negative exacerbations treated with prednisolone (8% vs. 15%, 95% CI -10 to 43; p=0.23). There was a greater recovery over 14 days in biomarker-positive exacerbations treated with prednisolone compared to biomarker-negative exacerbations treated with prednisolone (area under the % change in VAS curve (95% CI) 516 (449 to 583) vs. 350 (241 to 458) p<0.01), as shown in figure 8.9.

Figure 8.9; Daily percentage visual analogue scale symptom (VAS) improvement (for the domains of cough, breathlessness, sputum purulence and sputum production) over time in exacerbations that were biomarker positive treated with prednisolone (●); biomarker negative treated with prednisolone (■); and biomarker negative treated with placebo (▼). Data points presented as mean (standard error of the mean).
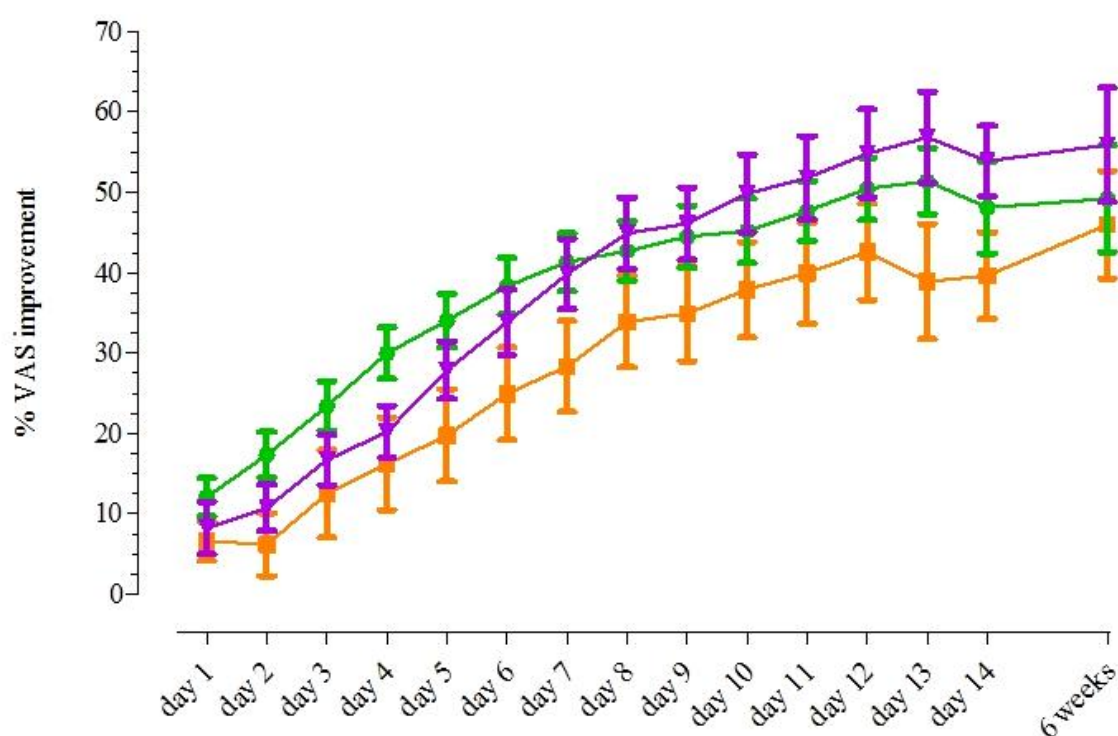
Blood biomarker phenotype stability

The blood eosinophil biomarker status at baseline had an OR (95%CI) of 5.5 (2.7 to 11.0) of predicting the blood eosinophil biomarker status at exacerbation. Specifically blood eosinophil biomarker negative status at baseline had an OR of 2.9 (1.6 to 5.0) for a blood eosinophil biomarker negative exacerbation and blood eosinophil biomarker-positive at baseline had an OR 2.2 (1.5 to 3.2, $p<0.01$) for a blood eosinophil biomarker-positive exacerbation. A blood eosinophil biomarker negative status at baseline was identified in 59% of all patients randomised. In the biomarker-directed

group, 80% of patients that were initially assigned prednisolone therapy would have been assigned prednisolone from the baseline blood eosinophil count. Similarly, 59% of patients assigned to placebo at exacerbation would have been assigned this treatment from the baseline blood eosinophil count. In patients with repeated exacerbation events, comparison of the first and second exacerbation event demonstrated that 22% switched biomarker status (from blood eosinophil biomarker negative to biomarker-positive or *vice versa*), whilst the remainder stayed in the same blood eosinophil biomarker group.

Airway inflammation and steroid responsiveness during exacerbations of COPD

Eosinophilic airway inflammation was present in 31 exacerbations and non eosinophilic airway inflammation was present in 135 exacerbations. All treatment failures had non eosinophilic airway inflammation at exacerbation. There was an improvement in the CRQ total score in exacerbations with eosinophilic airway inflammation compared to exacerbations with non eosinophilic airway inflammation after 14 days treatment of prednisolone (mean difference (95% confidence interval) 1.1 units (0.6 to 1.6) $p<0.0001$).

In exacerbations with non eosinophilic airway inflammation, there was a non statistical trend to improvement in the total CRQ score after 14 days of placebo treatment compared to prednisolone and a clinical improvement at the 6 week recovery visit (total CRQ score mean difference (95% confidence interval) at 14 days 0.4 units (0 to 0.9), p=0.07; 6 week recovery 0.6 units (0.1 to 1.1), p=0.08. figure 8.10).

Figure 8.10; CRQ score across stable and exacerbation visits in exacerbations that had airway eosinophilic inflammation treated with prednisolone (●); airway non-eosinophilic inflammation treated with prednisolone (■); and airway non eosinophilic inflammation treated with placebo (▼). Data points presented as mean (standard error of the mean). CRQ was significantly reduced from baseline to exacerbation and was significantly improved from exacerbation following two weeks treatment for all groups. The greatest reduction and improvement in CRQ occurred in subjects with sputum eosinophilia.
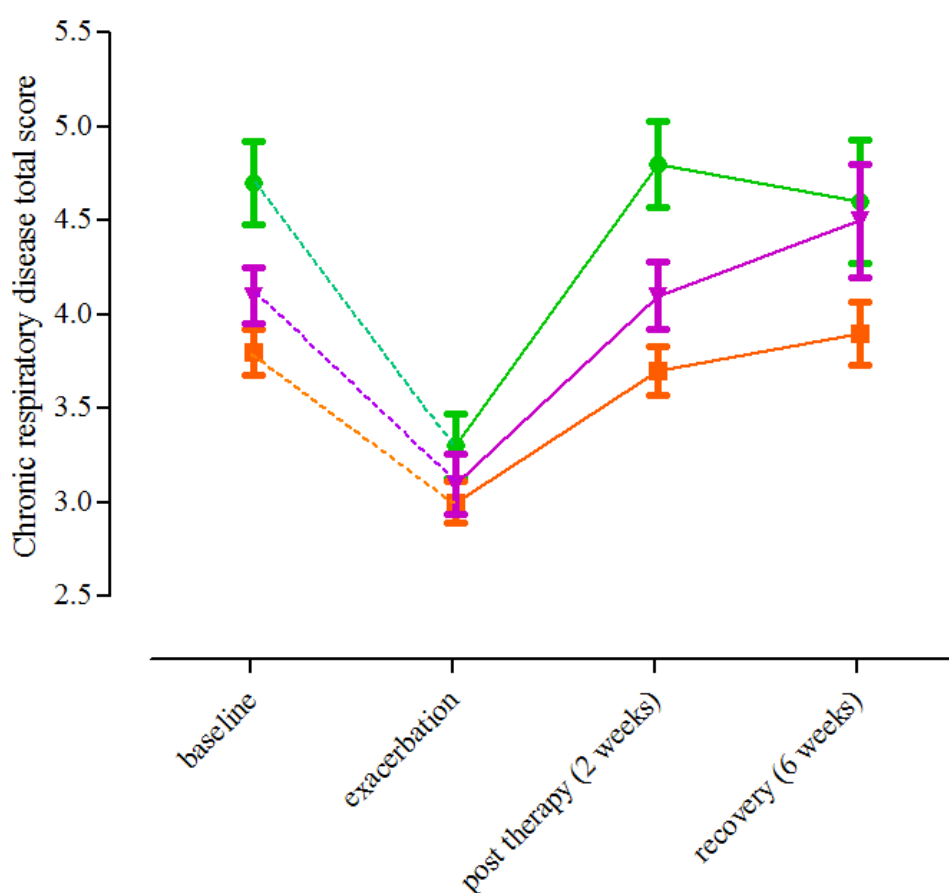
Mean improvement in FEV_1 (95% CI) after 14 days for exacerbations with airway eosinophilic inflammation given prednisolone, airway non eosinophilic exacerbations given placebo and airway non eosinophilic exacerbations given prednisolone was 335mL (224 to 446), 116mL (29 to 203) and 102mL (53 to 151) respectively (see figure 8.11).

