The Mechanisms and Consequences of Bacterial Colonisation in Chronic Obstructive Pulmonary Disease

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Abstract

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory condition of the lung caused by an abnormal response to noxious gases and particles, particularly cigarette smoke. There is increasing evidence that bacteria contribute to the pathophysiology of COPD both during stable and exacerbation states, and the use of molecular diagnostic techniques have increased our ability to detect and thus investigate their relationship with clinical features, particularly during periods of stability. The main aim of this thesis was to investigate the hypothesis that airway pathogenic bacteria presence, load and species, as detected by qPCR, in stable COPD drives a differential inflammatory response, contributing to systemic inflammation and altering the natural history of the disease, including exacerbation susceptibility and characteristics, and may be related to defective innate immunity, particularly impaired macrophage phagocytosis.

The work in this thesis has demonstrated that in stable COPD there is an apparent bacterial load threshold, at which airway inflammation is significantly greater than in patients with either low bacterial loads or no pathogenic bacteria. Patients with pathogenic airway bacteria above this load threshold have a shorter time to next exacerbation, suggesting bacterial load is an important modulator of exacerbation susceptibility. *H. influenzae* has been consistently shown to play an important role in the pathogenesis of COPD, with a species-specific inflammatory response observed not only in the stable state, but also associated with its increased load at exacerbation, independent of other bacterial or viral pathogens, although this inflammatory response was discordant with patient reported outcomes. No species-specific or bacterial load

effect was seen in systemic inflammation, except with changes in *M. catarrhalis* load at exacerbation, and there were generally poor relationships between the same biomarkers measured in both airway and systemic compartments. In addition, bacterial colonisation appears to be influenced by bacterial load at exacerbation although a prior stable sputum sample with pathogenic bacteria detected is a good predictor of subsequent stable state bacterial presence.

Using monocyte-derived macrophages (MDMs) as a model of alveolar macrophages, impaired phagocytosis was investigated as a mechanism for bacterial colonisation. Although decreased MDM phagocytosis to *H. influenzae* at stable state was associated with higher exacerbation frequency, this could not be explained by the relationship between phagocytosis and bacterial presence, load or species. Furthermore, MDM phagocytosis is a stable phenomenon and cannot provide a biological explanation for changes in colonisation status in stable disease or the heterogeneity of exacerbation aetiology. However, differential activation of MDMs occurs at exacerbations, which may represent different monocyte populations.

The findings from this thesis have important implications in the management of COPD to modifying exacerbation risk. The evidence provided should encourage clinical trials to investigate the use of prophylactic antibiotics specifically for COPD patients with high bacterial loads and those at risk of *H. influenzae* infection at both stable and exacerbated states, and the future development of novel treatments such as specific anti-cytokine agents or those designed to improve macrophage phagocytosis, thereby improving the clinical outcomes for patients with COPD.

Author's Declaration

I, Richa Singh, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. In particular I am grateful for the contributions from the following individuals, in the specific areas described (in alphabetical order):

- Dr Kylie Belchamber, Dr Kiran Chana and Mr Peter Fenwick processed blood samples for the culture of MDMs, the data of which is presented in Chapter 7 and 8.
- Dr Gavin Donaldson assisted with the multiple linear regression statistical analysis of the data presented in Chapter 6.
- Dr Davinder Garcha and Ms Sarah Thurston tested sputum samples for the bacterial qPCR, the results of which are included in all Chapters.
- Dr Siobhan George tested sputum samples for the human rhinovirus PCR, the results of which were included in Chapters 5, 6 and 8.
- MedImmune Pharmaceuticals UK funded the provision of MSD multiplex assays for sputum and serum biomarker analysis, presented in Chapter 6.
- Dr Anant Patel, Dr Alex Mackay, Dr Simon Brill, Dr James Allinson and Ms Beverly Kowlessar, with whom I co-ran the London COPD cohort, assisted with the sample collection and processing. These colleagues, along with the previous research fellows, recruited patients into the cohort and collected data that enabled the analysis of the data included in this thesis.
- Pfizer Pharmaceuticals UK generated heatmaps of biomarkers measured using the MSD multiplex assays, presented in Chapter 6.

- Mr Ray Sapsford assisted with sample processing from patients collected by the study team.

Richa Singh, April 2016.

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Publications and Abstracts

Original articles – first author

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- **The relationship between airway bacteria and impaired macrophage phagocytosis in stable and exacerbated COPD. Singh R,** Belchamber K, Mackay AJ, Thurston SJ, Donaldson GC, Barnes PJ, Wedzicha JA, Donnelly LE.

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- **COPD and co-morbidity: Infection and co-morbidity. Singh R**, Mackay AJ, Wedzicha JA. Eur Respir Monogr 2013;59.

- **Acute COPD Exacerbations.** Wedzicha JA, **Singh R**, Mackay AJ. Clin Chest Med. 2014; 35(1):157-63.

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- **The species-specific inflammatory response of colonising bacteria in stable COPD. Singh R**, Patel ARC, Mackay AJ, Garcha DS, Kowlessar BS, Brill SE, Sapsford R, Donnelly LE, Barnes PJ, Donaldson GC, Wedzicha JA. *The European Respiratory Society* (2013).

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List of abbreviations

AAT	□1-antitrypsin
AECOPD	Acute exacerbation of COPD
AM	Alveolar macrophage
ATS	American Thoracic Society
BAL	Broncho-alveolar lavage
BCSS	Breathlessness, Cough and Sputum Scale
CAT	COPD Assessment Test
CBA	Columbia blood agar
CCL	C-C motif ligand
cfu	Colony-forming units
CHOC	Chocolate agar
COBA	Columbia blood agar base with streptococcus selective supplement agar
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CT	Computerised tomography
CTGF	Connective tissue growth factor
CXCL	CXC-chemokine ligand
D-PBS	Dulbecco's phosphate buffered saline
DMSO	Dimethylsulphoxide
EDTA	K3ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERS	European Respiratory Society
EXACT	Exacerbations of Chronic Pulmonary Disease Tool

FCS	Foetal calf serum
FE	Frequent exacerbator
FEV1	Forced expiratory volume in 1 second
FVC	Forced expiratory volume
g	Gram
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOLD	Global Initiative for Chronic Obstructive Lung Disease
GWAS	Genome-wide association study
HBSS	Hank's balanced salt solution
HCU	Healthcare utilisation
HDAC	Histone deacetylase
HI	*H. influenzae*
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HRV	Human rhinovirus
IAC	Internal amplification control
ICS	Inhaled corticosteroids
IE	Infrequent exacerbator
IFN	Interferon
IL	Interleukin
IQR	Interquartile range
l	Litres
LABA	Long acting □2-agonists
LABC	Lower airway bacterial colonisation
LAMA	Long acting muscarinic antagonists

LPS	Lipopolysaccharide
LT	Leukotriene
MAC	MacConkey agar
MALDI-TOF	Matrix assisted laser desorption/ionization – time of flight
MARCO	Macrophage receptor with collagenous structure
MC	*M. catarrhalis*
MDM	Monocyte-derived macrophage
mg	Milligram
ml	Millilitres
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
MRC	Medical Research Council
MSD	Meso Scale Discovery
MTT	methylthiazoyldiphenyl-tetrazolium bromide
N	Number
NE	Neutrophil elastase
NF-□B	Nuclear factor-□B
ng	Nanogram
NO	Nitric oxide
Nrf2	Nuclear erythroid related factor 2
OD	Optical density
OUT	Operational taxonomic unit
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PEF	Peak expiratory flow
PEFR	Peak expiratory flow rate
pfu	Plaque-forming units
pg	Picogram
PPM	Potentially pathogenic microorganisms
PRRs	Pattern-recognition receptor families
PSB	Protected brush specimen
PYH	Pack year history
QC	Quantitative culture
qPCR	Quantitative polymerase chain reaction
RPMI	Roswell Park Memorial Institute medium
SD	Standard deviation
SGRQ	St George's Respiratory Questionnaire
SP	*S. pneumoniae*
TGF	Transforming growth factor
TLR	Toll-like receptors
TNF-□	Tumour necrosis factor - □
v/v	Volume by volume
w/v	Weight by volume
□	Change in
□	Wavelength
µg	Microgram
µl	microlitres

Chapter 1:

Introduction

1.1 Definition of chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is a debilitating condition, with a rising global incidence, and is now the third most common cause of death globally (Lozano et al., 2012). The Global Initiative for Chronic Obstructive Lung Disease (GOLD) defines COPD as:

'*a common preventable and treatable disease, characterised by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and comorbidities contribute to the overall severity in individual patients.*' (Vestbo et al., 2013)

The considerable morbidity and mortality associated with this disease is well recognised by respiratory physicians. However, despite increasing research and interest in COPD, available therapeutic interventions remain limited, and its importance frequently remains under-recognised in both public and government domains, and under-diagnosed in the wider medical community (van den Boom et al., 1998).

1.2 Diagnosis and clinical features of COPD

The diagnosis of COPD relies on the combination of three important features; a clinical history of chronic respiratory symptoms, history of exposure to noxious particles or gases and post-bronchodilator spirometry. However, COPD is a heterogeneous condition, with different sub-groups, or phenotypes, representing different presentations and underlying pathogenesis (Barker and Brightling, 2013). In addition, many of these core features of COPD may be complicated by co-morbid medical conditions, which both contribute to the under-diagnosis of COPD.

Characteristic symptoms of COPD include chronic and progressive dyspnoea, cough and/or sputum production. In some cases, cough and sputum production can precede the onset of airflow obstruction, whereas in others, significant airflow obstruction may occur with only dyspnoea (Vestbo et al., 2013). However, many symptoms may be under-reported by patients due to the belief that the symptoms are part of the normal ageing process, especially by smokers who are most at risk, or be attributed to other known medical conditions (Halpin and Miravitlles, 2006). The respiratory symptoms may contribute to decreased physical inactivity, so as to minimise the patients' discomfort, and this can lead to further deconditioning, weakness and worsening of the patients' symptom burden contributing to poorer quality of life (Hassett et al., 2014).

A reasonable history of exposure to noxious particles or gases should be evident to elicit the abnormal airway inflammatory responses characteristic of COPD. For the majority of patients diagnosed with COPD in developed countries, tobacco smoke is the most important risk factor associated with the disease, observed in up to 95% of cases, with the remaining cases due to secondary tobacco, occupational and environmental exposure (Barnes et al., 2003). However, the absence of a smoking history should not preclude a diagnosis of COPD. In developing countries, biomass fuels are frequently used in poorly ventilated areas, and biomass smoke is recognised as a risk factor for the development of COPD (Perez-Padilla et al., 1996; Ramirez-Venegas et al., 2006; Salvi and Barnes, 2009). In addition, as only approximately 50% of smokers develop COPD (Rennard and Vestbo, 2006), and there is evidence of increased risk of COPD in smokers who have a first-degree relative with the disease (Silverman et al., 1998), genetic factors clearly play a role. The best understood

genetic factor associated with COPD is □1-antitrypsin (AAT) deficiency, which occurs in 1-3% of patients with COPD, and should be considered in cases with a strong family history of early and severe airflow obstruction (Ioachimescu and Stoller, 2005). These genetic and genomic risk factors for the development of COPD are discussed further in section 1.4.

Post-bronchodilator forced expiratory volume in 1 second (FEV_1)/forced expiratory volume (FVC) of <70% provides confirmatory, objective evidence of abnormal airway obstruction, and the severity of airway obstruction is measured using FEV_1% predicted, based on gender, age and height (□□□□□□□□). However, spirometric readings alone may be inaccurate for the diagnosis of COPD. Not only are these measurements effort-dependent, but also the fixed FEV_1/FVC ratio may underestimate the disease in younger patients with true disease, but a normal ratio, and overestimate the disease in normal, older patients (Hardie et al., 2002; Roberts et al., 2006). In addition, comorbidities such as obesity and kyphosis may cause extra-thoracic restriction and therefore may normalise an otherwise obstructed airflow pattern.

GOLD Stage	Description	FEV_1
I	Mild	□80% predicted
II	Moderate	50%□FEV_1<80% predicted
III	Severe	30%□FEV_1<50% predicted
IV	Very Severe	<30% predicted or FEV_1<50% predicted plus chronic respiratory failure

Table 1.1. Grading of severity of airflow limitation in COPD, as defined by GOLD (Vestbo et al., 2013).

1.3 Epidemiology and burden of COPD

COPD mortality is increasing despite advances in the management of both stable and exacerbated disease, which is in contrast to a reduction in mortality rates observed in other chronic conditions such as cardiovascular disease and stroke (Jemal et al., 2005). There is considerable variability in the prevalence, morbidity and mortality from COPD across both different countries, and within different populations within the same country (Vestbo et al., 2013). In England, COPD is the fifth leading cause of death (Halpin, 2011) and the second largest cause of emergency admissions (National Institute for Health and Clinical Excellence (NICE), 2010). The high socioeconomic costs associated with COPD are attributable not only to the direct healthcare costs coming from healthcare provision and pharmacotherapy, but also from indirect costs related to the effects on the working age population (Fletcher et al., 2011), with an estimated 24 million working days lost in the UK resulting from costs related to social security and loss of productivity estimated at £600 million and £1.5 billion per year, respectively (Calverley and Sondhi, 1998); Halpin, 2006). Furthermore, the disease severity and associated comorbidities correlate with increasing socioeconomic costs (Fletcher et al., 2011).

COPD is one of few diseases whose global prevalence continues to rise (Mannino et al., 2006). The true prevalence rate of COPD is difficult to determine due to differing diagnostic criteria, methods and interpretation of spirometry (Diaz-Guzman and Mannino, 2014). The global prevalence of physiologically defined COPD in adults aged 40 years and over is reported at approximately 10% (Halbert et al., 2006). However, this figure is likely to be an under-estimate of the true prevalence, as an estimated 45-65% of patients with COPD do not seek medical intervention, and thus

obtain a formal diagnosis, despite having symptoms consistent with COPD. In addition, while historically COPD was considered as a disease primarily affecting males, it is clear that there has been increasing prevalence observed in women (Diaz-Guzman and Mannino, 2014; Halpin and Miravitlles, 2006), although again, the true prevalence is likely to be an under-estimate as the diagnoses is often less frequently considered in women than in men, despite having the same risk factors and symptomatology (Miravitlles et al., 2006).

1.4 Genetic and genomic risk factors for COPD

Although the major risk factors for the development of COPD are environmental in nature, (Barnes et al., 2003), it is evident that there is marked variability in susceptibility to these risks, suggesting that genetic factors also play a role (Postma et al., 2015). To date, AAT deficiency is the only established genetic cause of COPD following a Mendelain pattern of inheritance (Eriksson, 1965), although genome-wide association study (GWAS) has been used to identify other potential target genes that influence the development of COPD.

Severe AAT deficiency is a rare but well-recognised cause of COPD, found in 1-2% of cases. AAT deficiency was first described in 1963 when it was associated with severe, early-onset basal panacinar emphysema (Laurell and Eriksson, 1963), which was later attributed to mutations in the *SERPINA1* gene (Schroeder et al., 1985). The *SERPINA1* codes for the AAT protein, which is synthesized predominantly by the liver, but also by neutrophils, monocytes, macrophages and epithelial cells. In healthy individuals, adequate levels of AAT act to counter the effects serine proteases, including neutrophil elastase (NE) (DeMeo and Silverman, 2004) and proteinase 3

(PR3) (Sinden and Stockley, 2013). As a result of AAT deficiency, an imbalance occurs between the protease-antiprotease proteins within the lung, especially during episodes of increased inflammation. This leads to destruction of the lung parenchyma and the development of emphysema (Abboud and Vimalanathan, 2008; Kao et al., 1988). Depending on the presence of different alleles, M, S and Z being the most common, AAT protein variants exist which alter the risk of development of emphysema. However, despite AAT deficiency being the only established genetic cause of COPD, it is clear that genetic modifiers also exist which lead to the variability seen in the development and clinical characteristics of patients with AAT-associated COPD.

GWAS studies have discovered potential genes and pathways that are associated with susceptibility to both COPD and to different COPD phenotypes, as well as with lung function within the general population, which may also confer susceptibility to COPD (Kim and Lee, 2015). The most well recognized candidate genes identified from several large cohort studies are the *CHRNA 3/5*, *IREB2*, *HHIP*, *FAM13A* and *AGER* (Castaldi et al., 2011; Cho et al., 2010; Pillai et al., 2009; Wilk et al., 2012). While these genes have been repeatedly isolated in GWAS studies from different populations, their function and the mechanism by which they impart susceptibility to COPD remains unknown, and they are likely to represent only a small proportion of genes which explain the variability in COPD phenotypes. Thus further research is needed before these genes may be used as future targets either as biomarkers of disease activity or targets for future treatment.

1.5 Pathophysiology of COPD

The hallmark of COPD is an abnormal inflammatory response to inhaled particles and gases, of which chronic tobacco smoke exposure is the most common aetiological factor (MacNee, 2005). This response is the result of a complex series of interactions between the immune cells of the airways and inflammatory mediators, of which recruitment of leukocytes into the airways and parenchyma plays a significant role (Barnes, 2008; Holloway and Donnelly, 2013) (□□□□□□□□). More recently, protease-antiprotease imbalance and oxidative stress have also been proposed to play a part in the pathogenesis of COPD (Barnes, 2008; Barnes et al., 2003; Fischer et al., 2015; Kukkonen et al., 2013; MacNee, 2005).