Figure 8.11; FEV$_1$ across stable and exacerbation visits in exacerbations that had airway eosinophilic inflammation treated with prednisolone (●); airway non-eosinophilic inflammation treated with prednisolone (■); and airway non eosinophilic inflammation treated with placebo (▼). Data points presented as mean (standard error of the mean)
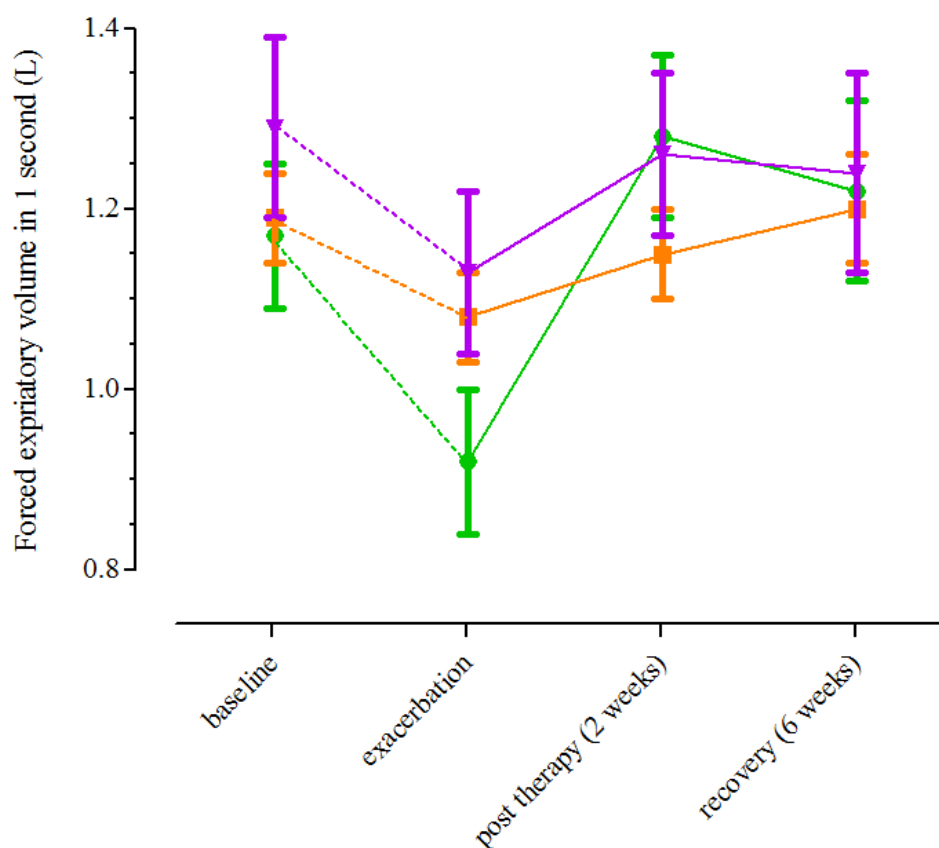
There was a significant difference in the proportions (95% CI) of exacerbations that reported increased symptoms following 14 days of treatment in patients with eosinophilic inflammation given prednisolone, non eosinophilic airway inflammation given prednisolone and non eosinophilic airway inflammation given placebo (3% (0 to 18) vs. 17% (11 to 26) vs. 5% (1 to 17%) respectively, χ^2 p=0.04).

The time (days) to return to baseline VAS symptoms in exacerbations with eosinophilic airway inflammation treated with prednisolone, exacerbations with non eosinophilic airway inflammation treated with prednisolone and exacerbations with non eosinophilic inflammation treated with placebo was significantly different between groups (log Gaussian regression analysis p=0.016) and is demonstrated in figure 8.12 using a Kaplan-Meier plot. Not all patients had a complete resolution of symptoms after 14 days.

Figure 8.12; Kaplan-Meier plot for the time taken to return to baseline symptom scores for subjects with eosinophilic airway inflammation treated with prednisolone (━); non-eosinophilic airway inflammation treated with prednisolone (━); and non-eosinophilic airway inflammation treated with placebo (━) using left and right censored data. Symptoms do not return to baseline in all subjects after 14 days.
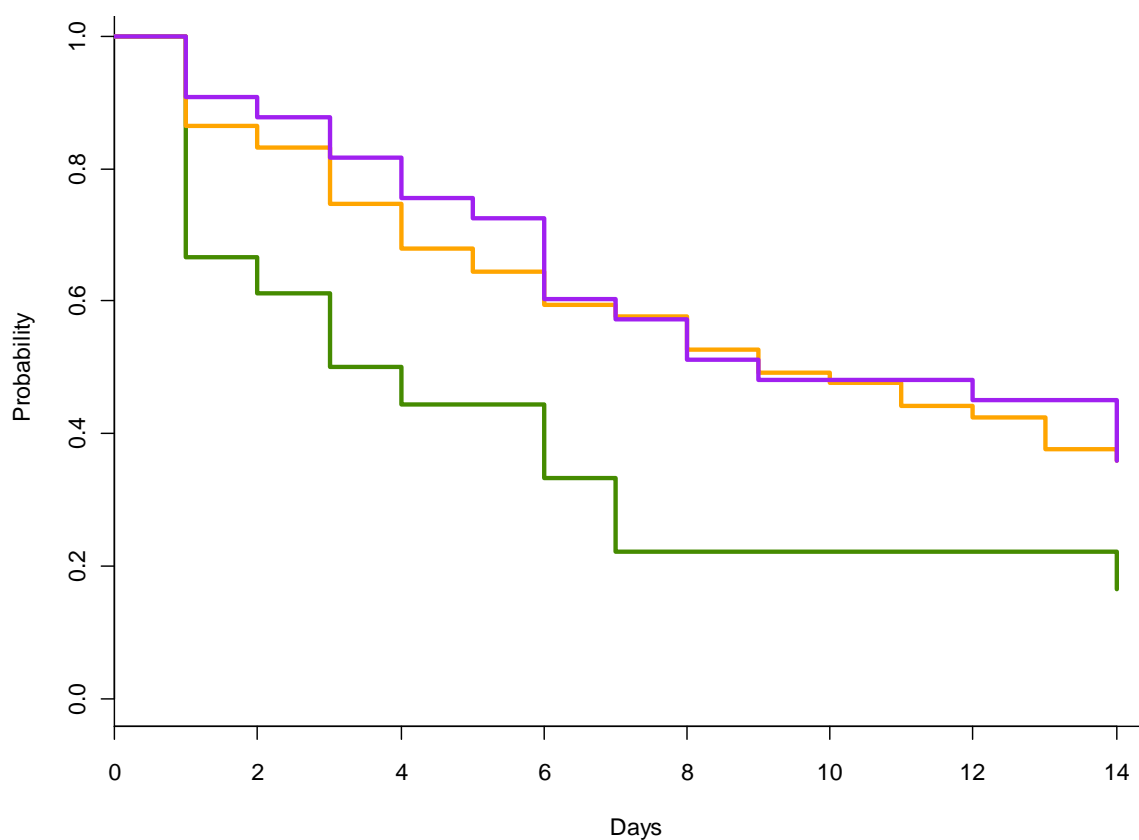
The mean (SEM) daily VAS symptom score following treatment is illustrated in figure 8.13. VAS symptom scores after treatment in some patients were better than baseline, but were not found to be significantly different at 14 days from baseline for any of the groups.

Figure 8.13; Daily VAS symptom scores following treatment for exacerbations that had airway eosinophilic inflammation treated with prednisolone (●); airway non-eosinophilic inflammation treated with prednisolone (■); and airway non eosinophilic inflammation treated with placebo (▼). Data points presented as mean (standard error of the mean). Dashed horizontal line represents mean baseline VAS symptom score.
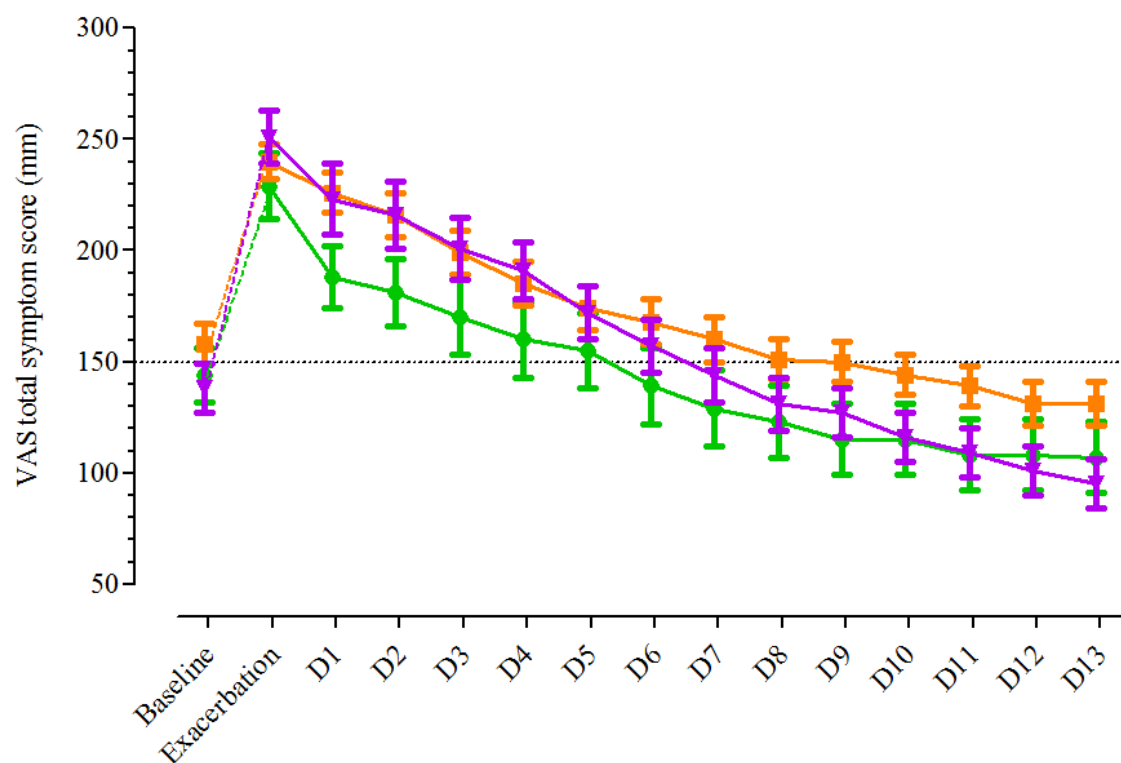
Validation of the biomarkers peripheral blood eosinophils, sputum IL-1β, and serum

CXCL10

For all patients randomised at exacerbation, peripheral blood eosinophil counts remained a highly sensitive and specific biomarker for a sputum eosinophilia, with an area under the ROC curve of 0.90 (95% CI 0.48 to 0.96, p<0.0001) (figure 8.14).

Figure 8.14; Receiver operator characteristic curve for peripheral blood eosinophils to identify sputum eosinophilia during COPD exacerbations in de-novo patients
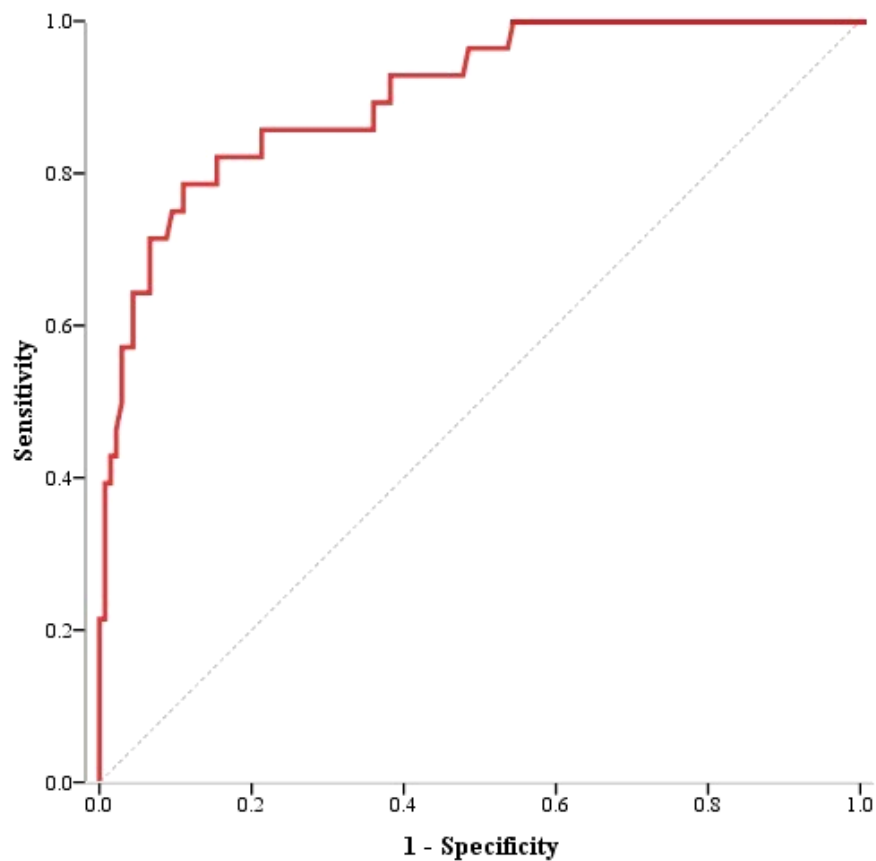
The area under the ROC curve (95% CI) for sputum IL-1β to identify a bacteria associated exacerbation was 0.73 (0.61 to 0.85); a sputum IL-1β cut-off of 130pg/mL had a sensitivity and specificity of 80% and 60%, (figure 8.15).

Figure 8.15; Receiver operator characteristic curve for sputum IL-1β to identify bacteria associated exacerbations of COPD in de-novo patients
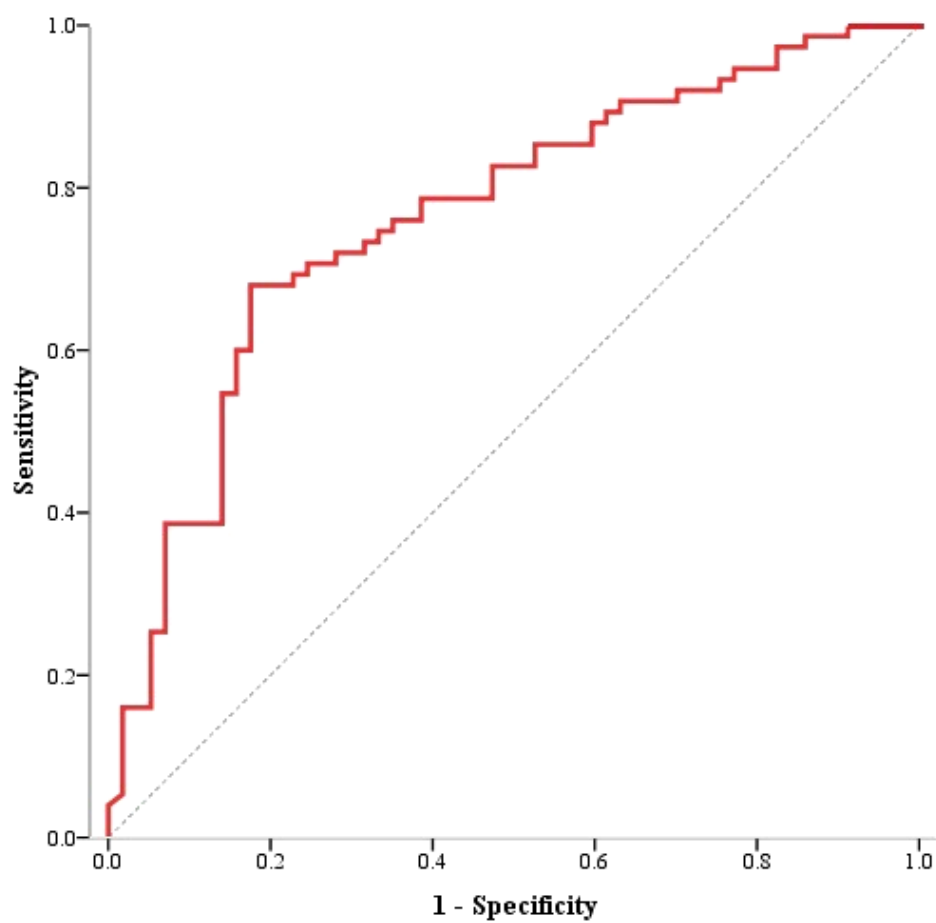
The area under the ROC curve (95% CI) for serum CXCL10 to identify a virus associated exacerbation was 0.65 (0.52 to 0.78) with a cut-off of 145pg/mL having a sensitivity and specificity of 70% and 60% (figure 8.16).