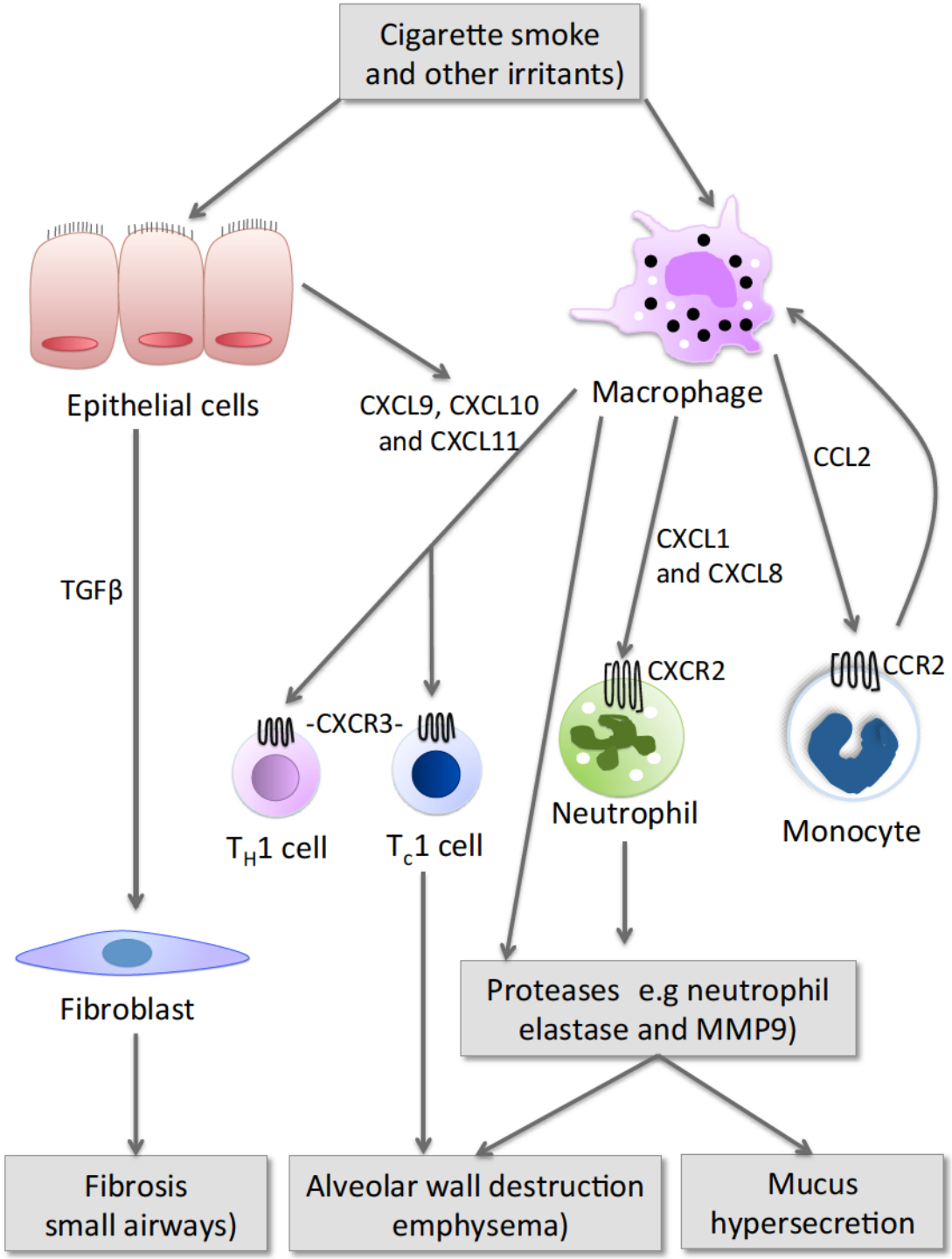


Figure 1.1. Overview of the immune cells involved in the pathogenesis of COPD. Adapted from (Barnes, 2008).

Three distinct, but overlapping pathophysiological processes have been described; emphysema, chronic bronchitis and bronchiolitis. While these processes often co-exist, the proportions of each present within an individual, contribute to the different clinical phenotypes and thus inter-patient variability observed in COPD.

Emphysema is a histological diagnosis, and results from the destruction of the normal lung parenchymal architecture by protease-antiprotease imbalance, (Abboud and Vimalanathan, 2008; Barnes et al., 2003). Proteolytic enzymes, including neutrophil elastase (NE) and matrix metalloproteinases (MMPs), contribute towards the destruction of lung elastin and matrix macromolecules. This increases airspace inflammation, with further infiltration and activation of neutrophils and macrophages (Stockley, 1999; Tetley, 2002), which release more proteolytic enzymes worsening the damage to parenchymal elastic tissue, and thus reducing lung compliance and increasing hyperinflation of the lung. There is a weak relationship between the degree of emphysema and smoking exposure, with only 40% of smokers developing substantial emphysema. Indeed emphysema is observed in some individuals with normal lung function (Hogg, 2004). Furthermore, there is only a weak relationship between the degree of emphysema and lung function, with a reduction in gas transfer often preceding any decrease in FEV_1, and only weak correlations observed between the degree of emphysema, as seen on computerized tomography (CT) scans and lung function testing (Gould et al., 1991; Nakano et al., 2000).

Chronic bronchitis is associated with accumulation of mucus within the airways due to increased production and secretion, as well as impaired mucociliary clearance and increased permeability of the airspace epithelium. This reflects the underlying

inflammatory process, and as a consequence there is remodelling of the airway epithelium, with hypertrophy and hyperplasia of mucus glands (Innes et al., 2006; Saetta et al., 2000). Unlike in healthy airways, luminal mucus accumulation occurs in both the large and small airways, and its volume relates to the severity of the underlying airflow obstruction (Hogg et al., 2004). Data from the Copenhagen Heart Study demonstrated that mucus hypersecretion was associated with a more rapid decline in FEV_1 in male but not female patients with COPD, after adjusting for age, height, weight changes and smoking and was also associated with a higher risk of hospitalizations due to COPD (Vestbo et al., 1996). In addition, chronic mucus production has been shown to be associated with higher loads of airway bacteria, which can further increase airway inflammation (Hill et al., 2000), worsen respiratory symptoms and increase the risk of acute exacerbations in COPD (Kim et al., 2011), the latter further accelerating the decline in FEV_1 (Donaldson et al., 2002). However, not all studies have shown similar effects on exacerbations, with both the ECLIPSE and PLATINO studies demonstrating no significant difference in exacerbation rates in patients with and without mucus hypersecretion (Agusti et al., 2010; de Oca et al., 2012), although these disparities in outcomes may be related to the different study designs and definitions used to define chronic mucus secretion.

Small airway bronchiolitis is found in the airways <2mm in diameter (MacNee, 2005) and is characterised by not only the underlying inflammation, but the subsequent thickening, fibrosis and remodelling of the airway wall. This leads to increased small airways resistance of up to 4-40 times higher than normal in patients with COPD (Hogg et al., 1968), and progression of COPD severity has been associated with increases in the volume of tissue within the small airways, accumulation of

inflammatory mucus exudates within the airway lumen and increased percentage of airways containing inflammatory cells (Hogg et al., 2004). A further consequence is that peribronchiolar fibrosis may be observed, contributing to the fixed airway obstruction seen in COPD (MacNee, 2005). The mechanisms by which peribronchiolar fibrosis occurs are not well understood, but may represent the host's mechanisms for repairing the damage caused by chronic inflammation via transforming growth factor (TGF)-□ induction of connective tissue growth factor (CTGF), which leads to the deposition of collagen within the small airways (Barnes et al., 2003).

1.5.1 Airway inflammation

The abnormal underlying airway inflammation observed in COPD patients, has been demonstrated in parenchymal and bronchial biopsies, sputum and post-mortem samples (Hogg et al., 2004; Keatings et al., 1996; O'Shaughnessy et al., 1997), and several inflammatory mediators and cells, including neutrophils, macrophages and T lymphocytes, play an important role in the pathogenesis of COPD (Barnes, 2008; Holloway and Donnelly, 2013; O'Donnell et al., 2006; Sethi et al., 2012).

Cigarette smoking exposes the lung to cyclical levels of a complex aerosol mixture of toxic gases and particles, damaging mucociliary clearance and resulting in activation of both innate and adaptive immune mechanisms, which drives inflammation. A similar pattern of inflammation is seen between patients with COPD and in healthy smokers, although in patients with COPD, this inflammatory process is amplified and persistent even if exposure to the noxious gases and particles is removed, suggesting a self-perpetuating mechanism (Barnes et al., 2003; Hogg, 2004; MacNee, 2005). This

amplification of inflammation may be in part driven by transcription factor nuclear factor-□B (NF-□B) (Barnes, 2008). NF-□B plays a critical role in the regulation of numerous pro-inflammatory chemokines and cytokines, and in healthy individuals, these pathways are tightly regulated to control airway inflammation. However, in COPD there is increased activation of NF-□B in airway epithelial cells and macrophages, resulting in the abundance of pro-inflammatory chemokines and cytokines observed in COPD, including CXC-chemokine ligand 8 (CXCL8), tumour necrosis factor (TNF)-□ and nitric oxide (NO) (Barnes, 2008; Caramori et al., 2003; Pasparakis, 2012).

A single puff of cigarette smoke has been quantified to contain between 4000-7000 constituents and 10^{15}-10^{17} free radicals (Church and Pryor, 1985; Pryor, 1987; Pryor, 1997), increasing the number of free radicals within the respiratory tract directly and/or by stimulating inflammatory cells to produce further free radicals (Cano et al., 2010). As a result, smoking shifts the balance between oxidants and antioxidants resulting in increased oxidative stress both within the lungs and also systemically (Fischer et al., 2015). However, smoking cessation does not eliminate the increased oxidative stress (Louhelainen et al., 2009). Furthermore, the increased numbers of neutrophils and macrophages within the lungs of patients with COPD can also generate further oxidative stress, and recruitment of more immune cells to the lung. Due to the accumulation of iron particulates from tar in macrophages from smokers, disrupted iron homeostasis results in a higher oxidant burden (Wesselius et al., 1994). There is increasing evidence that oxidative stress plays a role in the pathogenesis of COPD. Lung matrix components including elastin and collagen can be directly damaged by cigarette smoke (Cantin and Crystal, 1985), and increased permeability

of the airspace epithelium is seen with increased concentrations of oxidants (Morrison et al., 1999). Additionally, the increased oxidative stress observed in COPD up-regulates the *MUC5AC* gene expression, resulting in increased mucus production.

1.5.2 Key inflammatory cells

1.5.2.1 Neutrophils

Neutrophils are produced from the bone marrow and are the predominant granulocyte in the circulation (Chaudhuri and Sabroe, 2008). They form the front line of defence at sites of infection, rapidly recruited to the site of injury by chemokines, including CXCL8 and leukotriene (LT)-B_4. However, they are usually short-lived and transient cells, and neutrophilic inflammation usually resolves once the cells become apoptotic and are subsequently cleared by macrophages (Luo and Loison, 2008; Rossi et al., 2007). Neutrophils have the potential to release potent mediators in response to stimulation by bacterial products or activation by other cytokines, such as TNF□. In COPD, there is consistent evidence that the underlying chronic inflammation is neutrophilic in nature (Fuke et al., 2004). Evidence for this comes from sputum and bronchoscopic studies which report that increased airway neutrophil chemokines (Hill et al., 2000) and increasing sputum neutrophilia and their associated products, correlate with disease severity (Pilette et al., 2007; Singh et al., 2010; Thompson et al., 1989), disease progression (Donaldson et al., 2005; O'Donnell et al., 2006), and to the development of emphysema (Parr et al., 2006; Stockley, 1999).

Further contributions of neutrophils to the pathogenesis of COPD comes from work demonstrating that migrating neutrophils from COPD patients, irrespective of disease severity, demonstrate faster but less accurate chemotaxis, which may result in greater

neutrophil elastase (NE) release and delayed pathogen clearance (Sapey et al., 2011). Phagocytosis in neutrophils is less well studied than in macrophages, but has also been shown to be impaired to bacterial pathogens in COPD (Prieto et al., 2001), and this may represent a common defect seen in phagocytic cells of COPD patients.

1.5.2.2 T Lymphocytes

T cells, which are part of the adaptive immune system, have also been shown to play a role in COPD pathogenesis, although they have been studied less extensively. $CD8^+$ T Lymphocytes are the predominant lymphocyte found in COPD. One of their usual functions is to minimise viral infections, by either cytolysis of infected cells or apoptosis (Lowin et al., 1994). $CD8^+$ T Lymphocytes have been postulated to contribute to the pathogenesis of emphysema, with the release of lytic substances such as granzyme, which causes destruction of the lung parenchyma (Garcia-Sanz et al., 1988) and has been shown to be related to disease severity (Freeman et al., 2010), by inducing structural cell apoptosis (Majo et al., 2001). In addition, studies in the HIV-infected population, have demonstrated differences in the number and function of $CD4^+$ T Lymphocytes from the lung but not the blood in patients with and without co-existing COPD, which correlated with FEV_1, suggesting these cells may play a significant role in HIV-associated COPD (Popescu et al., 2014). Kalathil and colleagues demonstrated that a subset of $CD4^+$ T Lymphocytes from peripheral blood mononuclear cells are suppressive and may contribute to the impaired immune response to *H. influenzae* (Kalathil et al., 2014). Therefore, it appears that deranged T Lymphocyte function may contribute to both HIV-associated and HIV-independent COPD, although further studies are needed.

1.5.2.3 Macrophages

Alveolar macrophages are the most prominent and highly adapted phagocyte within the lung, and play a vital role in the clearance of inhaled particles and bacteria from the respiratory tract. They account for up to 95% of the cells identified in broncho-alveolar lavage (BAL) samples (Gordon and Read, 2002), and alveolar macrophages can have a long life span of up to 2 years (Marques et al., 1997). Macrophages were originally believed to exist as two different phenotypes (Gordon and Taylor, 2005). Pro-inflammatory, or classically activated, M1 macrophages are produced during cell-mediated immune responses. These M1 macrophages are activated by bacterial products such as lipopolysaccharide (LPS) and the combination of inflammatory cytokines including TNF□ and interferon-□ (IFN-□), but are characteristically less phagocytic in nature. Alternatively activated, M2 macrophages are driven by interleukin (IL)-4 and IL-13, or IL-10, and are more phagocytic and play a role in immunoregulation and tissue remodelling. However, it is increasingly recognised that there is a spectrum of macrophage phenotypes, rather than solely these two distinct populations, and the phenotype may change depending on the local environment (Mosser and Edwards, 2008)□(Porcheray et al., 2005).□However, the macrophage phenotype predominantly found in healthy lung is still open to debate, with conflicting findings in studies (Pechkovsky et al., 2010; Shaykhiev et al., 2009).

In COPD patients, macrophage numbers are increased 10 to 20-fold, either due to increased recruitment of their precursor monocyte, or due to increased longevity of resident macrophages (Barnes, 2004a). Alveolar macrophages are derived from circulating monocytes and migrate to the lung in response to chemoattractants such as the chemokine (C-C motif) ligand 2 (CCL2) and CXCL1 (Traves et al., 2004). There

is increasing evidence that due to the diverse responses of alveolar macrophages, they play a pivotal role in orchestrating the underlying inflammatory response seen in COPD (Barnes et al., 2003) (Barnes, 2008) (□□□□□□□□□).

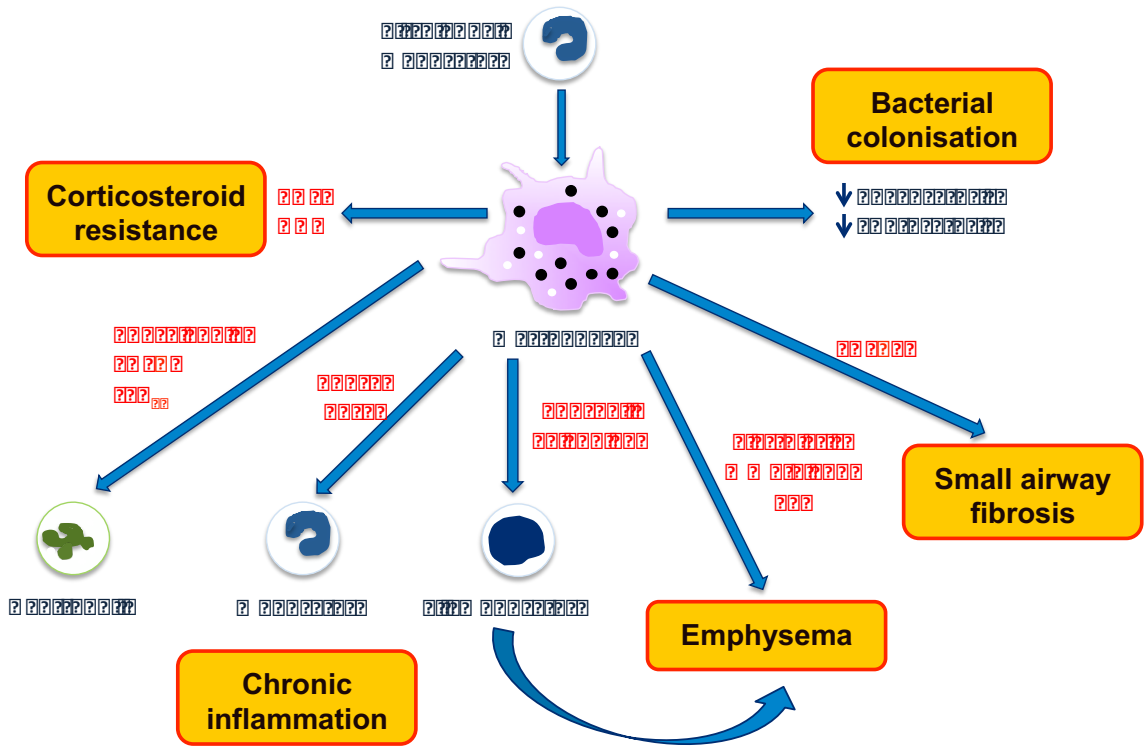


Figure 1.2. The role of macrophages in the pathogenesis of COPD. Adapted from (Barnes et al., 2003).

□
□
Cigarette smoke has been shown to activate macrophages, releasing chemokines to further recruit neutrophils and T lymphocytes into the lung (Barnes et al., 2003). Both *in vitro* studies of inflammatory mediator release from macrophages and *in vivo* studies in COPD patients have demonstrated higher levels of inflammatory cytokines (Culpitt et al., 2003)□(Keatings et al., 1996), suggestive that M1-like alveolar macrophages may contribute to the underlying chronic inflammation seen in COPD.

In addition, macrophages synthesise and release MMPs in response to their cellular environment, which is influenced by cytokines, endotoxin, phagocytosis and growth factors (Barnes et al., 2003; Tetley, 2002). The predominant MMP is MMP-9, which has been found at sites of macrophage accumulation in emphysematous lungs (Ohnishi et al., 1998; Russell et al., 2002). These secreted MMPs need to be activated, and once taken place, the MMPs can undergo auto-activation and also inactivate inhibitors of other proteinases, contributing to the proteinase/anti-proteinase imbalance and subsequent damage to parenchymal elastic tissue.

In addition, macrophages contribute to the corticosteroids resistance observed in COPD. Acetylation of core histones regulates gene expression, and thus enabling transcription to initiate, but co-activator molecules are also needed. Expression of inflammatory genes is regulated by the acetylation of histone 4 (Barnes et al., 2005), and in COPD, there is increased acetylation of inflammatory genes such as CXCL8, which is regulated by NF-□B. This histone acetylation is reversed by one of 11 histone deacetylases (HDACs) isoenzymes (Thiagalingam et al., 2003). In alveolar macrophages from COPD patients, HDAC activity is reduced, thought to be a consequence of increased oxidative stress (Barnes, 2004b), and as a result there is increased expression of inflammatory genes.

Numerous studies have also demonstrated defective bacterial phagocytosis in patients with COPD (Vecchiarelli et al., 1991) (Ferrara et al., 1996) and *E. Coli* (Berenson et al., 2006a; Berenson et al., 2006b; Prieto et al., 2001; Taylor et al., 2010) (Taylor et al., 2010). Defective phagocytosis may contribute to the persistence of airway

pathogens in COPD, and hence play a role in the pathogenesis of COPD, and this is discussed in detail in section 1.9.

1.5.3 Systemic inflammation

In addition to the pulmonary inflammatory consequences of COPD, it is increasingly recognised that COPD has several systemic features and effects on comorbidities, which have been linked to increased systemic inflammation (Barnes, 2010; Sinden and Stockley, 2010) (Boschetto et al., 2012; Thomsen et al., 2012) (□□□□□□□□□). Studies have demonstrated elevated levels of CRP, leucocyte count, fibrinogen, serum IL-6 and TNF□ in stable COPD patients, compared to 'healthy' smokers or non-smokers (Agusti, 2007; Fabbri and Rabe, 2007; Gan et al., 2004). However, the majority of these studies were cross-sectional in nature, small and did not account for confounders, such as smoking status or the use of anti-inflammatory medications. More recent longitudinal studies have shown that although there is considerable intra- and inter-patient variability in systemic inflammation (Agusti et al., 2012), increases in systemic inflammation are associated with FEV_1 decline (Donaldson et al., 2005) and persistently elevated levels were associated with higher all-cause mortality (Agusti et al., 2012) and exacerbation frequency (Agusti et al., 2012; Fu et al., 2015), highlighting the effects on clinically important outcomes. Furthermore, systemic inflammation has been shown to further increase during times of acute exacerbation (Gan et al., 2004; Hurst et al., 2006b), which may worsen comorbidities, particularly cardiovascular disease (Donaldson et al., 2010; Patel et al., 2012), resulting in poorer outcomes in these patients.

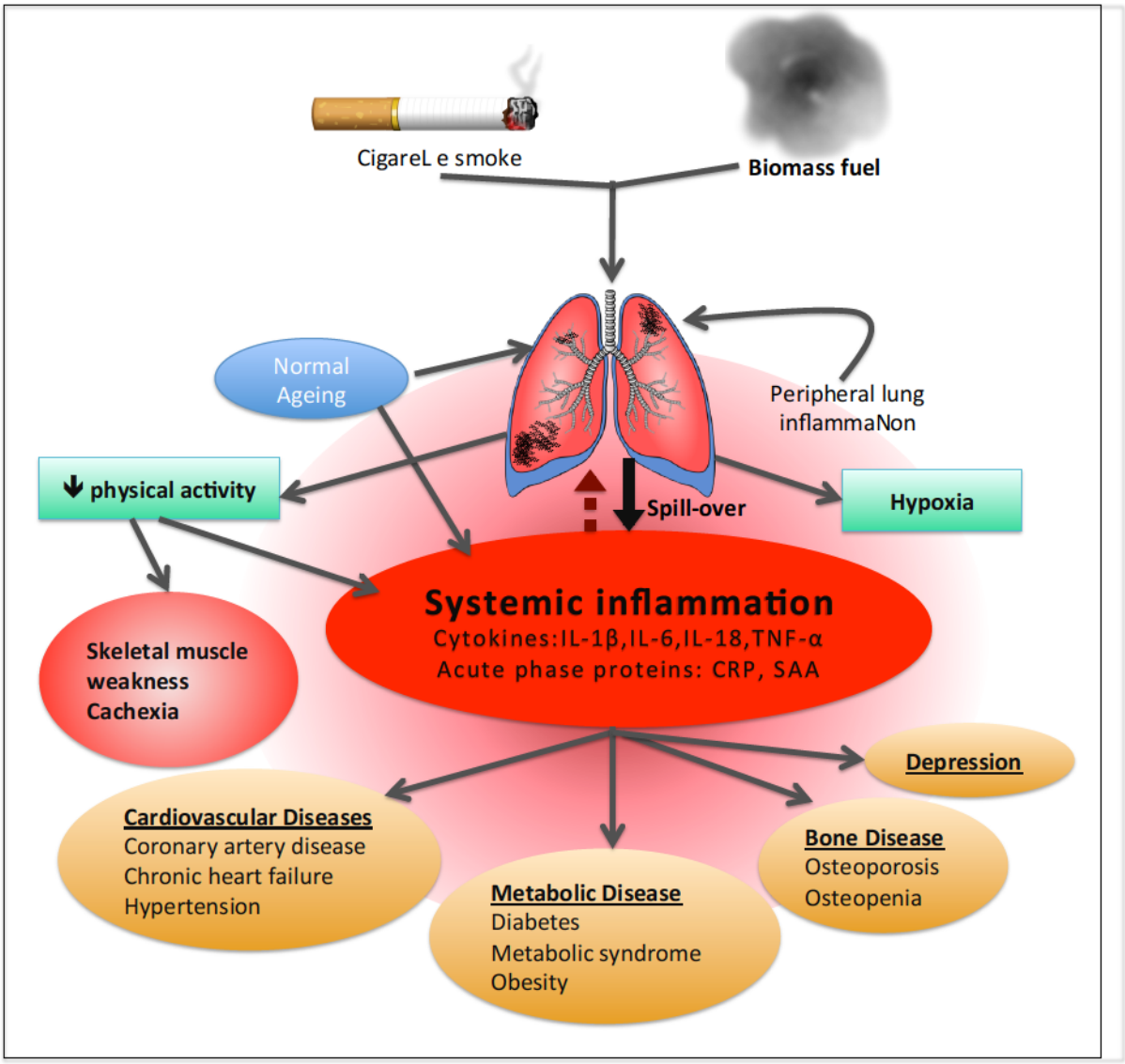


Figure 1.3. The effects of systemic inflammation in COPD and the link with co-morbidities. Adapted from (Boschetto et al., 2012).

It has been hypothesised that systemic inflammation results from an 'over-spill' of airway inflammatory cytokines into the systemic circulation, as the cytokines that are increased within the circulation are also elevated in airway samples (Barnes, 2010; Sinden and Stockley, 2010). Although there is evidence of direct protein movement between the lung and systemic circulation in animal studies (Vogelmeier et al., 1990), there has been a clear lack of correlation between airway and systemic inflammatory

markers in human studies (Sapey et al., 2009; Vernooy et al., 2002). Furthermore, these studies were limited by small numbers and the inflammatory markers measured are common to other inflammatory conditions, and thus it is difficult to verify over-spill as the likely mechanism involved, although it is likely that other mechanisms would contribute to this effect.

TNF is thought to play a central role in both airway and systemic inflammation (Churg et al., 2004; Sinden and Stockley, 2010), particularly at exacerbation (Aaron et al., 2001). Not only are its levels elevated in in both stable and exacerbated disease (Karadag et al., 2008), it is also associated with disease severity and in COPD patients with weight loss (Di Francia et al., 1994; von Haehling et al., 2009). TNF may be derived from circulating monocytes and can induce cell apoptosis in several cell systems (Agusti et al., 2003) in addition to inducing other pro-inflammatory cytokines, up-regulating adhesion molecules and facilitating migration of leucocytes via CXCL8 induction (Berkow et al., 1987). Unfortunately, to date TNF antagonists have failed to show clinical efficacy in randomised controlled studies (Aaron et al., 2013), and this is probably due to the fact that COPD is a highly complex inflammatory disorder, with numerous cytokines playing a role, and thus the inhibition of one of these systemic cytokines will unlikely lead to a significant clinical effect.

1.6 Host defence of the lung: the innate immune system and its failure in COPD

The body's natural dense mechanisms to protect the host from invading organisms rely on a co-ordinated response by the both the innate and adaptive immune systems, and failure of these mechanisms contribute to infections and disease.

Innate immunity is a multifaceted system whose primary role is antimicrobial defences, through barrier and humoral immunity and phagocytic inflammatory cells (Chaudhuri and Sabroe, 2008; Martin and Frevert, 2005). This system maintains the homeostasis of the lung environment, preventing the inflammatory and infective consequences of inhaled environmental particles and pathogens. The upward propulsive beating of the mucociliary system continually clears trapped particles and pathogens in the fluid lining the lung towards the digestive tract. Particles <1μm, the size of bacterial and viral particles, are carried to the alveolar surface where alveolar fluid components and macrophages act to minimise their damage. The fluid lining the airways and alveoli contains many endogenous antimicrobial polypeptides (Sethi and Murphy, 2008). These include such as collectins, which bind to the bacterial surface protein lipopolysaccharide (LPS), defensins and cathelicidins, both which bind to and kill bacteria, lactoferrin, which prevents iron from being used by bacterial metabolism and lysozyme, which is lytic to bacterial membranes (Chaudhuri and Sabroe, 2008; Martin and Frevert, 2005). Furthermore, humoral factors such as complement and antibodies can be delivered through increased capillary permeability and the recruitment of specific inflammatory cells (Chaudhuri and Sabroe, 2008).

In COPD, there are several disruptions of these barrier functions of the innate immune system, including the universal, albeit variable impairment of mucociliary clearance in moderate-heavy smokers, reduction of antimicrobial polypeptides within the lining fluid of the lungs and alveoli (Sethi and Murphy, 2008). As a consequence of failure of barrier immunity, a pathogen or inflammatory stimulus must be recognised by the specialist inflammatory cells to allow subsequent clearance of the insult (Chaudhuri and Sabroe, 2008). These cellular responses involve both the innate and adaptive

immune systems, with the former being non-specific and activated on host exposure to the pathogen, whereas the latter requires days to initiate the pathways to eliminate the pathogen but enables the generation of immunological memory.

To initiate the innate cellular responses, host pattern-recognition receptor (PRRs) families, of which the Toll-like receptors (TLRs) are the most extensively studied. These PRRs must recognise a wide range of pathogen molecules, broadly defined as pathogen-associated molecular patterns (PAMPs). This activates downstream signalling pathways, including those regulated by NF-□B, activating an inflammatory response, which as previously discussed in 1.5.1, is amplified in COPD. Furthermore, alveolar macrophages play a key role in the homeostasis of the lung environment, phagocytosing inhaled particles and pathogens. In patients with COPD, alveolar macrophages show impaired phagocytosis, contributing to the persistence if airway pathogens and particles and thus inflammation. Defective phagocytosis in COPD is discussed in detail in section 1.9.

1.7 Bacteria in COPD

It is well recognised that bacterial infections can cause a range of both upper and lower respiratory infections in healthy individuals, which usually resolve over a relatively short period of time without significant lasting consequence. However, in COPD, the role of bacteria has been more controversial.

The role of bacteria in the pathogenesis and natural history of COPD was first described in 1950s and 1960s by British investigators, who proposed that recurrent bronchial infections, resulting in chronic cough and sputum production i.e. chronic

bronchitis, were the cause of some smokers to develop airway and alveolar damage, leading to airflow obstruction (Anthonisen, 2004; Fletcher, 1959). However, their role was disputed following a seminal review which found insufficient evidence to support the role of bacterial infections in chronic bronchitis (Tager and Speizer, 1975), and classical work by Fletcher and colleagues, which although found that both COPD and chronic bronchitis were associated with smoking, they were not related (Fletcher, 1976).

Over the last two decades our understanding of COPD mechanisms has considerably improved, and more recent studies have supported the British Hypothesis. The Copenhagen City Heart Study Group demonstrated a significant association between chronic mucus hypersecretion with both FEV_1 decline and COPD morbidity, as measured by an increased risk of subsequent hospitalisation from COPD (Vestbo et al., 1996). Analysis of the Lung Health Study further supported the association of chronic bronchitis with increased lower respiratory tract infections and FEV_1 decline in mild COPD patients (Kanner et al., 2001). More recently, the COPDgene Study found that in COPD patients with symptoms of chronic bronchitis had worse respiratory symptoms and higher risk of exacerbations (Kim et al., 2011). These findings, together with studies investigating the microbiology of airway samples from stable patients with chronic bronchitis (Monso et al., 1999) (Hill et al., 2000), further strengthen the argument that bacteria play a significant role in the pathogenesis of COPD.

The bacteria most commonly isolated in COPD, irrespective of patient state, are *Haemophilus influenzae* (*H. influenzae*), *Streptococcus pneumoniae* (*S. pneumoniae*),

and *Moraxella catarrhalis* (*M. catarrhalis*) (Sethi and Murphy, 2008) (□□□□□□□□), referred to as typical airway bacteria, and are exclusively human pathogens. In patients with worse disease severity and repeated hospitalisations, *Pseudomonas aeruginosa* is also an important bacterial pathogen. To understand the role these typical airway bacteria play in COPD, it is important to understand their mechanisms of virulence.

Bacteria	Role in exacerbations	Role in stable disease
H. influenzae	20-30%	Major role
S. pneumoniae	10-15%	Minor role
M. catarrhalis	10-15%	Minor role
Pseudomonas aeruginosa	5-10%, increased in worsening disease severity	Role in worsening disease severity
Chlamydophila pneumoniae	3-5%	Unlikely
Mycoplasma pneumoniae	1-2%	Unlikely

Table 1.2. Microbial pathogens in stable and exacerbated COPD. Adapted from (Sethi and Murphy, 2008).

□

1.7.1 *Haemophilus influenzae*

H. influenzae is a fastidious, Gram-negative coccobacillus, which is a member of the Pasteurellaceae family, that can grow either aerobically or anaerobically (King, 2012), and strains are sub-divided into typeable or non-typeable strains on the presence or absence of a polysaccharide capsule respectively. Typeable strains predominantly cause systemic infections, such as meningitis, whereas non-typeable strains are primarily respiratory mucosal pathogens (King, 2012).

H. influenzae colonises the nasopharynx early in childhood (Fontanals et al., 2000), and this provides a source for on-going or recurrent lower respiratory tract disease

which occurs through spread from the nasopharynx (King, 2012). Unlike in the nasopharynx, once within the lower respiratory tract, *H. influenzae* has a number of specific virulence mechanisms to evade the host immune response inducing a subsequent inflammatory response via the NF-□B intracellular signalling pathways (Finney et al., 2014; King and Sharma, 2015). Phase variation describes changes in *H. influenzae* cell structures to minimise antibody-mediated host responses (van Ham et al., 1993), and some surface antigens can undergo permanent changes in their amino acid sequences, akin to antigenic drift seen in influenzae viruses (Duim et al., 1996). *H. influenzae* can also form biofilms, which are microbial communities within a matrix of secreted polymeric compounds, which offer protection against recognition by the host immune system. *H. influenzae* may evade mucosal immunity by adhering to the respiratory mucosa (King, 2012), and in some studies *H. influenzae* has been shown to reside between the epithelial and subepithelial tissues (Read et al., 1991). Furthermore, some strains of *H. influenzae* reduce both complement and IgA host mechanisms to clear the bacteria from the lower airways (Hallstrom et al., 2008; Kilian et al., 1988). In COPD, as previously discussed, structural and functional changes within the airway alter the host immunity and thus contribute to respiratory disease pathogenesis (Finney et al., 2014).

1.7.2 *Streptococcus pneumoniae*

S. pneumoniae is a Gram-positive, □-haemolytic diplococcus, and is a facultative anaerobe. It is the leading cause of bacterial pneumonia as well as causing otitis media and meningitis (Mitchell and Mitchell, 2010). As with *H. influenzae*, *S. pneumoniae* produces several difference virulence factors, which influence the development of disease (Mitchell and Mitchell, 2010; Sethi and Murphy, 2001). The

most important virulence factor is its polysaccharide capsule. This prevents immunoglobulins and complement from binding to the cell surface and inhibiting subsequent phagocytosis by leucocytes (Musher, 1992), prevents mechanical removal by mucus (Nelson et al., 2007) and also limits autolysis and exposure to antibiotics (van der Poll and Opal, 2009). *S. pneumoniae* also releases the toxin pneumolysin, which contributes to a pro-inflammatory response by interacting with the TLR-4 receptor on inflammatory cells and this toxin presence is associated with sepsis (Malley et al., 2003). Surface pili present on some *S. pneumoniae* strains mediate adherence to epithelial cells (Bagnoli et al., 2008) and stimulate pro-inflammatory cytokines (Barocchi et al., 2006). Other important virulence factors comprise of biofilm formation, surface proteins, including pneumococcal surface protein A, which interferes with both complement and lactoferrin binding to prevent subsequent bacterial lysis (Hammerschmidt et al., 1999; Ren et al., 2003) and genetic polymorphisms for surface proteins and pili.

1.7.3 *Moraxella catarrhalis*

M. catarrhalis is a Gram-negative, aerobic diplococcus, and is a common cause of otitis media and respiratory tract infections (Murphy and Parameswaran, 2009). Similarly to both *H. influenzae* and *S. pneumoniae*, *M. catarrhalis* has a number of virulence factors contributing to infection and disease. An important feature of *M. catarrhalis* is its ability to adhere to epithelial cells via adhesion molecules, and intracellular invasion in some cell types, including bronchial and small airway epithelial cells and type 2 alveolar cells (Slevogt et al., 2007). Sero-resistant strains, which form a distinct lineage, are more strongly associated with virulence (Wirth et al., 2007). Although *M. catarrhalis* is also able to form biofilms, this appears to be

more associated with otitis media rather than respiratory tract infection (Pearson et al., 2006).

1.8 Airway bacteria in stable COPD: Lower airway bacterial colonisation

Much of the current understanding of the role bacteria play in the pathogenesis of COPD is based on studies using classical culture methods for diagnostic microbiology and the previously held belief that the tracheobronchial tree and lung parenchyma are sterile in healthy, non-smoking individuals (Lees and McNaught, 1959). However, in patients with COPD, potentially pathogenic bacteria are frequently cultured from bronchial specimens during periods of stability (Sethi and Murphy, 2008).