Figure 8.16; Receiver operator characteristic curve for serum CXCL10 to identify virus associated exacerbations of COPD in de-novo patients
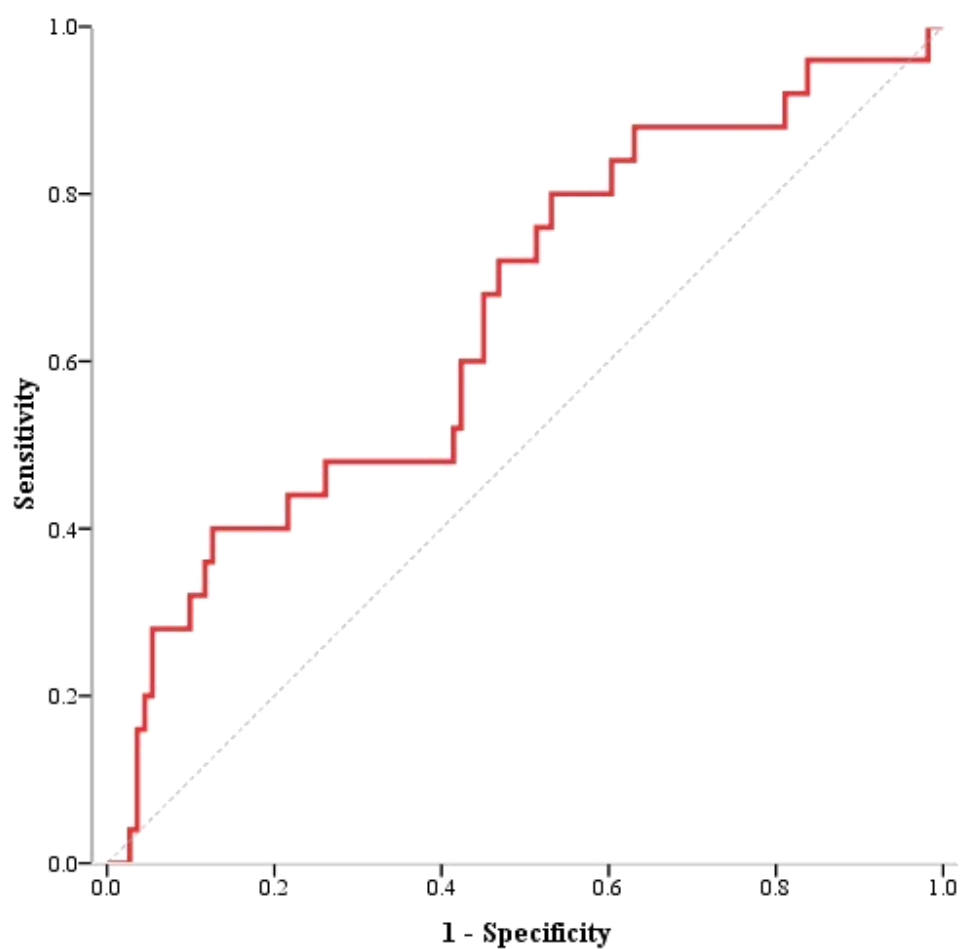
Discussion

In this chapter I have shown that the peripheral blood eosinophil count can be used as a biomarker to guide treatment with corticosteroids at the onset of COPD exacerbations. This strategy was not associated with an increase in treatment failure or a reduction in health status compared to current standard therapy. I have shown that in a sub-group of patients that exhibit the peripheral blood biomarker negative phenotype there is a worsening in health status with prednisolone treatment in comparison to placebo treatment and a significantly increased number of treatment failures. I have also established that there is stability of the blood eosinophil biomarker at stable state; and have demonstrated that this predicts the blood eosinophil biomarker status at exacerbation. Furthermore, I have also shown that an airway eosinophilia during exacerbations of COPD was associated with the most improvement in lung function, health status, improvement of symptoms and rate of recovery following treatment with corticosteroid therapy. And finally, in a separate cohort of COPD exacerbations I have replicated the biomarker findings that sputum IL-1β, serum CXCL10 and peripheral blood eosinophils can accurately determine bacteria, virus and sputum eosinophil associated exacerbations of COPD identified in Chapter 5.

Biomarker-directed therapy versus standard therapy

This is the first work to use a biomarker-directed strategy to direct corticosteroid therapy during exacerbations of COPD. Currently systemic corticosteroid therapy during COPD exacerbations is the recommended treatment strategy globally.

The Cochrane review of systemic corticosteroids for acute exacerbations of COPD concluded that the use of corticosteroids was associated with reduced rates of treatment

failure and length of hospital stay (Walters 2009). However the use of corticosteroids was also associated with a significant increase in the number of adverse events; a five times increased risk of hyperglycaemia and a number needed to harm of 5. Only 2 studies in the meta-analysis were out-patient based (Thompson 1996; Aaron 2003). These out-patient RCTs showed that prednisolone treatment compared to placebo was associated with a reduction in treatment failure rates; however the treatment failures rates between prednisolone and placebo did not reach statistical significance in the study conducted by Aaron *et al* (27% vs. 43%, p=0.05). The studies by Thompson *et al* and Aaron *et al* also showed that prednisolone compared to placebo improved FEV_1 by 50mL (Thompson, 1996) and 140mL (Aaron, 2003). All the RCTs that have investigated corticosteroid use during exacerbations of COPD have randomised patients into the study groups based on a random generator allocation; however there has never been any published work using a biomarker-directed strategy to guide corticosteroid treatment during COPD exacerbations. The use of PCT biomarker-directed antibiotic therapy during exacerbations of COPD has been advocated by the Swiss group (Christ-Crain 2004, Stolz 2007, Schuetz 2009) and has showed that an antibiotic biomarker-directed treatment strategy was non-inferior to current standard therapy, could reduce antibiotic prescription and was not associated with an increase of treatment failures.

In this chapter, the study was designed to demonstrate non-inferiority for biomarker-directed therapy for corticosteroid use. In non-inferiority studies, the new treatment should be shown not to be worse than the existing treatment. The null hypothesis in this study was that patients treated in the biomarker-directed treatment arm would not feel worse after two weeks treatment compared to the standard treatment strategy and would not have an increase in treatment failure rates. Health status was measured using the

CRQ (Guyatt, 1998) and the modified CRQ score at two weeks following an exacerbation and has been validated as an acute health status tool in a study by Aaron *et al* (Aaron 2003). The minimally important change of the CRQ score was 0.5 and non-inferiority was calculated to power this, based on observed confidence intervals. A feature of this non-inferiority study is that it confirms that the biomarker-directed arm is no worse than current standard clinical practice; whilst strength of this study was that power was achieved.

In this chapter I have showed non-inferiority in health status outcomes in the biomarker-directed arm compared to the standard arm. Interestingly the trend favoured the biomarker-directed arm (CRQ mean score change from exacerbation to 2 week post therapy biomarker-directed arm versus standard therapy arm, 0.8 vs. 1.1, mean difference 0.3, 95% CI 0.0 to 0.6, p=0.05). This was similar to CRQ improvements in the prednisolone and placebo groups demonstrated by Aaron *et al* (Aaron 2003), which suggests that the COPD patient population the two studies are similar. A trend in reduced treatment failure rates in the biomarker-directed arm compared to the standard arm was also detected. Treatment failure was defined as death, hospitalisation or failure of treatment requiring the commencement of therapy within 30 days. The definition of treatment failure although arbitrary, was based on definitions used by the Cochrane systematic review for the previous RCTs. Double-blind randomisation was ensured in the study by a blinded clinical team performing all assessments and medications for treatment failures. Patients were randomised by minimisation (Treasure, 1998) in order to reduce differences in the groups that may be of importance that could arise from random allocation, namely exacerbation frequency, FEV_1 and sputum eosinophil counts. Patients were not different in gender, age, atopy and systemic or pulmonary

inflammation. These findings make it very unlikely that an important difference of outcome in favour of standard, non-biomarker directed therapy was missed. Furthermore, falls in FEV_1 at exacerbation and improvements after treatment were very similar in the biomarker-directed and standard therapy arm and similar to mean improvements found by Thompson and Aaron *et al* (Thompson, 1996; Aaron, 2003). Symptom scores recorded on the VAS for the domains of cough, breathlessness, sputum production and purulence also improved by similar proportions in these two arms. Corticosteroid use was reduced by 50% in the biomarker-directed arm and was in part driven by the design of the study. However, this reduction demonstrated that outcomes were not worse in the biomarker-directed arm and despite not having health utilisation outcome analysis, it is clear that cost savings can be associated with reductions in the risk of hyperglycaemia, osteoporosis and cardiovascular disease in a population already at risk of significant harm.