Early studies identified these pathogens in 18-50% of stable patients with COPD, and this prevalence increases with worsening severity of airflow obstruction (Cabello et al., 1997; Monso et al., 1999; Zalacain et al., 1999). Lower airway bacterial colonisation (LABC) was the term used to describe the detection of bacteria in the stable state, as the patients had been free from symptoms of an exacerbation or antibiotic therapy for a minimum of 4 weeks prior to sampling. However, this definition does not take into account the different cut-off values used to define a positive culture, which in most cases is arbitrary. Thus the rates of positive cultures will vary depending on both the sampling technique used and the cut-off value used to define a positive culture (□□□□□□□□).

Author	Bronchial sample	Bacterial load used to define LABC (cfu/ml)	Pre-defined bacterial load used in study and patient details	Frequency of colonisation (%)
Cabello *et al.*	PSB	$\square 10^2$	Pre-defined	PSB: 83
	BAL	$\square 10^3$	n=18 COPD	BAL: 12
Zalacain *et al.*	PSB	$\square 10^3$	Pre-defined n=88 COPD n=20 controls	COPD: 31 Controls: 0
Monso *et al.*	PSB	$\square 10^3$	Pre-defined n=41 COPD	Overall: 22 (included non-pathogenic bacteria)
Soler *et al.*	PSB	$\square 10^2$	Pre-defined PSB: n=52 COPD, n=12 smoking control	PSB: 34 in all groups
	BAL	$\square 10^3$	BAL: n=24 COPD, n=9 smoking controls	BAL: 27 in all groups
Patel *et al.*	Induced sputum	Mean value used	No pre-defined values n=29 COPD	Overall: 51
Sethi *et al.*	BAL	$\square 10^2$	Pre-defined n=26 COPD, n=20 ex-smokers and n=15 non-smokers	COPD: 35, Ex-smokers: 0 Non-smokers: 7

Table 1.3. Bronchial samples, bacterial thresholds and patient groups used in studies to define lower airway bacterial colonisation (LABC). PSB: protected specimen brush. BAL: broncho-alveolar lavage.

1.8.1 Inflammatory consequences of LABC

In these early studies (Cabello et al., 1997; Monso et al., 1999; Zalacain et al., 1999), inflammatory markers were not measured, and subsequent studies have shown that the presence of bacteria in patients with stable COPD is not an innocuous event, but is associated with increased inflammation, which may contribute to the chronic inflammatory process characteristic of COPD.

Sputum studies in stable patients with chronic bronchitis and COPD have demonstrated that in samples with bacteria detected, significantly higher airway inflammation is observed than in those patients without evidence of bacteria, and this inflammatory response is related to the load of the pathogen (Banerjee et al., 2004; Hill et al., 2000; Marin et al., 2010; Patel et al., 2002). Greater airway inflammation has also been noted in studies using bronchoscopic samples, either broncho-alveolar lavage (BAL), or protected specimen brush (PSB), to define bacterial colonisation status (Rosell et al., 2005; Sethi et al., 2006; Soler et al., 1999). Importantly, the study by Hill and colleagues suggested that the increase in airway inflammation over the underlying chronic inflammatory process only occurred at loads of between 10^6 to 10^7 cfu/ml (Hill et al., 2000). This is suggestive of a threshold between the ability of the host defences to maintain the homeostasis of the lung environment and to minimise the underlying inflammation, which is crossed with higher pathogen loads. Airway inflammatory responses also appear to be dependent on the different bacterial species, with *Pseudomonas aeruginosa* associated with a greater inflammatory response than *H. influenzae*, which in turn is higher than with *M. catarrhalis* (Hill et al., 2000; Marin et al., 2012).

Neutrophilia has been shown to be a hallmark of the inflammatory pathogenesis of COPD (Fuke et al., 2004). Recruitment of neutrophils to the airway with resultant purulent sputum has been shown to be a key feature of active bronchial infections in bronchiectasis (Hill et al., 1986) as well as in patients with chronic bronchitis (Stockley et al., 2001b). The purulent sputum colour reflects the underlying airway inflammation due to the green heme-containing myeloperoxidase (MPO), which is contained within the azurophil granules of neutrophils and pro-inflammatory

monocytes (Kargi et al., 1990; Owen et al., 1994). Sputum colour has been shown to be a non-invasive marker of LABC (Miravitlles et al., 2010), and thus could be used in future clinical studies to monitor LABC, particularly in interventional studies.

While the majority of studies of LABC in COPD have focused on airway inflammation, some reports have demonstrated an increased systemic inflammatory response (Banerjee et al., 2004; Hurst et al., 2005b; Marin et al., 2012). This is believed to be a consequence of a 'spill-over' of airway inflammatory mediators (Barnes, 2010), although no clear correlations between airway and systemic inflammatory markers, or between bacterial loads and systemic inflammation, have been observed. Systemic inflammatory markers may be difficult to measure, as their concentrations in the circulation are often below the lower limit of detection of commercially available enzyme-linked immunosorbent assay (ELISA) kits, and high performance electrochemiluminescence immunoassays for Meso Scale Discovery (MSD) are not readily available due to costs. Acute-phase proteins, such as C-reactive protein (CRP) and fibrinogen, which are easily measured, are more likely to significantly increase in response to higher airway inflammation seen during acute exacerbations. However, in view of the conflicting results, further study of the potential systemic inflammation is warranted in view of the known influence on co-morbidities.

1.8.2 Clinical outcomes in LABC

The airway inflammatory process associated with LABC, further damages the airway epithelial cells and thereby worsens the already defective host innate immune system, leading to a self-perpetuating cycle, known as the vicious-circle hypothesis of

infection and inflammation. This hypothesis was originally proposed as a model of chronic lung infection in bronchiectasis (Cole, 1997), but also applies to LABC in COPD (Sethi and Murphy, 2008), (□□□□□□□□□), and explains the important clinical outcomes seen in patients with LABC.

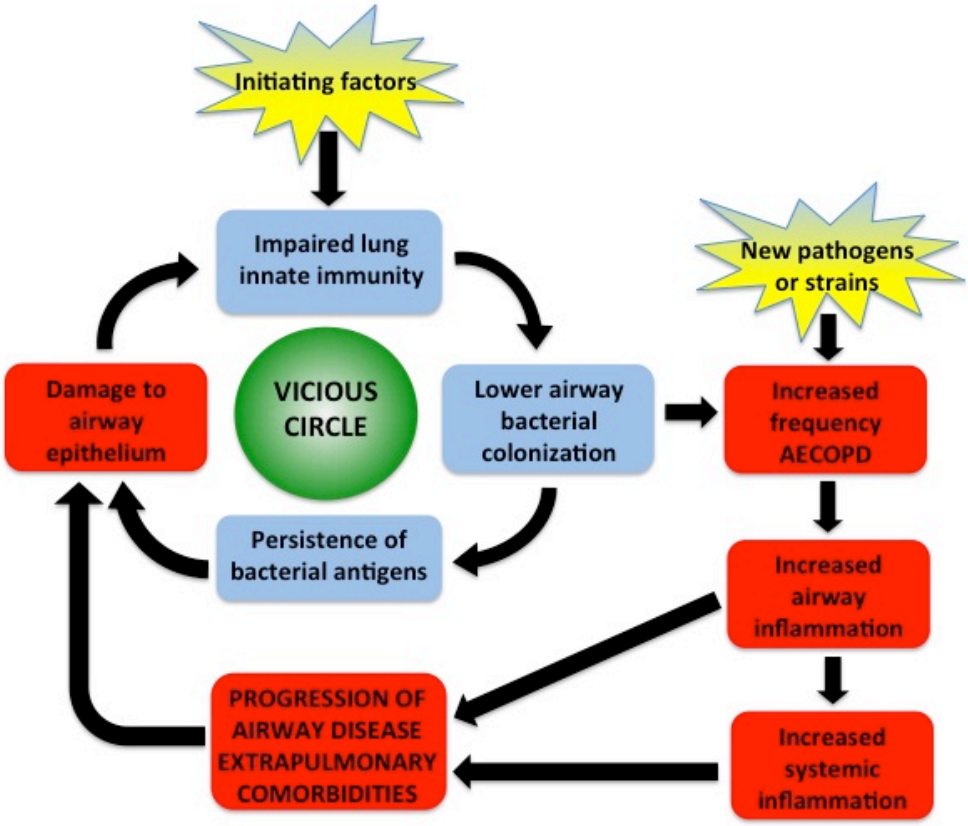


Figure 1.4. The vicious-circle hypothesis of infection and inflammation in COPD. Adapted from (Sethi and Murphy, 2008).

LABC status has been shown to be associated with increased susceptibility for acute exacerbations (Patel et al., 2002; Rosell et al., 2005), which as previously stated, contribute significantly to the morbidity and mortality of the disease. Acute exacerbations are associated with an even greater inflammatory response, both airway and systemic, than observed in the stable state (Gompertz et al., 2001; Sethi et al., 2008; Wilkinson et al., 2006b), and this further damages the airway epithelial cells

and leading to progression of airway disease. Wilkinson and colleagues demonstrated this in the first longitudinal study of the effects of LABC (Wilkinson et al., 2003). In this study, 30 patients from the East London COPD Cohort were followed up over a 12 month period, and over this time, FEV_1 decline was significantly related to increasing bacterial load in the colonised patients, and was greater in those patients who exhibited a change in the colonising bacterial species compared to those with the persistence of a single bacterial species. In addition, further damage to the airway epithelium can lead to concomitant bronchiectasis, observed in up to 50% of patients (Martinez-Garcia et al., 2011; Patel et al., 2004), and which is in itself associated with persistence of bacteria within the airways (Patel et al., 2004).

Both severity of disease and exacerbation frequency contribute to the significant impairment of quality of life seen in patients with COPD (Seemungal et al., 1998), and patients with LABC have been shown to have clinically worse scores on the St George's Respiratory Questionnaire (Banerjee et al., 2004; Marin et al., 2012). In addition, a recent longitudinal study, which collected daily symptoms together with fortnightly sputum samples for identification of LABC status, demonstrated significantly higher daily symptom scores during periods of LABC, after controlling for the effects of exacerbations, seasonal variations, lung function and other demographic variables (Desai et al., 2014).

LABC may also contribute to the increased risk of pneumonia identified in large prospective studies (Calverley et al., 2007; Calverley et al., 2011; Crim et al., 2009), meta-analyses (Drummond et al., 2008; Singh et al., 2009) and in a recent large population based cohort (Suissa et al., 2013) of COPD patients treated with inhaled

corticosteroids (ICS). While the pathophysiological mechanisms are unclear, there have been limited studies investigating this relationship. Previous experimental work has shown the importance of the NF-□B, which mediates downstream signalling pathways generating pro-inflammatory cytokines, such as TNF□ and IL-6 (Edwards et al., 2009), which play an important role in the pathogenesis of COPD (Di Stefano et al., 2002). While ICS inhibition of NF-□B is beneficial as an anti-inflammatory response, it may also inhibit the immune response to airway bacteria. Garcha and colleagues demonstrated that in stable COPD patients, the total airway bacterial load detected using molecular techniques was shown to be significantly higher with increasing ICS dose, independent of age, smoking status and disease severity (Garcha et al., 2012). Furthermore, in a mouse model of pneumonia using *Klebsiella pneumoniae* challenge, both airway and systemic bacterial load were higher in animals treated with 8 days of inhaled fluticasone compared to those without fluticasone (Patterson et al., 2012), adding further weight that increasing stable airway bacterial load may play a role in the increased risk of pneumonia with ICS therapy.

In view of increased inflammatory processes, alteration of the natural history and the associated damage to the lung observed in patients with evidence of LABC, there is an argument that the term ‘colonisation’ is misleading, as it suggests an innocuous process. As this is not the case, there is an argument to use the term ‘chronic infection’ instead of colonisation to reflect these important consequences (Matkovic and Miravitlles, 2013). However, further research is needed into several aspects of the role of bacteria in the pathogenesis of COPD. The presence of airway bacteria in stable COPD is likely to be a dynamic process, with periods of isolation of different pathogens or co-infection with differing loads, as well as periods without the isolation

of bacteria, resulting in a waxing and waning of inflammation (Marin et al., 2010; Murphy et al., 2004; Sethi and Murphy, 2008; Wilkinson et al., 2003). How this process affects exacerbation susceptibility and relates to exacerbation treatment, especially systemic antibiotic therapy, has not been fully addressed. These aspects are important to understand to enable the development of effective treatments to target LABC early in the course of COPD, thereby breaking the cycle of infection and inflammation, with the ultimate aim of improving clinical outcomes for patients.

1.9 Mechanism of bacterial colonisation: defective macrophage phagocytosis

Phagocytosis is the complex process whereby a phagocytic cell recognises and removes a foreign particle or pathogen (Donnelly and Barnes, 2012), thereby helping to maintain the balance of the lung microbiome. In humans, 'professional' phagocytes, macrophages and neutrophils, are the most effective phagocytic cells (Rabinovitch, 1995), and in the lung, alveolar macrophages are the predominant phagocyte (Martin and Frevert, 2005), although neutrophils may be rapidly recruited to the lung following an inflammatory insult (Zhang et al., 2000).

Foreign particles and pathogens are commonly recognised by the phagocyte through a process known as opsonisation. This involves serum complement or immunoglobulins binding to the foreign particle or pathogens, which are subsequently recognised by specific receptors on the phagocyte leading to rearrangement of the cytoskeleton to enable either 'sinking' phagocytosis associated with complement recognition, or pseudopod formation associated with immunoglobulin recognition (Allen and Aderem, 1996). This generates a phagosome, which then fuses with the lysosomes to form a phagolysosome, where acidification destroys the particle or pathogen.

However, the lung is not considered a serum-rich environment, and non-opsonised phagocytosis occurs through scavenger receptors, of which the macrophage receptor with collagenous structure (MARCO) is the most important in alveolar macrophages (Arredouani et al., 2005).

As previously discussed, lower airway bacterial colonisation is frequently observed in bronchial samples from stable COPD patients using culture microbiological detection. This persistence is suggestive of failure of the phagocytic process to remove the foreign pathogen. Studies investigating the phagocytic capacity of alveolar macrophages from stable COPD patients and healthy smokers have demonstrated an attenuated response to different pathogens, including fungal species and both Gram-negative and Gram-positive bacteria. Early work focused on the phagocytosis of non-respiratory pathogens, including *Candida* spp. (Ferrara et al., 1996; Vecchiarelli et al., 1991) and *E. Coli* (Prieto et al., 2001; Taylor et al., 2010). Importantly, more recent studies have demonstrated defective macrophage phagocytosis in response to respiratory pathogens commonly isolated from COPD patients, including *H. influenzae* and *S. pneumoniae* (Berenson et al., 2006a; Berenson et al., 2006b; Taylor et al., 2010). Furthermore, *in vitro* exposure of alveolar macrophages to cigarette smoke impaired *H. influenzae* phagocytosis without cigarette smoke having an effect on either macrophage or bacteria viability (Marti-Lliteras et al., 2009). However, in smokers and COPD patients, macrophage phagocytosis to inert beads does not show a similar impairment (Marti-Lliteras et al., 2009; Taylor et al., 2010). Therefore, macrophages from COPD patients have the potential ability to phagocytose but their response to different pathogens is diminished, possibly due to an intrinsic defect in pathogen recognition and subsequent formation of the phagolysosome. Despite this

impairment in macrophage phagocytosis of respiratory pathogens, to date, no studies have examined this relationship and the microbiology of bronchial samples, including both bacterial presence and load.

Although, the cause of this intrinsic defect has not yet been fully elucidated, reversal or at least improvement in macrophage phagocytic function may provide a novel therapeutic option for patients with COPD. Studies by Hodge and colleagues have demonstrated an improvement in alveolar macrophage phagocytosis to both apoptotic bronchial epithelial cells (Hodge et al., 2008) and to *E. coli* (Hodge and Reynolds, 2012) following azithromycin administration, although monocyte-derived macrophages (MDMs) from COPD patients did not show such an improvement (Taylor et al., 2010). Sulforaphane, a nuclear erythroid related factor 2 (Nrf2) activator, has been shown to improve bacterial clearance by alveolar macrophages and reduce inflammation in a mouse model (Harvey et al., 2011), although this has not been studied using human macrophages. Recently, physiological levels of extracellular calcium was shown to up-regulate bacterial recognition receptors, including MARCO, on MDMs and significantly improve phagocytosis to *H. influenzae* (Provost et al., 2014). However, these findings were from small studies and therefore, before their potential use as novel treatments for COPD can be identified, these studies need to be replicated and performed in larger populations of COPD patients.

1.9.1 The monocyte-derived macrophage model of alveolar macrophages

The previous studies described above used alveolar macrophages from COPD patients, which necessitated the invasive procedure of a flexible bronchoscopy and

BAL. Although bronchoscopy has been shown to be safe in appropriately assessed patients with COPD (Hattotuwa et al., 2002), it does carry significant residual risk of oxygen desaturation and impaired respiratory function. In addition, BAL return and subsequent viable alveolar macrophage isolation may be poor. In order to bypass the need for invasive sampling, MDMs are increasingly used to model alveolar macrophages, which derive from circulating monocytes (Gordon and Taylor, 2005), and can be easily sampled by peripheral venepuncture.

MDMs that have been differentiated from monocytes using granulocyte-macrophage colony-stimulating factor (GM-CSF) have similar molecular and functional properties to alveolar macrophages (Winkler et al., 2008), particularly a similar reduction in phagocytic capacity to pathogens (Taylor et al., 2010; Winkler et al., 2008). Although in a study by Berenson and colleagues (Berenson et al., 2006a), defective phagocytosis was only observed in alveolar macrophages, and not MDMs, which the authors suggested was due to a compartmentalised defect, the blood macrophages used in this study did not appear to have been differentiated using GM-CSF. In addition, the study from our research group by Taylor and colleagues used both confocal and electron microscopy, to validate the phagocytosis process carried out by MDMs (Taylor et al., 2010). Therefore, due to potentially large sample sizes needed to investigate defective macrophage phagocytosis as a mechanism for lower airway bacterial colonisation, the MDM model will be used for the studies carried out in this thesis.

1.10 Acute exacerbations of COPD

The natural history of COPD is interrupted by acute episodes of respiratory symptom worsening, termed exacerbations, (Wedzicha and Seemungal, 2007), that is beyond the normal daily variation and necessitates a change in medication (Vestbo et al., 2013). Exacerbations are heterogeneous but important clinical events, with a negative impact on both health status (Seemungal et al., 1998) (Mackay et al., 2012) and disease progression (Donaldson et al., 2002), and also contribute to the significant mortality (Wouters, 2003) and socioeconomic costs (National Institute for Health and Clinical Excellence (NICE), 2010). associated with the disease. Therefore, the importance of preventing exacerbations has been stressed in the recent GOLD guidelines (Vestbo et al., 2013).

1.10.1 Definition of exacerbations

The definition of exacerbations can be complicated, especially as there is considerable respiratory symptom variation when patients are stable, and thus no standardised definition has been used in research studies and clinical practice (Sapey and Stockley, 2006).

The seminal study carried out by Anthonisen and colleagues defined exacerbations symptomatically, in order to determine the effects of antibiotics on exacerbations rather than to guide clinical management (Anthonisen et al., 1987). Three different exacerbation groups of were defined according to the associated symptoms:

- Type I exacerbations – defined as increased or new onset dyspnoea, sputum volume and sputum purulence
- Type II exacerbations - □2 of the above symptoms

- Type III exacerbations – defined as 1 of the above symptoms with at least one of the following symptoms
    - Upper respiratory tract symptoms in previous 5 days
    - Increased wheeze
    - Increased cough
    - Fever

These criteria have formed the basis of exacerbation definitions by many other research groups, and have been previously validated by our research group for the study of health status, inflammation and lung function decline associated with exacerbations using daily symptom diary cards (Bhowmik, 2000; Donaldson et al., 2002; Hurst et al., 2006a; Seemungal et al., 1998).

Many pharmaceutical trials have defined and measured exacerbations based on healthcare utilisation (HCU), where patients are prescribed short-term systemic therapy of antibiotics and/or steroids, at the discretion of reviewing clinician (Rodriguez-Roisin, 2000). However, this HCU definition is not without problems. Patients' access to healthcare professionals may vary considerably between different healthcare systems, and clinicians may have different prescribing thresholds. In addition, previous studies have suggested that up to two-thirds of all exacerbations may not be reported to healthcare professionals for evaluation and treatment, either being self-treated with emergency packs of systemic therapy, an increase in inhaled therapy only, and thus would be missed on this HCU definition, or remain untreated (Langsetmo et al., 2008; Seemungal et al., 1998; Xu et al., 2010). While these

‘unreported exacerbations’ may be of lesser severity than the HCU reported exacerbations, they still have a considerable impact on health status.

As a result, there is substantial interest in the use of patient-reported outcomes for the study of exacerbations. The Exacerbations of Chronic Pulmonary Disease Tool (EXACT) has been specifically designed to study exacerbations, and has been shown to be an effective tool to evaluate exacerbations, although its ability to detect exacerbations still requires patients to report their symptoms (Mackay et al., 2014).

Therefore, in this thesis, the use of daily symptom diary cards to capture symptom-defined exacerbations will be used.

1.10.2 Aetiology and pathogenesis of exacerbations

As previously described, exacerbations are heterogeneous events, and likely caused by complex interactions between the host, potential pathogens, including respiratory viruses and airway bacteria, and environmental factors (Wedzicha and Seemungal, 2007) (□□□□□□□□□). The commonest causes of exacerbations are shown in □□□□□□□.

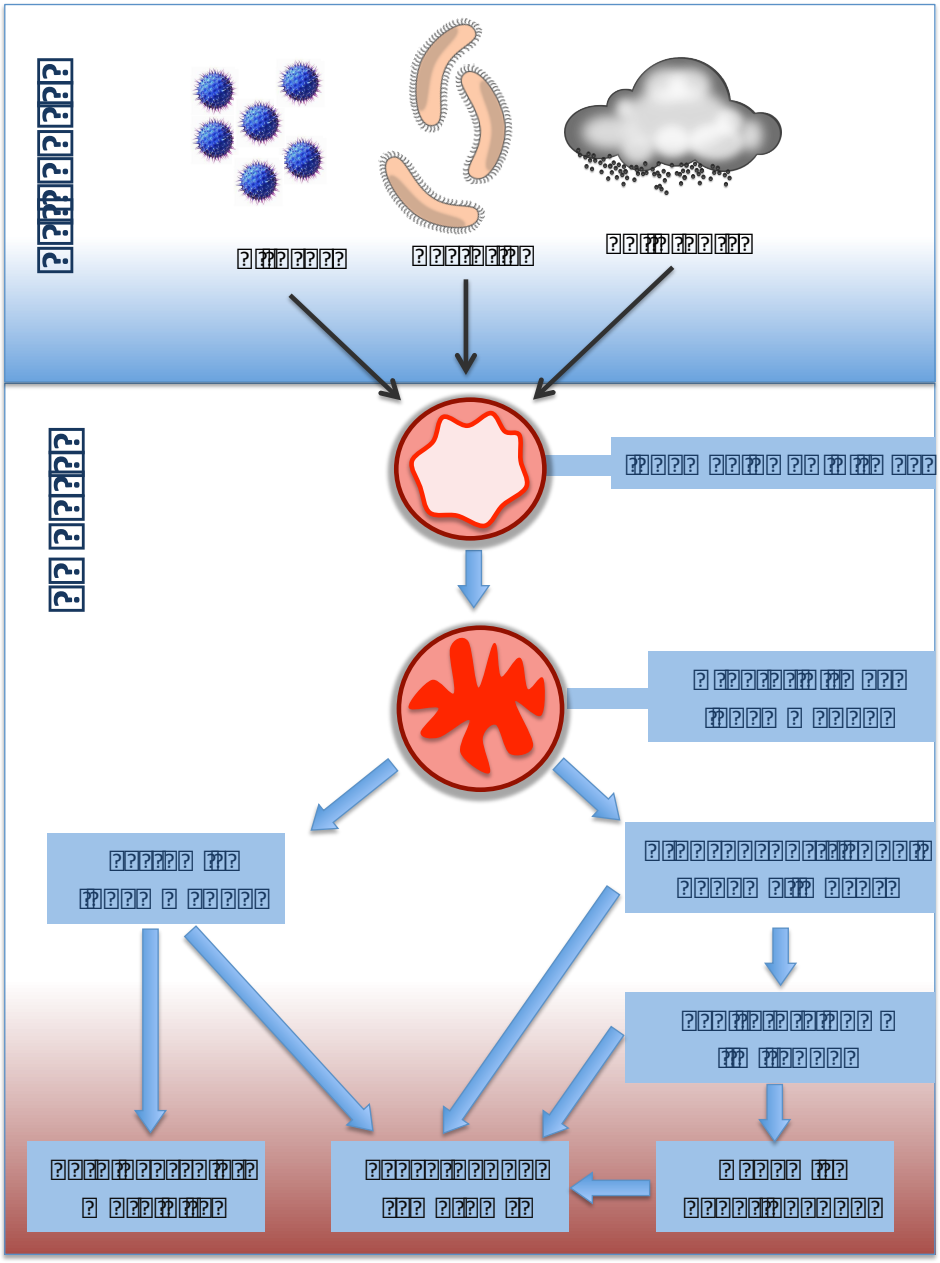


Figure 1.5. Triggers of COPD exacerbations and associated pathophysiological changes leading to increased exacerbation symptoms. Adapted from (Wedzicha and Seemungal, 2007).

Bacteria	Viruses	Environmental
Haemophilus influenzae *Streptococcus pneumoniae* *Moraxella catarrhalis* *Staphylococcus aureus* *Pseudomonas aeruginosa*	Human rhinovirus Influenza Parainfluenza Coronavirus Adenovirus Respiratory syncytial virus Picornavirus Metapneumovirus	Ozone Particulate matter Nitrogen dioxide Sulphur dioxide Biomass fuels

Table 1.4. Causes of COPD exacerbations. Adapted from (Sapey and Stockley, 2006; Sethi and Murphy, 2008).

1.10.3 Bacteria and COPD exacerbations

There is controversy over the role that bacteria play in the pathogenesis of COPD exacerbations and results from studies have been conflicting. It is likely bacteria play a complex role in exacerbations, particularly as bacteria are present during stable disease, although the primary infective trigger is now considered to be viral infection (Wedzicha et al., 2014).

Early studies examined inflammation and sputum characteristics at exacerbation and showed that increased airway and systemic inflammation were related to exacerbations with purulent sputum, which had detectable pathogens at high bacterial loads compared to exacerbations without detectable pathogens or mucoid sputum (Sethi et al., 2000; Stockley et al., 2000). This elevated inflammatory response observed at exacerbation subsequently decreased in those patients following bacterial eradication (White et al., 2003) or following convalescence (Wedzicha et al., 2000a).

However, none of these early studies compared the microbiology or inflammatory responses observed at exacerbation to those seen in stable COPD. Subsequent studies demonstrated that the detection of bacteria in sputum or bronchoscopic samples at exacerbation was significantly higher than during the stable state (between 50-70% at exacerbation, compared to 30-50% when stable) (Hurst et al., 2006b; Rosell et al., 2005; Wilkinson et al., 2006b), and this prevalence was seen to increases further in patients with severe exacerbations requiring hospitalisation and intubation (Bandi et al., 2001). However, studies by both Papi and Bafadhel and colleagues did not demonstrate such an increase in detection at exacerbation (Bafadhel et al., 2011; Papi et al., 2006).

Some exacerbations are associated with changes in bacterial strains from stable state (Chin et al., 2005; Murphy et al., 2007; Sethi et al., 2002). In one prospective study, molecular typing demonstrated that new strains of typical airway bacteria was associated with an increased risk of exacerbation, and the authors postulated that this was a mechanism to explain recurrent exacerbations (Sethi et al., 2002). However, even within this study the results are inconsistent, with not all exacerbations associated with a strain change and not all strain changes associated with exacerbation, and thus, strain change alone is not sufficient to be the only causative factor for exacerbation risk.

Furthermore, increasing load of pre-existing bacterial pathogens detected at stable state has been demonstrated in some studies of exacerbations associated with a heightened inflammatory response and greater symptom burden (Garcha et al., 2012; Hurst et al., 2006b; Wilkinson et al., 2006b). However, in a study by Sethi and

colleagues, although significant increases in load of new strains of *H. influenzae* and *M. catarrhalis* at exacerbation were observed, the absolute difference was small, with less than a log difference (Sethi et al., 2007). In addition, in patients with the same pre-existing strains at exacerbation as at stable state, the only significant change were in *M. catarrhalis* and *S. pneumoniae* which demonstrated a decrease in bacterial load (Sethi et al., 2007).

1.10.4 Virus infections in COPD exacerbations

The detection of viruses has considerably improved since the development of PCR-based techniques (Perotin et al., 2013), and as a result the role they play in COPD exacerbations has been re-evaluated. Respiratory viral infections are detected in up to 60% of exacerbations, with human rhinovirus (HRV) being the most common species identified (Rohde et al., 2003; Seemungal et al., 2001), and many patients report cold-like symptoms during exacerbations (Hurst et al., 2005a), which has been validated as a surrogate marker of viral infection (George et al., 2014). The experimental HRV infection model of exacerbations demonstrated increases in respiratory symptoms, and inflammatory markers with a concomitant fall in peak expiratory flow (PEF) with HRV infection in COPD patients, supporting a causal relationship between HRV and exacerbations (Mallia et al., 2011).

It is now considered that viruses are usually the first trigger for an exacerbation, which may result in a secondary bacterial infection (Wedzicha et al., 2014). Both the experimental rhinovirus model and HRV-associated, naturally occurring COPD exacerbations have demonstrated synergy between viral and bacterial pathogens (George et al., 2014; Mallia et al., 2012). While those exacerbations in the

experimental model were mild and did not require additional systemic treatment (Mallia et al., 2012), the naturally occurring exacerbations all required systemic therapy and the subsequent rise in bacterial load following HRV infections were associated with increases in respiratory symptoms, suggesting this interaction is an important clinical outcome (George et al., 2014). Furthermore, co-infection with both HRV and bacteria are associated with greater bacterial load and airway inflammation than in those without one or both pathogens (Wilkinson et al., 2006b) and increases specifically in *H. influenzae* bacterial burden (Molyneaux et al., 2013). One possible mechanism for this synergy may be due to viral modulation of the host immune system, which subsequently increases the pathogenicity of the bacteria (Avadhanula et al., 2006; Oliver et al., 2008), but further research is needed to fully elucidate this important finding.

1.10.5 Frequent exacerbator phenotype

Although exacerbations become more frequent as disease severity increases, the most reliable indicator of exacerbation risk appears to be a history of prior exacerbations (Donaldson and Wedzicha, 2006; Quint et al., 2011). However, it is increasingly recognised that a distinct group of patients are particularly susceptible to exacerbations. In the large ECLIPSE cohort, this phenomenon was seen irrespective of disease severity and these patients showed a relatively stable phenotype over the follow-up period of the study (Hurst et al., 2010). These patients have been described as ‘frequent exacerbators’ and have been shown to have increased susceptibility to future exacerbation events, faster FEV_1 decline, worse quality of life and considerably higher morbidity, both from their underlying COPD and also comorbidities ( ) (Wedzicha et al., 2013a). Therefore identifying these patients to target

appropriate therapy so as to minimise their risk of further exacerbations is vital to improve clinical outcomes in this high-risk group.

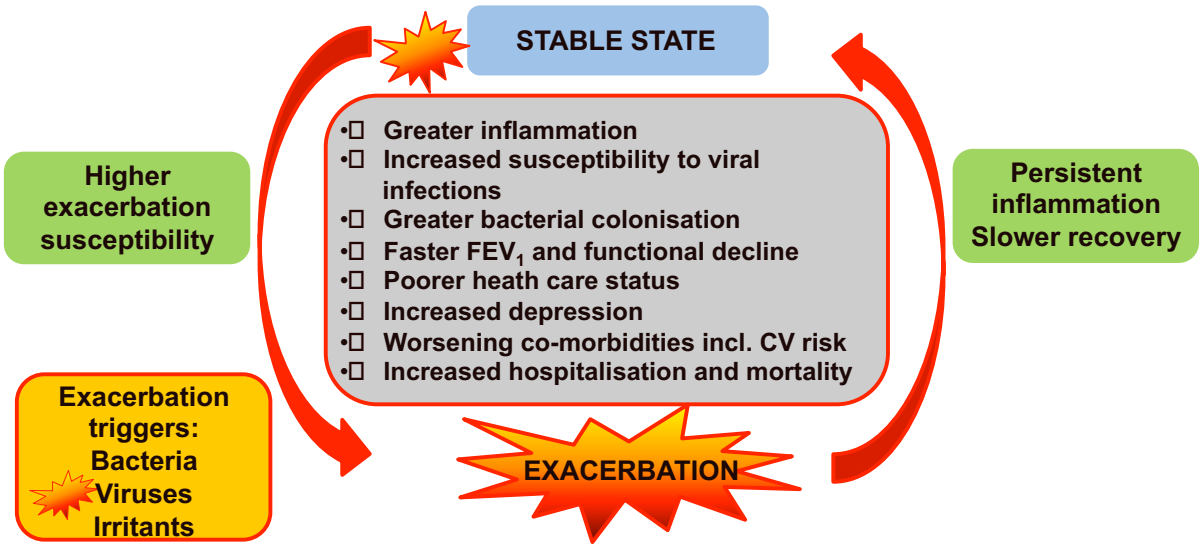


Figure 1.6. Schematic illustration of the frequent exacerbator phenotype. Adapted from (Wedzicha et al., 2013a).

1.11 The respiratory microbiome

With the introduction of culture-independent techniques over the past decade, an increased understanding of the role of microorganisms, and predominantly the bacterial community in the lung has been sought. It is clear from these techniques that a diverse microbial community exists within the lung in both health and disease, and this environment is defined as the microbiome (Sze et al., 2014). The previous, traditionally held belief that the tracheobronchial tree and lung parenchyma were sterile in healthy, non-smoking individuals was based on the use of classical culture methods for the detection of bacteria, and much of our current understanding of the role of bacteria in COPD has been gained from studies using these methods.

However, as 1% of bacteria can be cultured using such methods (Staley and Konopka, 1985), culture techniques are no longer considered the gold standard for the study of airway bacteria.

Molecular, culture-independent diagnostic techniques rely on the highly conserved genomic evolutionary relationships between bacteria. The majority of techniques are based on the polymerase chain reaction (PCR) amplification of the 16S ribosomal RNA gene from a biological sample. This is a component of the 30S subunit of prokaryotic ribosomes and is highly conserved amongst bacterial species, and can be used to differentiate species based on sequence differences, which are clustered in the hypervariable region (Ashelford et al., 2005). Primers are used to amplify the conserved sequences, which flank the hypervariable regions, enabling identification of specific bacteria within the community, and universal PCR primers are used to enable identification of all microorganisms in a given sample in one PCR reaction (Chambers et al., 2014). Sequencing is performed using cloneless, next generation sequencing technologies, such as 454 pyrosequencing or Illumina sequencing, and the sequences trimmed and clustered into operational taxonomic units (OTUs), which can then be identified against a reference database. The sequencing enables the identification of both bacterial species richness, i.e. the relative number of different species, and also species diversity, which in addition to species richness also takes into account the species evenness within the environment.

One of the first studies to use molecular techniques were carried out by Hilty and colleagues who analysed bronchoscopic brushings on 24 patients, including 4 with COPD and 8 healthy controls (Hilty et al., 2010). They demonstrated a wide range of

bacterial species in the healthy controls, and also found pathogenic Proteobacteria, were more common in patients with underlying respiratory disease. Further work has also noted considerable overlap between the bacteria in both COPD and healthy controls, suggestive of a core airway microbiome associated with Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria phyla (Erb-Downward et al., 2011; Hilty et al., 2010; Pragman et al., 2012; Sze et al., 2012). However, in patients with stable COPD, there is a difference in the clustering of the microbiome compared to healthy individuals, with increases in Firmicutes (Erb-Downward et al., 2011; Sze et al., 2012), and some studies have demonstrated changes in the microbiome depending on disease severity (Garcia-Nunez et al., 2014), although this has not been consistently shown in other microbiome studies (Pragman et al., 2012). Studies investigating how the microbiome changes at exacerbation are few, but although none showed a decrease in bacterial community diversity, there was a shift in community composition towards bacteria associated with exacerbations and the phylum Proteobacteria (Huang et al., 2014; Millares et al., 2014; Molyneaux et al., 2013).