Peripheral blood biomarker positive and negative exacerbations

From the results presented in chapter 5 the peripheral blood eosinophil count was chosen as the biomarker to direct corticosteroid therapy in the biomarker-directed arm. This biomarker was the most sensitive and specific for determining a sputum eosinophilia during exacerbations of COPD and a 2% cut off was associated with 90% sensitivity and 60% specificity for determining a sputum eosinophilia. This cut off was chosen in order to enrich the number of true positives and ensure prednisolone treatment in subjects with a sputum eosinophilia in the biomarker-directed arm. Only 1 patient in the biomarker-directed therapy group with a sputum eosinophilia at exacerbation was not treated with prednisolone. Changing the peripheral blood eosinophil cut-off

threshold to a higher sensitivity would be associated with a potential increase in the number of subjects being over-treated (false positives). Although studies have shown that corticosteroids can improve lung function and dyspnoea scores in the short term (Davies, 1999) these improvements are marginal (Aaron, 2003) and need to be weighed against the potential harm in a population who often have significant co-morbidities (Niewoehner, 1999; Walters, 2009). Conversely reducing the peripheral blood eosinophil cut-off may miss identifying patients who have a sputum eosinophilia. Whether treating a patient with a sputum eosinophil associated exacerbation is important is currently unknown and has never been studied. However evidence in stable COPD has showed that a sputum eosinophilia was associated with a positive response to corticosteroid therapy (Chin 1978, Pizzichini 1996, Brightling 2000) and stable data extrapolation may suggest that this is also important during exacerbations.

A peripheral blood eosinophilia has been shown to be associated with an increase in all-cause mortality in subjects with airways disease (Hospers, 2000). Although not powered to study mortality, biomarker positive exacerbations had a greater reduction in FEV_1 at exacerbation and improved beyond baseline at 6 weeks. No previous work has been undertaken to investigate the prednisolone response in health status and lung function using the blood eosinophil counts during COPD exacerbations. However in biomarker positive and biomarker negative subjects treated with prednisolone, there was a statistical and clinically significant difference in the CRQ improvement and recovery of daily symptoms over 14 days. These differences were not associated with changes in lung function (absolute FEV_1 recovery similar to meta-analysis) nor in treatment failure rates.

Additionally in the biomarker negative sub-group corticosteroid treatment resulted in worse outcomes compared to placebo. This finding was not associated with differences in bacteria detection rates, although colony forming units were higher in biomarker negative exacerbations. Corticosteroids reduce the ability of macrophages to phagocytose antibody coated particles and decrease the secretion of pro-inflammatory cytokines such as IL-1, IL-6 and TNF. Corticosteroids can also suppress polymorphonuclear vascular endothelial adherence, phagocytosis, degranulation, cytokine production, chemotaxis, oxidative burst and free radical generation, with inhibition of apoptosis and prolonged survival of dysfunctional neutrophils. There is increasing evidence that inhaled corticosteroids are associated with an increased risk of pneumonia in COPD (Calverley, 2007; Ernst, 2007; Kardos, 2007). My work shows that the peripheral blood biomarker is able to identify COPD phenotypes during exacerbations that are likely to be harmed from corticosteroid therapy. The elevated CFU's suggest that in patients with blood eosinophil biomarker-negative COPD exacerbations, infection maybe a primary driver and treatment with corticosteroids maybe detrimental. An alternative mechanism might be that the biomarker negative sub-group have an inherent reduction in macrophage phagocytosis and bacterial clearance, as demonstrated by a study by Taylor *et al* (Taylor, 2010), which would account for the increased CFU's and presumed bacterial infection frequency during the exacerbation. The detection of the blood biomarker-negative phenotype at stable state and its stability during longitudinal analysis would favour identification of a COPD phenotype associated with a defect in innate immunity.

Peripheral blood biomarker phenotype

In this chapter it was determined that the blood eosinophil biomarker status at stable state could predict the exacerbation blood biomarker status. Approximately 80% of subjects that were assigned prednisolone in the biomarker-directed arm (thus biomarker positive at exacerbation) would have been allocated prednisolone from the baseline blood eosinophil count. The biomarker positive and negative subjects differed at baseline by the peripheral blood eosinophil counts, whilst at exacerbation the differences included an increased sputum neutrophil count and CRP. Furthermore, few patients with captured repeated exacerbations switched between biomarker status and consequent treatment strategies, suggesting that this simple blood biomarker can define a phenotype of COPD.

In the proposed classification by Han *et al* (Han, 2010) a phenotype is defined as *'a single or combination of disease attributes that describe differences between individuals with COPD as they relate to clinically meaningful outcomes'*. Very few studies have examined the stability of phenotypes in COPD whilst none have examined predictors of the exacerbation phenotype or the stability of the exacerbation phenotype. The body mass index, airflow obstruction, dyspnoea and exercise capacity (BODE) index has been used as a predictive tool for mortality in COPD. In the study by Celli *et al*, these predictive variables were identified in 200 COPD subjects and then validated in a further 600 COPD subjects. The cachectic, breathless and functionally limited COPD phenotype was associated with a worse prognostic outcome (Celli, 2004). The ECLIPSE results showed that a history of previous exacerbations was predictive of an increased risk of future exacerbations and developing the frequent exacerbation

phenotype (Hurst, 2010). No studies have examined the stability and predictability of biomarkers to identify exacerbation phenotypes or treatment responses. The peripheral eosinophil blood biomarker is an attractive tool to use in clinical practice as it is simple to measure, available at the time of an exacerbation in current hospital practice and reliable. This blood biomarker fulfils the criterion for the National Institute of Health (NIH) biomarker definition workshop (Atkinson, 2001). It is a characteristic that can be objectively measured and can be evaluated as an indicator of the pathogenic and pharmacologic responses to treatment. The identification of a stable blood biomarker phenotype and the predictability of the exacerbation biomarker status lend to hypothesise that i) treatment responses at exacerbation of COPD can be predicted at stable state and ii) that biomarker status treatment of COPD at stable state may reduce exacerbation events. This work however would require further investigation. Whether these findings and biomarker-directed strategies can be performed in severe exacerbations of COPD also needs to be investigated further.

Sputum eosinophil counts and corticosteroid response

A sputum eosinophilia in stable COPD has been shown to be associated with corticosteroid responsiveness and important clinical outcomes, such as improvements in lung function, exercise capacity and health status (Shim, 1978; Pizzichini, 1998; Brightling, 2000). In this study, I have shown for the first time that this is also true for exacerbations of COPD. Subjects with eosinophilic airway inflammation during exacerbations of COPD had significantly better health status, lung function, and recovery with corticosteroids compared to subjects with non-eosinophilic airway inflammation given corticosteroids. The mean improvements in lung function observed for the whole group given corticosteroids versus those given placebo is similar to

previously published work (Davies, 1999; Niewoehner, 1999; Maltais, 2002; Aaron, 2003). Lung function improvements in this chapter was 335mL in sputum eosinophil associated exacerbations treated with corticosteroids, 102mL in non-sputum eosinophil associated exacerbations treated with corticosteroids and 116mL in non-eosinophil associated exacerbations treated with placebo. As the peripheral blood eosinophil biomarker was set at a high sensitivity to capture and treat all sputum eosinophil associated exacerbations, my results cannot provide information on whether lung function or health status would improve in sputum eosinophil associated exacerbation treated with placebo. The greater improvement in lung function in the sputum eosinophil sub-group suggests that this represents a corticosteroid responsive phenotype. This may highlight why previous studies of corticosteroids in COPD exacerbations have not been fully conclusive and furthermore indicative of the heterogeneity of the populations studied (Walters, 2009). The rate of recovery presented in the Kaplan-Meier plot (figure 8.12) demonstrated clear differences between the 3 groups. These differences in recovery are more apparent than in previously published studies. In the RCT by Davies *et al* there was a reduction in the median length of stay by 2 days favouring prednisolone treatment (Davies, 1999). In work by Niewoehner *et al*, the prednisolone group had an increase total length of stay compared to placebo because of non COPD exacerbation related causes (Niewoehner, 1999). In this chapter, the mean recovery rate is similar to previously published studies by Seemungal and Donaldson *et al* (Seemungal, 1998; Donaldson, 2005); but my work has shown a sub-group that perform significantly better with corticosteroids.