The study of the microbiome in both stable and exacerbated COPD remains in its infancy, with the majority of published studies cross-sectional in nature, and further research is needed to fully explain the complexities and the dynamics of the lung microbiome in COPD pathogenesis, exacerbations and disease progression. Furthermore, the use of these techniques is limited by several factors. High-throughput, genomic techniques are expensive research tools, not only for performing the PCR itself, but also for the additional, intensive computational bioinformatics analysis of the data, which often take weeks to analyse, limiting their use to small sample numbers. In addition, the highly sensitive nature of the techniques raises

concerns about contamination during sample acquisition, particularly from the oropharynx (Charlson et al., 2011), although on-going studies may help to address these technical issues.

However, limited molecular techniques have been used to identify the common pathogens isolated in COPD. In a large study by our research group, quantitative PCR (qPCR) was used to identify *H. influenzae, S. pneumoniae* and *M. catarrhalis*, as well as evaluating the presence of atypical bacteria (*Chlamydia pneumoniae, Legionella pneumophila* and *Mycoplasma pneumoniae*) from COPD patients at both stable and exacerbation states (Garcha et al., 2012). In this study, qPCR was shown to have a higher rate of detection for typical bacteria than culture, and the detection rate of atypical bacteria was low at 1.3%. As this technique does not rely on the analysis of the 16S rRNA gene, but instead uses specific primers and probes to identify these three bacteria, the expense is minimised. The average expense for samples costing in the hundreds, rather than the tens of thousands of pounds needed for genomic microbial analysis, making this much more widely accessible for research groups. Therefore, in this thesis, qPCR will be used as the primary method for the identification of typical bacteria.

1.12 Management of stable and exacerbated COPD

A holistic approach is important for the management of COPD, including non-pharmacological and pharmacological treatments to alleviate symptoms and improve clinical outcomes such as FEV_1 decline and exacerbation frequency. Previous GOLD guidelines have recommended treatment options based on FEV_1 as a measure of disease severity. However, the most recent GOLD guidelines have stressed the

importance of tailoring management to individual patient's symptoms as well as risk of exacerbations, using a combined assessment approach (Vestbo et al., 2013) (Figure 1.7). However, in reality, few patients are likely to fulfil the high exacerbation risk but few symptom group C, as the exacerbation history is often a major determinant of patients' symptoms (Seemungal et al., 1998). Thus, resources should be targeted at the frequent exacerbators and patients with significant symptom burden.

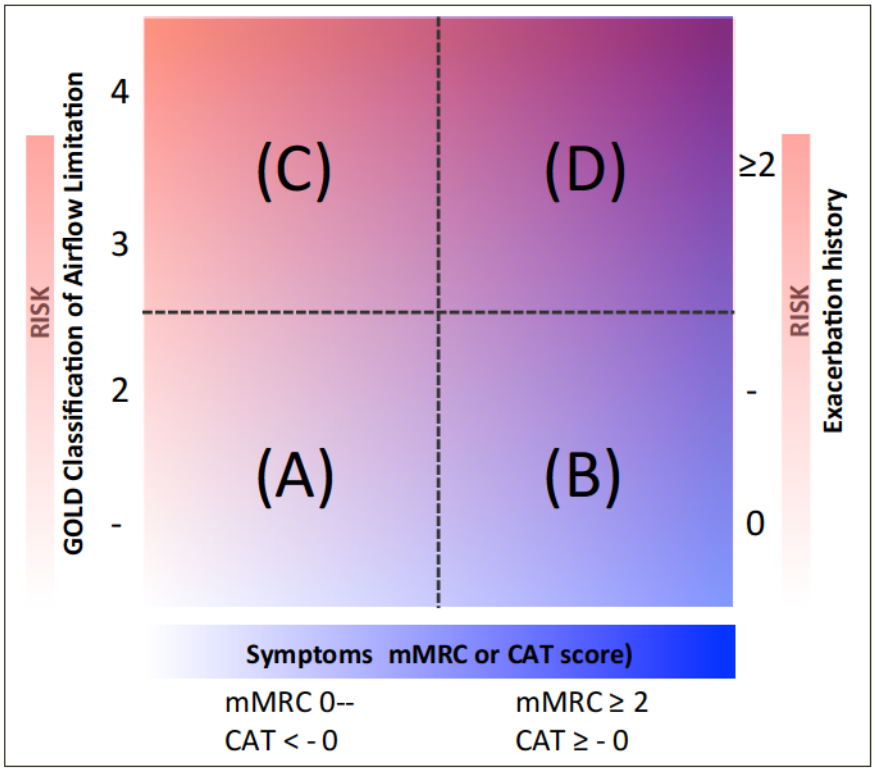


Figure 1.7. Combined COPD assessment, using both symptoms and risk. Assessment of risk should be done using the highest risk according to GOLD spirometric grade or exacerbation history. Adapted from (Vestbo et al., 2013).

Non-pharmacological treatment includes smoking cessation, vaccination and regular physical activity including pulmonary rehabilitation. Smoking cessation has been shown to be the single most effective intervention in the long-term to reduce the rate of lung function decline (Anthonisen et al., 2002), although it has not been shown to

be an effective measure to significantly alter exacerbation frequency (Hurst et al., 2010). Both influenza and pneumococcal vaccine are routinely offered to COPD patients. Influenza vaccination has been shown to confer a 27% reduction in risk of hospitalisation with either influenza or associated pneumonia and a 48% reduction in mortality in a large retrospective cohort study (Nichol et al., 2007). The evidence for pneumococcal vaccination in COPD is less strong but there has been increasing interest in the role of extended serotype vaccinations, which offer improved immunogenicity (Dransfield et al., 2012). Furthermore, OM-85 BV an oral immunomodulatory agent containing antigens from common respiratory pathogens, has been investigated for its effects on reducing exacerbation frequency and clinically important outcomes in COPD, although at present the evidence supporting its use is lacking and further studies are recommended (Pan et al., 2015; Sprenkle et al., 2005). Pulmonary rehabilitation has been shown to significantly improve respiratory symptoms, exercise capacity, mental health state and thus overall quality of life, and should be offered to all COPD patients (McCarthy et al., 2015)

The mainstay of maintenance therapy in stable COPD are bronchodilators (preferably long acting β_2-agonists and muscarinic antagonists, LABA and LAMA respectively) and inhaled corticosteroids in those patients with a FEV_1 <50% predicted and/or frequent exacerbations, to minimise symptoms and reduce exacerbation risk, and thereby improving overall health status. However, no current existing medications for COPD have been conclusively demonstrated to reduce long-term lung function decline or mortality (Calverley et al., 2007; Tashkin et al., 2008). Both LAMA and ICS/LABA combination inhalers have been shown to reduce exacerbation frequency (Calverley et al., 2007; Tashkin et al., 2008), and the LAMA tiotropium has been

shown to be more effective at reducing exacerbation frequency than the LABA salmeterol (Vogelmeier et al., 2011). However, despite this reduction in exacerbation frequency, paradoxically, ICS use has been associated with an increased risk of pneumonia (Calverley et al., 2007; Calverley et al., 2011; Crim et al., 2009; Drummond et al., 2008; Singh et al., 2009; Suissa et al., 2013), and therefore there has been growing concern about the safety of treatment. Dual bronchodilators are seen as a potential alternative to ICS-containing regimens and have shown to have significant benefit on exacerbation frequency (Wedzicha et al., 2013b). The recent WISDOM study was conducted to determine whether a step-wise withdrawal of ICS was possible without subsequently increasing exacerbation risk (Magnussen et al., 2014). No significant increase in risk of exacerbation was seen, although ICS withdrawal was associated with a decrease in FEV_1 of 43ml over the study period and no reduction in the incidence of pneumonia. Therefore, while the use of ICS in COPD has been challenged, further studies are needed to determine whether ICS therapy may be beneficial in some phenotypes and in these circumstances whether the benefits out-weigh the associated risks.

The novel, oral anti-inflammatory phosphodiesterase-4 inhibitor roflumilast has been shown to decrease neutrophilic inflammation associated with COPD (Grootendorst et al., 2007). Roflumilast has been shown to be of benefit when added to inhaled bronchodilators in patients with chronic bronchitis and high risk of exacerbations, although it appears less beneficial if the patient is co-prescribed an inhaled corticosteroid (Fabbri et al., 2009). In a pooled analysis from two large randomised, placebo-controlled, double-blind multicentre studies in patients with severe or very severe COPD with chronic bronchitis, roflumilast demonstrated a significant

reduction in moderate and severe exacerbations, although patients were not on maximal preventative therapy as ICS and LAMA therapies were not allowed (Calverley et al., 2009). This was readdressed in the REACT study where severe COPD patients with chronic bronchitis, a history of frequent exacerbations and on triple preventative therapy (LAMA, ICS/LABA) were included, roflumilast reduced moderate and severe exacerbations, although adverse events, predominantly gastrointestinal, were significantly higher in the roflumilast treated group (Martinez et al., 2015).

There has been increasing research into the use of long-term antibiotics, particularly macrolides, for the reduction of exacerbation frequency (Albert et al., 2011; Seemungal et al., 2008; Sethi et al., 2010), although presently there is insufficient evidence for their routine use in management of stable COPD (Vestbo et al., 2013). In a 12-month study, once daily erythromycin was shown to reduce the exacerbation frequency and prolong the time to first exacerbation, although no significant differences in airway inflammatory markers were seen (Seemungal et al., 2008), although other studies demonstrated reduction of inflammatory markers with 6-months erythromycin therapy (He et al., 2010). In a large US-based study, azithromycin was associated with a decrease in exacerbation frequency and prolonged time to first exacerbation, although the effects were more pronounced in patients not on maximal preventative inhaled therapy, and of concern, there was a higher rate of macrolide-resistant respiratory pathogens from the nasopharynx (Albert et al., 2011). The development of antibiotic resistance with long-term antibiotic therapy is of understandable concern. Previously published data using pulsed moxifloxacin was not associated with an increase in antibiotic resistance (Sethi et al., 2010), although a

recent randomised-controlled trial of three different antibiotic classes, including pulsed moxifloxacin, over a three-month period in stable COPD demonstrated considerable antibiotic resistance in respiratory isolates from sputum in all treatment groups, which has important implications for future use (Brill et al., 2015).

Outpatient management of acute exacerbations may include only an increase in bronchodilator therapy especially if symptoms are mild, although typically it includes the prompt use of short-courses of antibiotics (Vestbo et al., 2013; Wilson et al., 2013). The seminal study by Anthonisen and colleagues investigated the effects of antibiotic therapy or placebo, in patients with severe COPD on resolving symptoms by day 21 (Anthonisen et al., 1987). The success rate was significantly higher with antibiotic therapy in all exacerbations, and when exacerbations were sub-grouped into types I, II and III, the success rate was only observed in groups I and II. However, the Anthonisen study was carried out in patients with severe COPD, and a large number of exacerbation presentations, especially to primary care physicians, occur in patients that have mild-moderate airflow limitation. Therefore, Llor and colleagues evaluated the efficacy of antibiotic treatment in such patients in a multi-centre, parallel, double-blind, placebo-controlled study. Patients presenting with an exacerbation and with an FEV_1>50% predicted were randomised to either amoxicillin/clavulanic acid or placebo for 8 days, and oral corticosteroids were allowed for 10 days (Llor et al., 2012). The clinical cure at end of treatment was significantly higher in the antibiotic group compared to the placebo group, and time to next exacerbation was significantly longer in patients who received antibiotics. However, a recent Cochrane review has not demonstrated any significant effects on mortality or length of hospital stay (Vollenweider et al., 2012).

Studies have found significant benefit of oral corticosteroids in the treatment of acute exacerbations, shortening exacerbation recovery time and reduce early relapse or treatment failure (Niewoehner et al., 1999; Thompson et al., 1996; Wood-Baker et al., 2005), and may also be prescribed, either together with antibiotics or alone depending on the individual exacerbation severity. Although GOLD guidelines recommend a 10-14 day course of oral steroids (Vestbo et al., 2013), the optimal dose and duration is unclear and there is evidence that shorter, 5-day courses may be as efficacious (Leuppi et al., 2013). Recent studies have suggested that the beneficial effects of corticosteroids are only observed in patients with an eosinophilic inflammatory profile, and they may impair recovery in non-eosinophilic exacerbations (Bafadhel et al., 2012b).

Chapter 2:

Aims and hypothesis

This overall aim of this thesis is to understand the links between lower airway bacterial colonisation, chronic inflammation and clinical outcomes in COPD with the aim of addressing the general hypothesis that defective innate and/or adaptive immunity results in persistence of airway bacteria in stable COPD. The hypothesis is that the presence of airway bacteria, which drives both a predominantly neutrophilic-dominant airway inflammatory response and contributing to systemic inflammation, is resistant to cues for inflammation resolution, thereby altering the natural history of the disease including exacerbation susceptibility. Furthermore, the hypothesis is that macrophages play a key role in the regulation of airway bacteria at both stable and exacerbated COPD states, and may demonstrate differential activation and thus cytokine release at stable and exacerbation states will be investigated.

Specifically, this thesis is aimed to address the following key questions:

1. How does qPCR compare to quantitative bacterial culture for the detection of airway bacteria (**chapter 4**)?

2. Using qPCR to detect airway bacteria, what is the relationship between airway bacterial presence and load and the inflammatory response in stable COPD (**chapter 4**)?

3. Is there evidence of a species-specific effect in the inflammatory profile at both stable and exacerbated states (**chapter 4**)?

4. Do airway bacterial presence, load and species at stable state modulate the risk of subsequent exacerbation and alter the exacerbation characteristics (**chapter 5**)?

5. Does the aetiology of exacerbations influence subsequent bacterial colonisation (**chapter 5**)?

6. Is there evidence of a close relationship between airway and systemic biomarkers (identified using Meso Scale Discovery) at both stable and exacerbated states to support the 'spill-over' inflammation hypothesis (**chapter 6**)?

7. How do airway and systemic biomarkers relate to each other and change over three consecutive, stable-exacerbation-subsequent stable visits (**chapter 6**)?

8. Are species-specific changes in airway and systemic inflammation seen in the exploratory biomarker analysis (**chapter 6**)?

9. Using MDMs to model alveolar macrophages, what is the relationship between macrophage phagocytosis and stable clinical characteristics (**chapter 7**)?

10. Is impaired macrophage phagocytosis related to airway bacterial presence and load in stable COPD and does this change over time to explain the dynamics of bacterial colonisation (**chapter 7**)?

11. Can changes in macrophage phagocytosis explain the heterogeneity seen in the aetiology of exacerbations and does this influence the characteristics of the exacerbation itself (**chapter 8**)?

12. Does macrophage activation and subsequent cytokine release contribute to the chronic inflammatory profile seen in COPD, and do clinical characteristics and patient state influence this response (**chapter 8**)?

Chapter 3:

Methods

3.1 Subject recruitment

Patients enrolled in the MRC (Patient Research Cohorts Initiative) London COPD cohort were recruited for this study. The patients form part of a rolling cohort, maintained with at least 200 patients, which is used to prospectively investigate the pathophysiology of COPD and its associated acute exacerbations (Seemungal et al., 1998). Patients were able to leave the study at any point following recruitment and to ensure confidentiality, each subject was assigned a unique study number. Ethical approval for the study was granted from the Royal Free Hospital research ethics committee (Ref. 09/H0720/8) and all patients gave written informed consent.

3.1.1 Inclusion criteria

COPD patients were included if they had a smoking history of >10 pack years, with evidence of persistent airflow obstruction; forced expiratory volume in one second (FEV_1) < 100% predicted from age, height and sex, and post bronchodilator (β_2-agonist) FEV_1/forced vital capacity (FVC) <0.7, in keeping with GOLD grades I-IV

3.1.2 Exclusion criteria

Patients were excluded if they had a history of asthma, primary bronchiectasis or any other significant respiratory diseases. Patients unable to complete the daily symptoms diary cards were also excluded.

3.2 Clinical measurements

Clinical measurements were performed by either by myself, or by the research nurse and clinical research fellows who are part of the London COPD research team.

3.2.1 Spirometry

Spirometry was performed on study participants at all clinic visits. FEV_1 and FVC were measured in accordance with ATS/ERS guidelines using a Vitalograph Gold Standard spirometer (Vitalograph Ltd, Maids Moreton, UK). Values were taken as the best of three reproducible attempts, and are expressed either as absolute volume or as a percentage predicted based on sex, weight and height (using Quanjer tables).

3.2.2 Health assessment questionnaires

At annual review, the St George's Respiratory Questionnaire (SGRQ)[1](Jones et al., 1992), the Medical Research Council (MRC) dyspnoea score, and the COPD Assessment Test (CAT) were completed (copies of the questionnaires are provided in the Appendix). In addition, the CAT was requested at all other clinic visits, including 3-monthly stable, exacerbation and exacerbation-follow-up.

3.3 Diary cards

At recruitment, patients were taught how to record on daily symptom diary cards, and are provided with written instructions for filling these in (a copy of the diary card used and the patient instructions for completing the dairy cards are given in the Appendix).

Patients were asked to record their daily morning post-bronchodilator peak expiratory flow rate (PEFR), recording the best of three attempts (using a Mini-Wright peak flow meter, Clement Clarke International Ltd, Harlow, UK). In addition, they were asked to record any increase in their usual respiratory symptoms using a letter-annotated system (). Patients were instructed not record any symptoms that they normally experience when they are well or stable, but only to document any perceived increase in symptoms over their normal, stable condition.

Letter	Major or Minor	Symptom/Symptom Change
A	Major	Increased breathlessness
B1	Major	Increased sputum colour
B2	Major	Increased sputum amount
C	Minor	A cold (e.g. runny or blocked nose)
D	Minor	Increased wheeze/chest tightness
E1	Minor	Sore throat
E2	Minor	Increased cough
F	Minor	Fever

Table 3.5. Letter-annotated system to record any increase in patients' usual respiratory symptoms, either major or minor, on daily diary cards. Example: if a patient has symptoms of a cold, 'C' should be recorded on the diary card. If on the subsequent day the patient has both cold and increased breathlessness symptoms, then 'C' and 'A' should be recorded.

Patients were asked to contact the study team, via a dedicated mobile telephone number, if they experienced a worsening in any symptoms to arrange a clinic assessment visit, and to do this before starting any antibiotic or steroid tablets.

3.3.1 Diary card definition of stable state

Stable state was defined as those patients without evidence of symptom-defined exacerbations in the preceding 4 weeks and the subsequent 2 weeks post-clinic visit.

3.3.2 Diary card definition of exacerbations

An exacerbation was defined using previously validated symptomatic criteria (Seemungal et al., 1998), as the presence for at least two days of an increase in either two major symptoms (as listed above), or one major and one minor. Exacerbation onset was defined as the first day on which these symptom criteria were met. To ensure any chronic symptoms were not included when identifying the onset, symptoms recorded daily for more than 5 days preceding the suspected exacerbation onset were excluded when making the diagnosis.

Calculation of exacerbation severity was assessed by the summation of the number of individual symptoms recorded at the onset e.g. A, B1, C = 3 symptoms or A, B1, B2, D, E1, E2 = 6 symptoms.

The exacerbation end date was taken as the last day that increased symptoms (either major or minor) were recorded, followed by two consecutive symptom free days. Exacerbation duration was the number of days from onset to the end date.

Exacerbations were classified as ‘physician reported exacerbations’ – those seen by the study clinical team or the patient’s general practitioner and ‘unreported exacerbations’ – those unseen by physicians but recorded on diary cards.

In cases of doubt, clinical assessment and the use of treatment, such as increased β_2-agonist use, provided additional information to help determine whether an episode was classified as an exacerbation.

3.3.3 Recording of exacerbation treatment on diary cards

Patients with any worsening in their daily symptoms were asked to record any changes to their usual treatment, using a letter-annotated system (□□□□□□□).

Letter	Treatment
H	Admitted to hospital
I	Increased use of inhaled corticosteroids (red/brown/purple inhalers)
R	Increased reliever medication and no. of puffs
S	Use of steroid tablets and no. taken
X	Use of antibiotic tablets and name

Table 3.6. Letter-annotated system to record any changes to patients' usual treatment on daily diary cards.

3.4 Clinic visits schedule

3.4.1 Recruitment and annual review

At recruitment and annual review, a full medical and smoking history was obtained, and clinical examination performed, including measurement of oxygen saturations (PureSAT®, Nonin Medical Inc, Plymouth, MN, USA) and height and weight. Completion of health assessment questionnaires were requested. Comorbid diagnoses were established using clinical history and examination findings, supported where

appropriate with a review of the available medical records. Medication history was reviewed and where necessary, treatment optimised. Patients were taught, or re-educated, on completing daily symptom diary cards. Spirometry was performed and sputum and venous blood samples were collected.

3.4.2 Stable visits

Following recruitment, patients were regularly seen at 3-monthly intervals when stable. Diary cards, medical history and concurrent medication were reviewed to ensure the patient was stable before spirometry was performed. Patients were requested to complete the CAT questionnaire, and sputum and venous blood samples were collected.

3.4.3 Exacerbation visits

Patients contacted the study team for review if they experienced any worsening of their respiratory symptoms and prior to commencing any additional systemic therapy. Exacerbations were treated according to the prevailing guidelines and clinical judgment with increased inhaled therapy, antibiotics and/or oral steroids. When patients attended for an exacerbation, medical assessment was performed by one of the clinical research team. Spirometry was performed and patients were requested to complete the CAT questionnaire. Sputum and venous blood samples were taken prior to commencing exacerbation treatment.

3.4.4 Exacerbation follow-up visits

Patients were reviewed in clinic at two- and five weeks following presentation for exacerbation. Clinical assessment was performed, and diary cards were reviewed to determine exacerbation end date, and if on-going symptoms were present, together with clinical history, to determine whether re-treatment was clinically recommended. Spirometry was performed, patients were requested to complete the CAT questionnaire, and sputum and venous blood samples were taken.

3.5 Calculation of exacerbation frequency

Exacerbation frequency was calculated for each patient using diary card data obtained between recruitment and onset of the sub-study. For recently recruited patients with less than one-year diary data, exacerbation frequency was based on the number of exacerbations the patient recalled for the year prior to recruitment. Previous work has shown a good correlation between the number of exacerbations recorded on diary cards and the number of exacerbations recalled by the patient over the same 1-year period (Quint et al., 2011), and has shown that exacerbation frequency represents a stable patient phenotype (Hurst et al., 2010).

3.6 Sputum sampling and processing

3.6.1 Spontaneous sputum

Patients were asked to spontaneously expectorate sputum samples into a sterile pot, after rinsing their mouth. Patients who were unable to spontaneously expectorate sputum, or if the sputum sample was of poor quality, underwent sputum induction.

Sputum samples were kept at 4°C for no longer than 2 hours prior to further processing to prevent RNA degradation.

3.6.2 Induced sputum

Sputum induction was carried out, as previously described (Bhowmik et al., 1998). Study participants performed post-bronchodilator (β2-agonist) spirometry and oxygen saturations were measured (PureSAT®, Nonin Medical Inc, Plymouth, MN, USA). Administration of 5ml 3% (v/v) saline was carried out using an ultrasonic nebuliser (output approximately 2ml/min), in three, five-minute cycles. If the participant was deemed to be high-risk, 0.9% (v/v) saline was used for the initial nebulization. If this failed to induce sputum, and the participant was asymptomatic and FEV_1 remained stable, the saline was increased to 3% (v/v).

After the initial 5 minutes, FEV_1, and if clinically indicated, oxygen saturations were re-measured. If FEV_1 fell by <10%, the nebulisation was continued with 3% (v/v) saline. If FEV_1 fell by >20%, or if the participant experienced any distressing symptoms, the process was discontinued. Study participants were allowed to expectorate sputum at any point during the induction process. Prior to expectoration, patients were requested to blow their nose and rinse their mouth out with water before attempting sputum expectoration.

3.6.3 Sputum appearance

Sputum plugs were separated from contaminating saliva by macroscopic examination, and the colour of the sputum plug was graded using the BronkoTest® colour chart

(Stockley et al., 2001b). A copy of the BronkoTest® colour chart is provided in the Appendix. Sputum plugs were then divided for processing for quantitative PCR and measurement of sputum biomarkers as a priority, and when of sufficient quantity, for bacterial culture.

3.6.4 Sputum processing

Sputum plugs were homogenised with Dulbecco's phosphate buffered saline (D-PBS), as previously published (Hurst et al., 2005b) (Wilkinson et al., 2006b). Selected sputum plugs were transferred into an empty, pre-weighed polypropylene centrifuge tube. The weight of the sputum was calculated (weight of tube and sputum – weight of the empty centrifuge tube). Eight volumes x sputum weight (in grams) of D-PBS was added, and the sputum dispersed by repeated gentle aspiration into a plastic pipette.

Approximately 0.5ml of glass beads (Glass Balls 2.5-3.5mm. VWR International Ltd Cat No. 33212 4G) was added and the tube was vortexed for 15 seconds (Whirlimixer IKA-Vibrax-VXR, Scientific & Chemical Supplies Ltd.) and then subsequently for 15 minutes on a bench rocker (Voltex Mixer, Bench rocker (variable speed) Scientific & Chemical Supplies Ltd.). The centrifuge tube was re-vortexed for further 15 seconds. A 500µl aliquot of the homogenized sputum samples was stored in 1.5ml Eppendorf tubes and frozen at -80°C for later batch analysis for qPCR.

The remaining homogenized sputum was filtered through a nylon filter mesh (Plastok (Mesh and Filtration) Ltd, Merseyside, UK), and centrifuged at 790*g* for 10 minutes with the brakes off (Benchtop refrigerated centrifuge. Hettich Universal 380 R. Wolf

Laboratories Ltd.). 500µl aliquots of the sputum supernatant were stored in 1.5ml Eppendorf tubes, taking care not to disturb the cell pellet. The aliquots were frozen at -80°C for later batch analysis for sputum biomarkers.

3.7 Diagnostic microbiology

3.7.1 Qualitative culture

Qualitative sputum bacterial culture was carried out in the Department of Medical Microbiology, Royal Free Hospital. Briefly, sputum was cultured onto suitable agar for 24 hours at 37°C and 5% CO_2. Agar plates used were Columbia blood agar (CBA) for general growth media, Chocolate agar (CHOC) for growth of fastidious bacteria, MacConkey (MAC) agar for growth of Gram-negative bacteria and Columbia blood agar base with streptococcus selective supplement (COBA) agar (growth of *Streptococcus spp*). *S. pneumoniae* was identified through optochin sensitivity testing.

3.7.2 Quantitative culture

Quantitative sputum bacterial culture was carried out in the Centre for Clinical Microbiology, University College London. Sputum samples were homogenised with an equal volume of sputasol and diluted to 10^{-5} before being plated under sterile conditions onto CHOC, CBA, COBA, and MAC agar plates for 18 hours at 37^{o}C (CBA and MAC), and 37^{o}C, 5% CO_2 (CHOC and COBA).

Purity plates were prepared from the agar plates for each phenotypically different organism and incubated for 18 hours at 37^{o}C (CBA and MAC) and 37^{o}C, 5% CO_2 (CHOC and COBA). Matrix assisted laser desorption/ionization – time of flight

(MALDI-TOF) has recently replaced biochemical testing (Carbonnelle et al., 2011), was used for the subsequent identification of aerobic bacteria.

From each purity plate, a colony was inoculated with a sterile wooden tooth pick (Prolab diagnostics, Wirral, Merseyside, UK) onto a MALDI-TOF target plate in duplicate and 1μL matrix solution, (alpha-cyano-4-hydroxycinnamic acid (Bruker Daltonik GmbH, Bremen, Germany) prepared with LC-MS CHROMASOLV® water, acetonitrile and trifluoroacetic acid (Sigma- Aldrich, St. Louis, MO, USA)) was added onto each target spot with an Eppendorf pipette overlaying the smeared colony. The matrix was dried at room temperature before loading onto a Bruker Microflex™ platform for MALDI-TOF analysis.

Profile spectrum was generated and analysed by the Maldi Biotyper™ software. A log score >2.0 was required to accept the species identification; scores between 1.7-2.0 were considered acceptable for identification at the genus level and scores <1.7 were considered unacceptable for identification and retested with the application of 1□L 70% formic acid to the target plate, prior to the addition of the matrix solution. For all *Streptococcus mitis* group isolates, optochin tests were undertaken due to the similarity between species. Plates yielding between 30-300 colonies were counted using a manual tally counter and the viable bacterial numbers present were expressed as colony forming units per millilitre (cfu/ml) of original sputum (Pye et al., 1995).

□

3.7.3 Quantitative PCR for typical bacteria

Quantitative PCR (qPCR) was performed on *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* using a multiplex PCR by the Centre for Clinical Microbiology, University College London (Garcha et al., 2012).

Homogenised sputum samples were thawed and processed using a heat-kill treatment at 90°C for 30 minutes before being centrifuged at 13 000*g* for 10 minutes. The cell pellet was washed in 1ml PBS and spun at 13 000*g* for another 10 minutes before removal of the supernatant and re-suspension of the pellet in 200ml of PCR-grade UV-sterilized water (Sigma-W4502). 200ml of 10% Chelex 100 (Sigma C-7901) was added to each sample and incubated for 20 minutes in a heat block at 56°C. Samples were heated at 95°C for five minutes prior to cooling on ice and subsequently the samples were spun in a microfuge at 16 000*g* for 10 minutes. Supernatant containing extracted DNA was transferred to a fresh UV-sterilized 1.5ml Eppendorf tube and stored at 4°C.

An internal amplification control (IAC) was used to detect any PCR inhibition in the individual samples. The IAC was incorporated in the PCR mastermix (□□□□□□□□), and as the IAC concentration is equal in all the samples, the Ct value should be identical for the control in the samples. Non-identical Ct values are suggestive of the presence of inhibitors within the sample (e.g. Chelex present in the DNA extract), which may inhibit the PCR reaction.

Reagents	Volume (µl)
Platinum qPCR Supermix	12.5
$MgCl_2$ (50mM)	1.5
Forward primers for bacterial DNA (50□M)	0.075
Reverse primers for bacterial DNA (50□M)	0.125
IAC forward primer (50□M)	0.125
IAC reverse primer (50□M)	0.125
Probes for bacterial DNA (50□M)	0.125
IAC probe (50□M)	0.1
IAC	1
H2O	3.675

Table 3.7. Reagents included in the mastermix, used for multiplex qPCR. IAC= internal amplification control.

20µl mastermix was added to each tube before the addition of 5µl of each sample in duplicate.

The qPCR conditions were: 95°C for 3 minutes; 45 cycles of denaturation (95°C for 10 seconds, 60°C for 45 seconds). Primers and probes for *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* were based on protocols developed previously in-house, ensuring no cross-reactivity with oral species (□□□□□□□□).

The minimum limit of detection of the qPCR was 10^4 colony-forming units (cfu)/ml.

Oligo Name	Sequence (written 5' - 3')
Typical primers	
SP - Forward	AGTCGTTCCAAGGTAACAAGTCT
SP - Reverse	ACCAACTCGACCACCTCTTT
HI - Forward	CCGGGTGCGGTAGAATTTAATAA
HI - Reverse	CTGATTTTTCAGTGCTGTCTTTGC
MC – Forward	GTGAGTGCCGCTTTTACAACC
MC - Reverse	TGTATCGCCTGCCAAGACAA
IAC - Forward	AACTTGGCTTTAATGGACCTCCA
IAC - Reverse	ACATTCATCCTTACATGGCACCA
Typical probes	
SP - Probe	ROX-TACATGTAGGAAACTATTTTCCTCACAAA-BHQ2
HI - Probe	6FAM-ACAGCCACAACGGTAAAGTGTTCTACG-DB
MC - Probe	6JOE-TGCTTTTGCAGCTGTTAGCCAGCCTAA-BHQ1
IAC - Probe	Cy5-TGCACAAGCTATGGAACACCACGT-BBQ

Table 3.8. Primers and probes used for the identification of *S. pneumoniae* (SP), *H. influenzae* (HI) and *M. catarrhalis* (MC), including IAC. IAC=internal amplification control.

3.8 Human rhinovirus detection

qPCR for human rhinovirus (HRV) was batch-performed on homogenised sputum samples using the single-step total RNA isolations TRI Reagent LS (Sigma T3809-LS) method (Chomczynski and Sacchi, 1987; George et al., 2014) by Dr Siobhan George from within the department. All tubes and pipette tips used in RNA extraction and PCR were RNase/DNase free and were placed under UV light for 30-40 minutes before use to prevent ribonuclease enzymes degrading RNA.

500µl aliquots of homogenised sputum were thawed at room temperature and 750µl of TRI reagent was added before vortexing for 4 minutes. After 5 minutes incubation at room temperature, 200µl chloroform (Sigma C2432) was added and then vortexed for a further 4 minutes. The samples were then incubated on ice for 10 minutes before

being spun in a microcentrifuge at 12 000*g* for 15 minutes. The top aqueous layer containing the RNA was removed and transferred to a UV-sterilised microcentrifuge tube and 500µl of isopropanol (Sigman I9516) was added. The samples were left on ice for a further 15 minutes and were subsequently re-centrifuged at 12 000*g* for 20 minutes. The supernatant was removed and 200µl of 80% ethanol (Sigma E7023) added to the RNA pellet. The sample was then re-centrifuged at 12 000*g* for 5 minutes and the pellet was air dried for 30 minutes before being re-suspended in 30µl of RNase free water. Positive and negative controls (virus spiked sputum sample and water respectively) were included in the RNA extraction.

The extracted RNA was immediately reversed to generate complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems 4368814, Carlsbad, California). 10µl RNA sample and 10µl reverse transcriptase (RT) mastermix (□□□□□□□□) were added to 96-well PCR plates and all samples were run in duplicates with controls. The plate was sealed and loaded into the PCR machine (Techne TC-412 Thermal Cycler, Kieson). The RT conditions to generate cDNA were: 25°C for 10 minutes; 37°C for 2 hours and 85°C for 5 minutes.

Reagents	Volume (µl)
10x RT Buffer	2.0
25x dNTP mix (100mM)	0.8
Multiscribe™ Reverse Transcriptase	1.0
10x RT Primers	2.0
PCR Grade Water	4.2
RNA sample	10

Table 3.9. Reagents included in the 2x RT mastermix for reverse transcription.

An IAC was used to detect any PCR inhibition, similar to the qPCR for bacterial detection (section 3.7.2). For each PCR reaction, 22.5µl of mastermix and 2.5µl of cDNA sample were used. The PCR conditions were: 95°C for 15 minutes, 45 cycles of denaturation (95°C for 15 seconds, 58°C for 80 seconds). The primers and probes for HRV RT-PCR were based on protocols developed previously in-house (□□□□□□ □□□).

Oligo Name	Sequence (written 5' - 3')
Primers	
HRV - Forward	TGADTCCTCCGGCCCCT
HRV - Reverse	AAAGTAGTYGGTCCCRTCC
IAC - Forward	AACTTGGCTTTAATGGACCTCCA
IAC - Reverse	ACATTCATCCTTACATGGCACCA
Probes	
HRV - Probe	6-FAM – AATGYGGCTAACCT - MGB
IAC - Probe	Cy5-TGCACAAGCTATGGAACACCACGT-BBQ

Table 3.10. Primers and probes used for the identification of HRV, including IAC primers and probes. IAC=internal amplification control.

HRV load was subsequently calculated in plaque forming units per mL (pfu/mL) using a standard curve prepared using a plaque assay from tissue culture grown HRV-1B (American Type Culture Collection, ATCC). The lower limit of detection was 10.23 pfu/ml.

3.9 Measurement of sputum biomarkers

3.9.1 Enzyme-linked immunosorbent assay

PBS-processed sputum supernatants were thawed and processed according to the manufacturer's instructions to measure levels of CXC-chemokine ligand 8 (CXCL8), interleukin (IL)-1□ and myeloperoxidase (MPO) using a high sensitivity enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Abingdon, UK).

Supernatants were diluted in the assay diluent as necessary (CXCL8: 1:100, IL-1□: no dilution, MPO: 1:100). The diluted samples or standards were added to the appropriate microplate wells, covered and left at room temperature for 2 hours (the MPO plate was placed onto a horizontal microplate shaker set at 500±50 rpm), before being washed three times (for IL-1□), or four times (for CXCL8 and MPO) in wash buffer.

The appropriate detection antibody was added to all wells, (200µl for IL-1□and MPO conjugate, and 100µl for CXCL8 conjugate), and the plates were re-covered and incubated for a further 1 hour (for CXCL8 and IL-1□) or 2 hours (MPO). Plates were washed three or four times as above, and 200µl of the substrate solution was added to each well (equal volumes of Colour reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine) mixed directly before addition to the plates). The plates were covered and incubated in the dark for 20 minutes (IL-□) or 30 minutes (CXCL8 and MPO), following which, the reaction was stopped by the addition of 50µl/well 1M H_2SO_4.