As demonstrated in Chapter 5, the presence of sputum eosinophilia was predictive of a sputum-eosinophil associated exacerbation of COPD. Work by Siva *et al* has

demonstrated that reduction in sputum eosinophil counts in COPD subjects at stable state can reduce severe exacerbation frequency compared to standard COPD management (Siva, 2007). These findings have also been shown in asthmatics (Green, 2000). The data by Siva *et al* and my findings do not however conclusively define the importance of the eosinophil in the pathogenesis of COPD, but do demonstrate a steroid responsive eosinophil associated phenotype and a non-eosinophil associated non steroid responsive phenotype during COPD exacerbations. The occurrence of the eosinophil phenotype during stable disease may imply that defects in adaptive and innate immunity give rise to these separate COPD phenotypes. Published work demonstrating cellular heterogeneity of COPD exacerbations supports this (Saetta, 1998; Jeffrey, 2000; O'Donnell, 2004). Identification of specific phenotypes of COPD that are outwardly clinically indistinguishable allows a personalised phenotypic approach to the management of COPD and is likely to promote a more individual exploration of pathogenesis.

Bacteria, virus and sputum eosinophil biomarker repeatability

Finally as part of this study I have replicated the biomarkers peripheral blood eosinophil count, sputum IL-1β and serum CXCL10 in a validation cohort. The peripheral blood eosinophil count remained a strong marker of a sputum eosinophilia. Sputum IL-1β and serum CXCL10 were measured using a different platform but remained significant albeit weaker predictive markers of identifying a bacteria or virus associated exacerbations.

A potential limitation is this population may have included patients who had fixed airflow obstruction as a result of asthma. However, I have used stringent criteria to exclude patients with characteristics of asthma and was careful to ensure that the population met current diagnostic criteria for COPD, using the available tools required in accordance with the GOLD COPD definition (Rabe, 2007). Furthermore, features such as atopy and bronchodilator responsiveness were not related to eosinophilic airway inflammation in this group. The use of specialised imaging such as computed tomography would provide further information to the pathological description of COPD. However, this is not currently a requirement for diagnosis and was not used in this study to define COPD patients.

Although bacteria are believed to play a role in up to 50% of exacerbations (Sethi, 2008), evidence for the benefit of antibiotics is conflicting (Puhan, 2007; Puhan, 2008). Therefore in this study, I have standardised the effects of a bacterial aetiology by prescribing open labelled antibiotic therapy to eliminate any confounding effects of bacteria within exacerbations. Whether this is appropriate or not is unknown. Approximately 97% of exacerbations were associated with increase in sputum purulence and sputum production. Thus, by standards and the Anthonisen criteria, these subjects would have the most benefit with antibiotic treatment. No differences in bacteria culture positive rates in the biomarker and standard arm were detected, so this variable is unlikely to have confounded the comparison between these groups. Treatment failure rates were low, which is likely a reflection of the moderate severity of the exacerbations. It is therefore important that the hypothesis is tested in larger studies including subjects hospitalised with severe COPD exacerbations. These studies should

also investigate whether outcomes of biomarker directed therapy are influenced by factors such as tapered prednisolone doses, duration of treatment and the presence of infection, emphysema and chronic bronchitis. This study was not powered to investigate health economic impacts of biomarker-directed corticosteroid therapy and this important potential benefit requires further study.

Conclusions

In conclusion, a biomarker directed strategy using the peripheral blood eosinophil count can be used to direct corticosteroid therapy during acute COPD exacerbations and resulted in a 50% reduction in prednisolone prescription without deleterious consequences. This simple stratification allows for the identification of important phenotypes of COPD and may identify groups where modified therapy is needed. This data suggests that in the outpatient treatment of exacerbations of COPD, systemic corticosteroids should be only be given to those that have a peripheral blood eosinophil count >2%.

9. Final conclusions

Summary of findings

In this thesis I have shown that the heterogeneity of COPD can be delineated. Firstly, I have validated a sputum processing method that increases recovery of previously difficult to measure mediators. Secondly, I have investigated the utility of the biomarkers PCT and CRP and also proposed concentrations that could be used to direct antibiotic therapy in COPD exacerbations. Thirdly, I have identified sputum and serum biomarkers that are sensitive and specific for clinical phenotypes of bacteria, virus and sputum eosinophil associated exacerbations and I have validated the biomarkers IL-1β, CXCL10 and the peripheral blood eosinophil count in a separate cohort of COPD exacerbations. I have used multivariate modelling statistical analysis to phenotype COPD exacerbations using biomarkers and I have determined that there are biological clusters of exacerbations that relate to inflammation and aetiology. Using longitudinal analysis I have demonstrated stability in the COPD phenotypes and that microbiological and airway eosinophilic phenotypes can both predict exacerbation phenotypes associated with bacteria and sputum eosinophils. Finally I have used the peripheral blood eosinophil count to guide corticosteroid therapy in a randomised placebo controlled clinical trial, further validating this biomarker and identifying phenotypes of COPD likely to have greatest benefit from corticosteroid therapy.

COPD exacerbations are recognised to be heterogeneous with respect to aetiology, airway inflammation and treatment responses. Studies have explored the heterogeneity using clinical variables and cluster analysis to determine COPD phenotypes. Results identified phenotypes of COPD similar to the clinical description of the classical COPD

'blue bloater' and 'pink puffer'. However, there is a lack of data in the literature to phenotype COPD exacerbations. The work performed in this thesis expands this and importantly categorises COPD exacerbations into aetiologically specific and treatment responsive phenotypes. One of the most significant findings in the thesis was that COPD exacerbation phenotypes could be predicted from the stable state using simple sputum and blood biomarkers. The microbiological and eosinophilic COPD phenotypes at stable state were associated with either a bacteria or a sputum eosinophilia associated exacerbation. The eosinophilic phenotype was associated with corticosteroid responsiveness, whilst it could be hypothesised that the microbiological phenotype would be associated with antibiotic responsiveness. The work in this thesis represents a paradigm shift into future methods to explore mechanisms and management of COPD.

COPD Homeokinesis

The achievement of equilibrium in organisms by dynamic processes is called homeokinesis. In health, there is equilibrium in inflammation and to responses to activation of immunity following insults such as exposure to antigen, virus or bacterial pathogen. These insults occur near continuously but it is the appropriate achievement in homeokinesis that prevents healthy individuals from an exacerbation during a cold for example. In COPD, it might be postulated that the homeokinesis is established at a higher threshold where there is a low, but perpetual risk of exacerbation (figure 9.1). In healthy individuals respiratory and environmental challenges are tolerated with little consequence, however in COPD the effects are greater resulting in disequilibrium of homeokinesis and increases in inflammation despite so called 'stable' disease. This may explain the existence of the microbiological and eosinophilic phenotypes during stable disease. This disequilibrium presents an increased risk of developing an exacerbation.

Figure 9.1; Altered homeokinesis in COPD
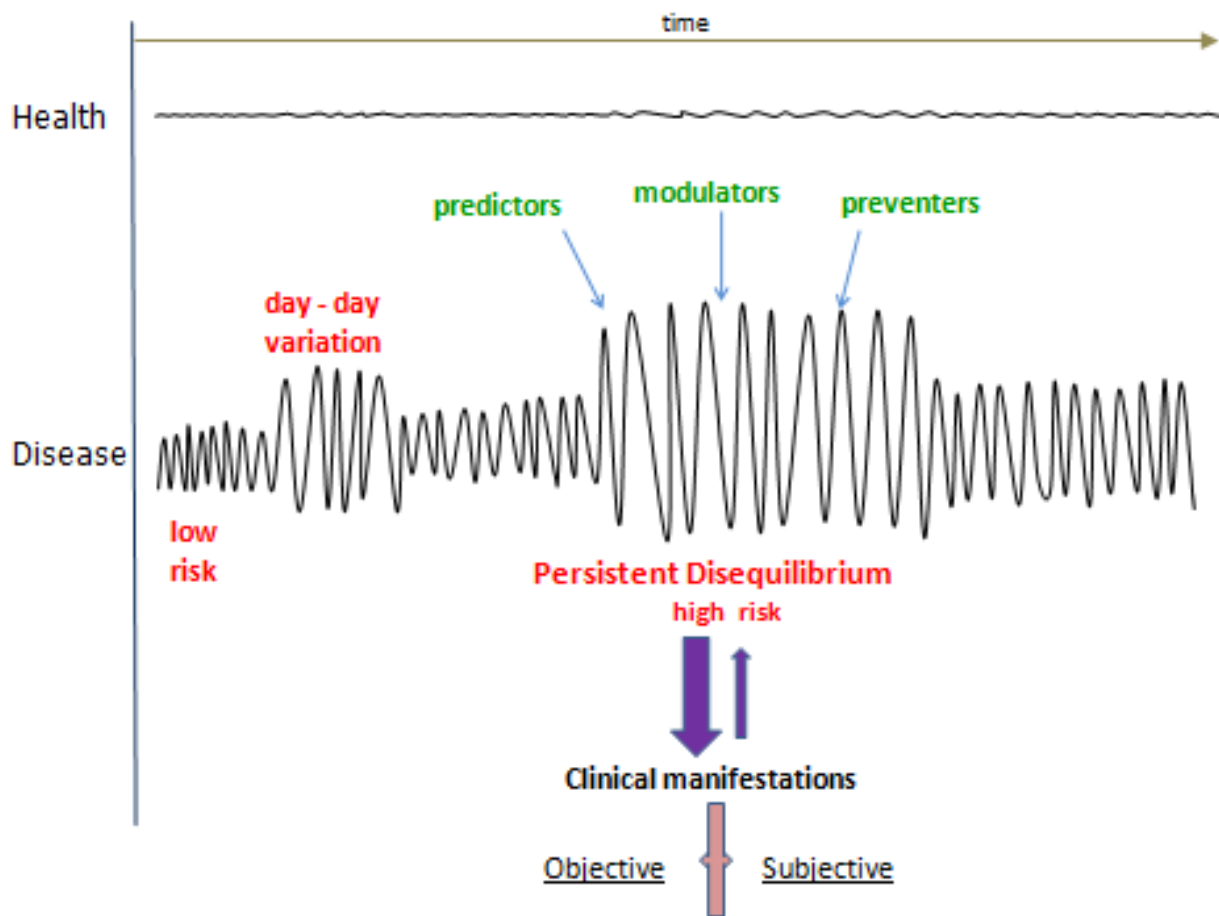
The work in this thesis concludes that the clinical manifestations of exacerbations are likely to be multifactorial and I suggest these can be categorised as intrinsic and extrinsic factors. The intrinsic factors for example are related to disease severity, inflammation and 'stable' state phenotypes. The extrinsic factors are linked to the source of the original insult, the concordance of symptoms and inflammation and social and psychological support. I have hypothesised and established that biomarkers can be used to predict the exacerbation phenotype, target treatments and propose that they can modulate outcomes. Preventing exacerbations by pre-emptive antibiotics or corticosteroids in the microbiological and eosinophilic phenotypes is likely to be of benefit and should be investigated.

Thesis limitations

Study limitations have been discussed within each chapter. However a general limitation of the biomarker studies is that the exacerbations were moderate and findings cannot be extrapolated to severe exacerbations. The inclusion criteria for the COPD population included a previous history of exacerbations, which may affect the bias of the biomarker phenotypes to frequent exacerbators. It has never been demonstrated that biological phenotypes and clinical COPD phenotypes in milder COPD subjects occur.

The definition of an exacerbation was based on patient reporting of symptoms which necessitated a change in medication. The definition used in this thesis was in accordance with current literature but this current definition is flawed. The description of an exacerbation relies on several parameters. Firstly, the patient has to experience a change in symptoms and recognise that these are different to background symptoms. Secondly the patient has to present to a medical practitioner whose role it is to i) assess that the reported symptoms are different to the patients baseline symptoms and ii) assess that these changes in symptoms require medical therapy with corticosteroids and antibiotics. The medical practitioner does not know whether these symptoms relate to a specific aetiology or whether the treatment will work.

Furthermore, the data captured by the exacerbation definition used in this thesis was from event-based exacerbations. It is widely accepted that a large proportion of exacerbations are unreported and can be retrospectively detected on diary card analysis. It however remains unclear why some exacerbations are unreported. Whether there are differences in the threshold of inflammation, host-pathogen response or biological expression in unreported exacerbations remains unknown. At present the basis of an

exacerbation relies on subjective reporting by patients and subjective assessments made by physicians. The currently used definition does not take into account the underlying cause of the exacerbation, the treatment response or the complex psychological or social influences that exist in patients with chronic respiratory disease; using an objective biomarker measurement will aid in the resolution of some of these complex issues.

A further limitation of this work is that MSD was only used to measure biomarkers at stable state on one occasion and further studies assessing the repeatability are required. Funding limitations precluded measurements of MSD biomarkers during the longitudinal stable visits. However the biomarkers IL-1β and CXCL10 were assessed in a different COPD cohort using different assays, whilst peripheral blood eosinophils were successfully used to guide corticosteroid treatment in a randomised clinical trial. Thus the biomarkers that were identified which were found to predict an exacerbation associated with bacteria, virus and sputum eosinophils were validated.

Finally, the biological cluster work and the identification of sensitive and specific biomarkers was performed using multivariate modelling statistical analysis and receiver operator characteristic curves. Factor and cluster analysis to be accurate have to observe strict statistical rules and do not necessarily imply causality. However, the observations of biological factors and clusters during COPD exacerbations are novel and do not rely on clinical variables to phenotype COPD. Further validity of these clusters is required during stable state, in repeated exacerbations and from different cohorts of COPD populations. This mathematical approach may help in identifying differences in genetic and cellular expression of COPD exacerbations and offer the prospect of characterising disease using biomarkers. ROC curves were drawn to identify sensitive and specific

biomarkers. This is a standard statistical method and can yield numerous cut-offs of sensitivity and specificity, with equal weighting designated as the Youden index. The critical requirement in using this method is exploration of these cut-offs to answer clinical questions. In this thesis, equal weighting was selected as an example for the biomarkers IL-1β and CXCL10. For the peripheral blood eosinophil count, a highly sensitive cut off was selected for use in the clinical trial. Similarly PCT and CRP levels were also chosen for their high sensitivity for future clinical trials.

Plans for future work

The findings of this project only apply to moderate exacerbations of COPD. It would be vital to study whether the identification of the biological clusters and the biomarkers, peripheral blood eosinophils, CRP, sputum IL-1β and serum CXCL10 in eosinophilic, bacterial and virus associated exacerbations could be reproduced in severe hospitalised exacerbations of COPD. Furthermore, the application of targeted treatment of COPD exacerbations in a randomised clinical trial using the biomarkers peripheral blood eosinophils and CRP could be applied to direct corticosteroid and antibiotic therapy in severe hospitalised exacerbations. Although the longitudinal work in this thesis develops key understandings in the stability of COPD phenotypes, further work to assess the prognosis and disease trajectory of the biological clusters and COPD microbiological and eosinophilic phenotypes are required.

The finding that stable state COPD phenotypes exist and that these were good predictors of the clinical exacerbation phenotype (exacerbations associated with bacteria, virus and sputum eosinophilia) provides a basis to investigate whether exacerbations can be prevented by treating the microbiological phenotype with prophylactic antibiotics and

similarly reduction of eosinophilic inflammation using corticosteroid therapy or novel biologics, with application of personalised treatments in primary and secondary care. In this project the biomarkers provided valuable information regarding the clinical phenotypes during stable state and exacerbations and indicate clinically important tools that could be used to direct therapy. Nevertheless it is important to investigate why these phenotypes occur in COPD and to use the biomarkers to isolate different groups and therefore progress our understanding in COPD pathogenesis.

The existence of a microbiological phenotype in COPD and the demonstration of microbes during stable COPD and during exacerbations may translate to the identification of abnormalities in the innate immune system. An important part of bacteria clearance from the lower airways in health involves the epithelial barrier function and the mucociliary clearance system. For the initiation of an inflammatory response, pattern recognition receptors including central toll-like receptors recognise pathogen-associated molecular patterns which create a critical cascade of inflammatory cytokines and chemokines, thereby recruiting neutrophils and macrophages to assist in the removal of bacteria. Impairment in any of these steps is likely to lead to the presence of a microbiological airway disease COPD phenotype. Primary investigation into the presence of cilial dysfunction, pattern recognition receptors deficiency or pathogen-associated molecular patterns defects as well as further analysis into degrees of impaired phagocytosis and the airway microbiome is important in our microbiological COPD phenotype.

The expression of an eosinophilic airway phenotype also demonstrates differences in the biology of the COPD phenotypes. Exploration of the role of environmental stimuli

including allergen exposure as a possible driver to Th2 mediated responses should be studied in the eosinophilic COPD phenotype. Assessments of eosinophil recruitment, function and activation in this phenotype may determine the role of eosinophilic airway inflammation during parenchymal destruction and airflow obstruction. A mechanism for eosinophilic COPD requiring further exploration is the unopposed upregulation of Th2 cell immunity, whether this is governed by a clonal proliferation of T-lymphocytes, over-expression of intracellular signalling pathways (e.g. STAT6) or aberrant T-cell mediated auto-immunity.

It is clear that interactions between the innate and adaptive immune systems are a dynamic and contiguous process. The use of biomarkers in COPD and the subsequent identification of different biological expressions during stable state which are then manifest during an exacerbation, will result in better understanding of the disease process and lead to superior management strategies.

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11. Appendices

Appendix 1: Systemic corticosteroids in acute exacerbations of COPD summary of published randomised control studies

Study	Number	Clinical setting	Dose and duration of corticosteroids	Inclusion criteria	Exacerbation severity	Primary outcomes	Results
Albert, 1980	N=44 M=44	Hospitalised	0.5mg/kg Methylprednisolone IV for 3 days	Chronic bronchitis; $PaO2<65mmHg$ $PaCO2>50mmHg$; $FEV_1<60\%$ or $FEV_1/FVC<60\%$; Reversibility $<30\%$	Severe	FEV_1	FEV_1 improved in study group in 3 days compared to placebo
Emerman, 1989	N=96 M=50 F=46	Hospitalised	Methylprednisolone 100mg IV	Emphysema or chronic bronchitis Age >50 $FEV_1<70\%$ and $FEV_1/FVC<60\%$	Severe	FEV_1 Length of stay	No difference for either outcome
Rostom, 1994	N=30 Gender unknown	Hospitalised	Methylprednisolone 40mg IV QDS for 3 days followed by 32mg oral Methylprednisolone over 15 days	Clinical COPD	Severe	FEV_1	No results

Bullard, 1996	N=113 M=97 F=16	Patients recruited from Emergency room	Stat Hydrocortisone 100mg Hydrocortisone 100mg QDS 4 days for in-patients OR Prednisolone 40mg for 4 days inclusive for hospitalised and discharged patients	Age >40 yrs; FEV_1 <60% or FEV_1/FVC <60%; Suspected airflow limitation	Moderate & Severe	FEV_1 Peak flow	FEV_1 and PEF improved within 6 hours
Thompson, 1996	N=27 M=26 F=1	Outpatients	Reducing 60mg Prednisolone course over 9 days	Clinical COPD >20 pack years FEV_1 <60% or FEV_1/FVC <65%	Moderate	FEV_1 VAS Treatment failure Arterial blood gases	Improved gas exchange, FEV_1, and fewer treatment failures
Wood-Baker, 1997	N=47 M=24 F=14	Hospitalised	Prednisolone 2.5mg/kg for 3 days Or Prednisolone 0.6mg/kg for 7 days	FEV_1<50% predicted >40 yrs age >10 pack years	Severe	FEV_1 Length of stay Arterial blood gases Treatment failure	Shorter length of stay

Davies, 1999	N=60 M=34 F=16	Hospitalised	Prednisolone 30mg for 14 days	Clinical COPD Age 40-80 FEV$_1$ <70% and FEV$_1$/FVC <75%	Severe Anthonisen	FEV$_1$ VAS symptom scores Length of stay	FEV$_1$ and shortened length of stay
Niewhoener, 1999	N=271 M=268 F=3	Hospitalised	Methylprednisolone 125mg QDS for 3 days and reducing course of 60mg prednisolone 60 days OR Methylprednisolone 125mg QDS for 3 days and reducing course of 60mg prednisolone 15 days	Clinical COPD Age >50 >30 pack years FEV$_1$ <1.5	Severe	Treatment failure Time to treatment failure Length of stay FEV$_1$	Early FEV$_1$ improvement Less treatment failure
Maltias, 2002	N=199 M=162 F=37	Hospitalised	Prednisolone 30mg BD for 3 days and Nebulised pulmicort	Clinical COPD Age >50 >20 pack years	Severe	FEV$_1$ Blood gases Dyspnoea Length of stay	FEV$_1$ improved

Aaron, 2003	N=147 M=84 F=63	Outpatients recruited from Emergency room	40mg prednisolone 10 days duration	Age >35 yrs; >15 pack yrs; FEV_1 <70% and FEV_1/FVC <70%; Reversibility <20%	Moderate	FEV_1 CRQ Transitional dyspnoea index Treatment failures	Shorter time to treatment failure FEV_1 improved at day 10
Chen, 2005	N=130 M=98 F=32	Not stated	Prednisolone 30mg 7 days and Placebo 7 days Or Prednisolone 30mg 10 days and 15mg for 5 days	Not stated	Not stated	FEV_1 Blood gas Length of stay Treatment failures Symptom scores	No differences

Appendix 2: Antibiotic therapy in acute exacerbations of COPD summary of published randomised control studies

Study	Participants	Clinical setting	Dose and duration of antibiotics	Inclusion criteria	Exacerbation definition	Primary outcomes	Results
Elmes, 1965	N=56 20M 36F	Hospitalised	7 days Ampicillin	Chronic bronchitis	Symptom based	Treatment failure Mortality	Reduced treatment failure
Petersen, 1967	Not stated	Hospitalised	8 days Chloramphenicol	Chronic bronchitis Age 45 – 75	Not stated	Not stated	Not stated
Pines, 1968	N=30 30M	Hospitalised	14 days streptomycin	Males Age >50 Chronic bronchitis PEF < 200L^{-1} Purulent sputum	Symptoms for 6 weeks	Treatment failure Mortality Sputum purulence	Reduced treatment failure Less sputum purulence

Pines, 1972	N=259 259M	Hospitalised	12 days Tetracycline or Chloramphenicol	Males Age >60 Chronic bronchitis PEF < 200L^{-1} Purulent sputum	Not stated	Treatment failure Mortality Sputum purulence	Reduced treatment failure Less sputum purulence
Nicotra, 1982	N=20 10M 10F	Hospitalised	7 days Tetracycline	Chronic bronchitis	Symptom based	Blood gas FEV_1	No differences
Anthonisen, 1987	N=116 Gender not stated	Out-patients	10 days Septrin, Augmentin or Doxycycline	Age > 35 Clinical COPD FEV_1 <70% and FVC <70% TLC > 80%	Symptom based	Treatment failure	Overall no differences
Manresa, 1987	Not stated	Hospitalised	8 days Ceflexin	Clinical COPD	Symptom based	Length of stay Blood gas	No differences
Hansen, 1990	Not stated	Hospitalised	7 days Amoxicillin	Clinical COPD	Not stated	Not Stated	Not stated

Jorgensen, 1992	N=133 56M 77F	Out-patients	7 days Amoxicillin	Age >18 Chronic Bronchitis	Symptoms based for 3 days	Treatment failure Sputum purulence	No differences
Nouira, 2001	N=47 42M 5F	Intensive care	10 days Ofloxacin	Age >40 Respiratory failure Clinical COPD	Not stated	Mortality Treatment failure Length of stay FEV_1	Mortality benefit Reduced length of stay

Appendix 3: Visual analogue scale used in biomarker studies

Visual Analogue Scale, COPD Symptoms   VAS

Regard the line as representing the full range of each dimension. Rate your symtoms as they are at the moment.

No cough   The worst cough
├──┤ ever imaginable

No dyspnoea   The worst dyspnoea
├──┤ ever imaginable

No sputum   The worst sputum
production   ├──┤ production ever
                                                                        imaginable

No sputum   The worst sputum
purulence   ├──┤ purulence ever
                                                                        imaginable

Appendix 4: Daily diary card used in biomarker studies

DIARY CARD

DATE AND TIME

NEXT APPOINTMENT…………………………………

What is normal for you?

Breathlessness

none ☐ occasionally ☐

Often ☐ very often ☐

Amount of phlegm

none ☐ teaspoon ☐

Tablespoon ☐ other ………

Colour of phlegm

clear ☐ cream ☐

Green ☐ other…………………..

Wheeze

never ☐ Occasionally ☐

Often ☐ Very often ☐

Cough

never ☐ Occasionally ☐

Often ☐ Very often ☐

Daily number of puffs of relief inhaler ....

Please tick the symptoms if it is worse than normal

Date:							
AM Peak Flow							
More breathless							
More phlegm							
Change in phlegm colour							
Wheeze							
Sore throat							
Nasal discharge							
Fever							
Number of puffs of reliever inhaler							