The OD of each well was determined using a spectrophotometer (BioTek Gen5 v2.0 All-In-One Microplate Reader Software), read at 450nm while subtracting values at 570nm to correct for any optical imperfections in the plate. Concentrations of cytokines were derived from extrapolation from standard curve graphs generated from the spectrophotometer (□□□□□□□□).

The lower limit of detection for the ELISA assays were 3.5 pg/ml, <1.0 pg/ml and 0.014 ng/ml for CXCL8, IL-1□and MPO respectively.□

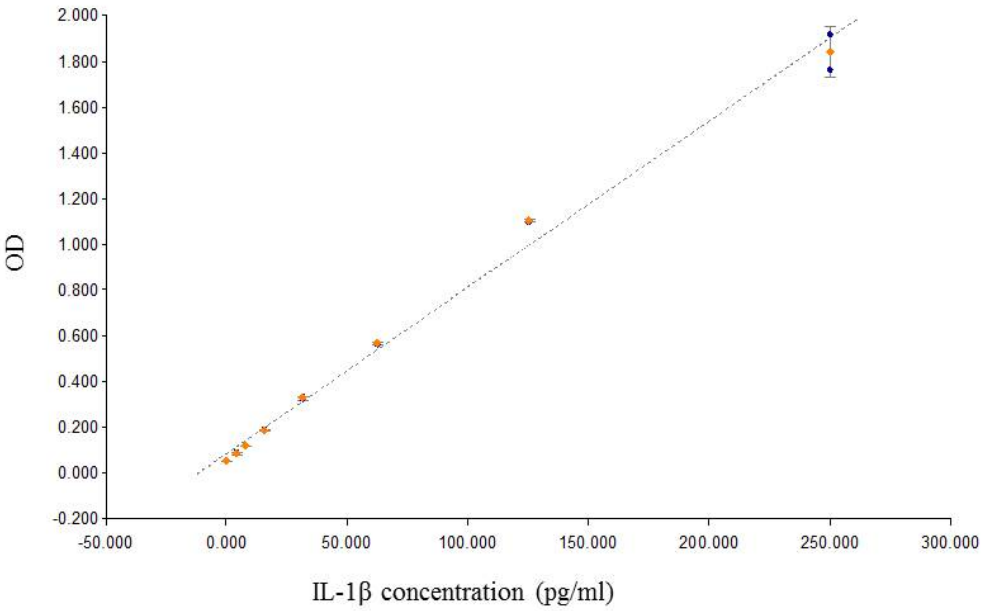


Figure 3.8. Example of standard curve generated using BioTek Gen5 v2.0 All-In-One Microplate Reader Software, to determine unknown concentrations of IL-1β in cell supernatants. The absorbance was read at □450nm. Similar standard curve graphs were generated for CXCL8 and MPO.

□ □□□

3.9.1 Meso Scale Discovery analysis

Meso Scale Discovery (MSD) V-PLEX Plus Human Biomarker 40-Plex kit (MSD, Rockville, MD, USA) was used to measure chemokines, cytokines, pro-inflammatory, angiogenesis and vascular injury biomarkers within PBS-processed sputum supernatants. MSD provides multiplex assays on 10-spot MULTI-SPOT® plates, as shown in , with individual assays provided on Small Spot plates, shown in .

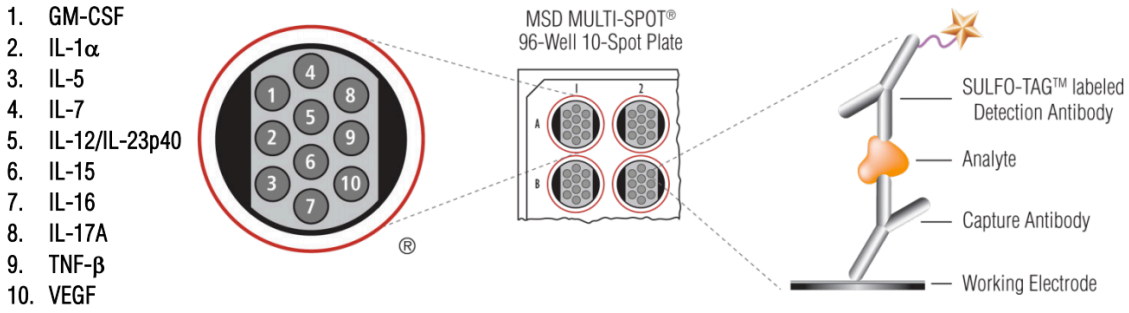


Figure 3.9. Schematic showing multiplex plate spot placement of analyte capture antibodies for the cytokine panel. The numbering convention for the different spots is maintained in the software visualisation tools, the plate packaging and data files. Diagram taken from www.mesoscale.com.

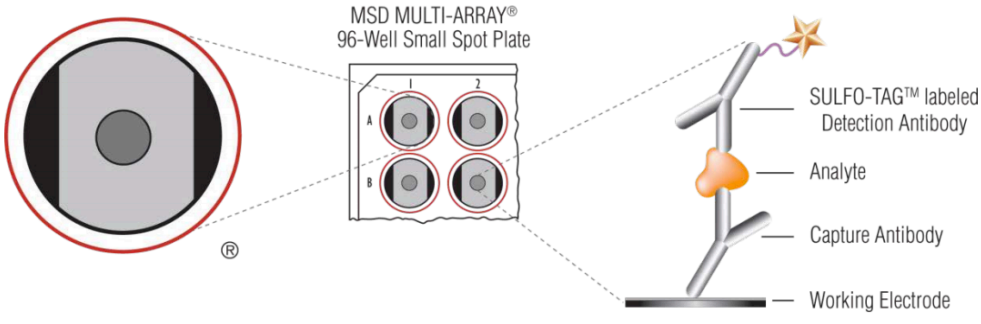


Figure 3.10. Schematic showing the small spot plate placement of analyte capture antibodies. Diagram taken from www.mesoscale.com.

PBS-processed sputum supernatants were thawed and processed, and MSD reagents and plates prepared according to the manufacturer's instructions for each panel. The sputum supernatant samples, calibrator or control were added to the appropriate microplate wells. The plate was sealed with an adhesive plate seal and left at room temperature for 2 hours on a microplate shaker set at set at 500±50 rpm, before being washed three times in wash buffer.

25µl appropriate detection antibody solution was added to all wells and the plates were re-covered and incubated for a further 1-hour (vascular injury panel) or 2 hours (chemokine, cytokine, pro-inflammatory and angiogenesis panels) at room temperature on the microplate shaker. Plates were washed three times with wash buffer and 150µl of either 1X Read Buffer T (vascular injury panel) or 2X Read Buffer T (chemokine, cytokine, pro-inflammatory and angiogenesis panels) was added to each well. Plates were read on the MSD reader (Sector S 600, Meso Scale Diagnostics, Rockville, MD, USA) immediately following the addition of Read Buffer for vascular injury, pro-inflammatory, cytokine and angiogenesis panels, and after 10 minutes of incubation at room temperature for the chemokine panel.

The calibration curves were generated from the MSD reader using MSD DISCOVERY WORKBENCH® analysis software (MSD, Rockville, MD, USA). [illegible] shows an example of the standard curves generated for the MSD cytokine panel.

The lower limit of detection for the biomarkers in each biomarker panel included are shown in [illegible].

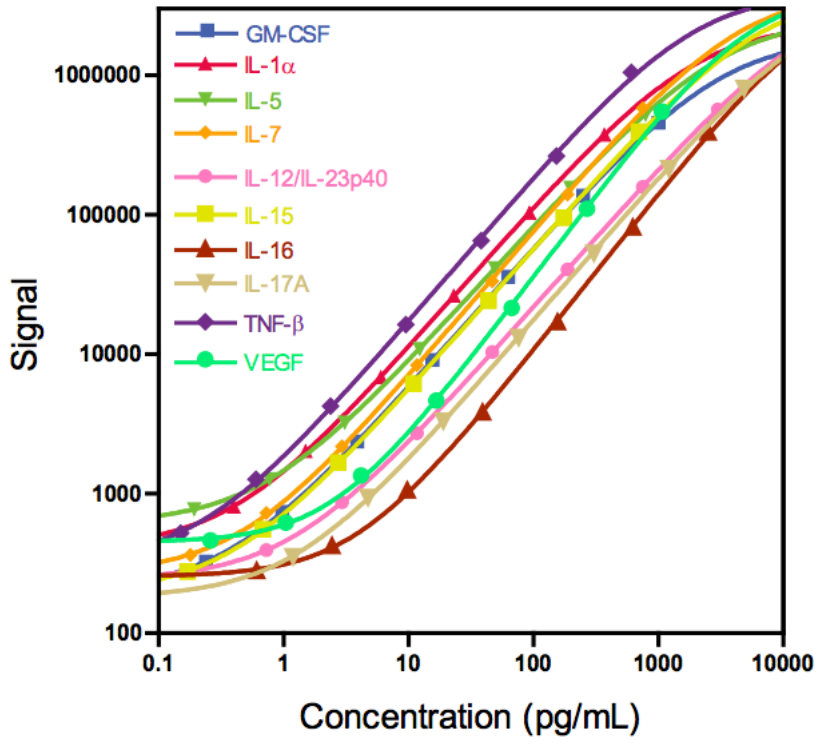


Figure 3.11. Example of the standard curve generated using MSD DISCOVERY WORKBENCH® analysis software to determine unknown concentrations of biomarkers in the MSD cytokine panel. Graph taken from www.mesoscale.com.

Chemokine biomarkers	Median LLOD (pg/ml)	Cytokine biomarkers	Median LLOD (pg/ml)	Pro-inflammatory biomarkers	Median LLOD (pg/ml)	Angiogenesis biomarkers	Median LLOD (pg/ml)	Vascular injury biomarkers	Median LLOD (pg/ml)
Eotaxin	3.26	GM-CSF	0.14	IFN-γ	0.2	VEGF	0.48	SAA	10.9
MIP-1β	0.37	IL-1α	0.09	IL-1β	0.04	VEGF-C	11.1	CRP	1.33
Eotaxin-3	1.77	IL-5	0.22	IL-2	0.09	VEGF-D	2.53	VCAM-1	6
TARC	0.22	Il-7	0.16	IL-4	0.02	Tie-2	21.1	ICAM-1	1.03
IP-10	0.37	IL-12/IL23p40	0.39	IL-6	0.06	Flt-1	0.56		
MIP-1α	3.02	IL-15	0.17	IL-8	0.04	PIGF	0.32		
IL-8*	95.6	IL-16	2.83	IL-10	0.03	bFGF			
MCP-1	0.09	IL-17A	0.74	IL-12p70	0.11				
MDC	1.22	TNF-β	0.05	IL-13	0.24				
MCP-4	1.69	VEGF	1.12	TNF-α	0.04				

Table 3.11. Median values for the lower limit of detection (LLOD) of the MSD biomarker panels included in the V-PLEX Human Biomarker 40-Plex kit. *High sensitivity assay

3.10 Blood sampling and processing

3.10.1 Full blood count

3ml of whole blood was collected from study participants by venepuncture in Vacutainer® containing liquid K_3ethylenediaminetetraacetic acid (EDTA) and spray-coated K_2EDTA tubes for total white blood cell count and differential neutrophil and eosinophil counts.

3.10.2 Serum for CRP

3.5ml of whole blood was collected from study participants by venepuncture in Vacutainer® SST tubes, containing spray-coated silica and a polymer gel for serum separation. Serum C-reactive protein (CRP) was measured using Modular Analytics E 170 Module (Roche, Burgess Hill, UK). The lower limit of detection was 1mg/L.

3.10.3 Plasma for fibrinogen

2.7ml of whole blood was collected by venepuncture in Vacutainer® Citrate tubes containing 3.2% buffered sodium citrate solution. Plasma fibrinogen was measured using the Clauss method (IL ACL Top Coagulation Analyzer, Lexington, MA, USA). The lower limit of detection was 0.1g/L.

3.10.4 Serum for biomarker analysis

5-10ml of whole blood was collected by venepuncture in Vacutainer® silicone coated tubes for clot activation. After a minimum of 30 minutes post-collection, the Vacutainer® was centrifuged and the serum stored in 500µl aliquots at -80°C until batch analysis for biomarker studies.

Serum samples were analysed using Meso Scale Discovery (MSD) V-PLEX Plus Human Biomarker 40-Plex kit (MSD, Rockville, MD, USA) to measure chemokines, cytokines, pro-inflammatory, angiogenesis and vascular injury biomarkers using the same protocol as described in section 3.9.1.

3.10.5 PBMC isolation

60ml of whole blood was collected by venepuncture in Vacutainers® containing liquid EDTA and spray-coated K_2EDTA. The whole blood was mixed with 20ml 6% (w/v) dextran and 10ml Hank's balanced salt solution (HBSS), and subsequently incubated at room temperature for 45 minutes to sediment erythrocytes. The top, leukocyte layer was aspirated and washed with HBSS by centrifugation (350*g* for 10 minutes).

Peripheral blood mononuclear cells (PBMC) were isolated from the resultant cell pellet, which contained a mixture of leukocytes, by discontinuous Percoll gradient (GE Healthcare, USA). A 100% (v/v) Percoll solution was prepared using 0.9% (v/v) saline. Using this solution, Percoll gradients of 81% (v/v), 68% (v/v) and 55% (v/v) were prepared using 0.9% (v/v) saline. The gradients were prepared by transferring 4ml of 81% (v/v) Percoll solution into a 15ml Falcon tube, followed by 4ml of 68% (v/v). The cell pellet was re-suspended in 4ml of the 55% (v/v) solution and was overlaid onto the previous two gradients. The cell fractions were separated according to density by centrifugation at 750*g* for 25 minutes, at room temperature. The PBMC fraction was harvested from the 55% (v/v)/68% (v/v) Percoll interface, and washed in 50ml HBSS (centrifugation at 1500*g* for 5 minutes). Cell counts were performed using a Neubauer haemocytometer, after staining with 1:100 Kimura stain (0.05% (v/v) toluidine blue, 0.03% (v/v) light green, 10% (v/v) saponin, 0.7M phosphate buffer).

3.10.6 Culture of MDMs

Following cell counts, as described above, the PBMC were re-suspended in complete media (Roswell Park Memorial Institute (RPMI) 1640 medium, 10% (v/v) foetal calf serum, 10mg/ml (1%(v/v)) penicillin/streptomycin and 2mM (1%(v/v) L-glutamine) at a concentration of $4x10^6$ cells/ml. 100µl/well of the re-suspended PBMC solution was seeded in a 4x4 well-layout on a 96 well back plate ($1x10^5$ monocytes/well). The plates were incubated at 37°C and 5% (v/v) CO_2 for 2 hours to enable monocyte adherence. Following this, the media and any non-adherent cells were aspirated from the wells. Fresh complete media containing GM-CSF (2ng/ml) was added to the cells and incubated at 37°C and 5% (v/v) CO_2 for 12 days. Media was replaced on days 4

and 8. □□□□□□□□□ shows the cellular differentiation of monocytes cultured to generate MDMs in the presence of GM-CSF from day 0 to day 12.

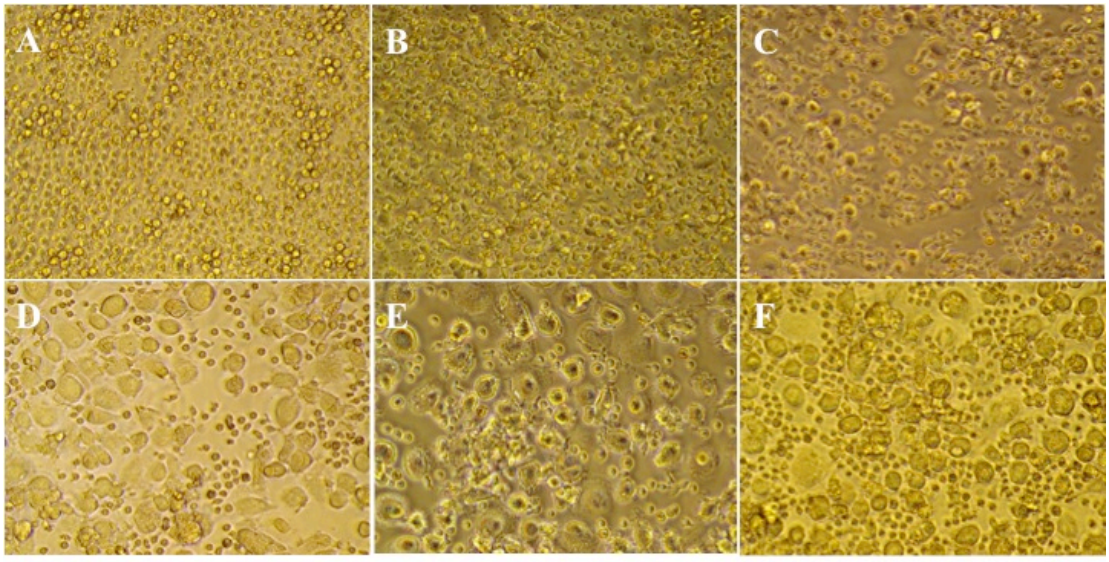


Figure 3.12. Morphology of monocyte-derived macrophages (MDMs) cultured in the presence of GM-CSF, showing cellular differentiation of cells at day 0 (A), day 2 (B), day 4 (C), day 7 (D), day 9 (E) and day 12 (F). Photographs were taken using an inverted microscope at 40x magnification.

3.11 Phagocytosis assay

3.11.1 Bacterial culture and labelling

Non-typeable *H. influenzae*, strain 1479 and *S. pneumoniae* serotype 9V, strain 10692 (taken from a COPD patient during exacerbation) (Taylor et al., 2010) were plated onto CBA plates and incubated at 37^{o}C, 5% CO_2 overnight. Single colonies of *H. influenzae* and *S. pneumoniae* were subsequently inoculated into brain heart infusion (BHI) broth with bovine hemin and NAD^{+} (10µg/ml) or Todd-Hewitt broth containing 5% (v/v) yeast extract for bacterial culture.

Following culture, the bacteria were harvested by centrifugation at 1600g for 15 minutes and re-suspended in D-PBS. The bacteria were placed in a water bath at 70°C for 2 hours, followed by washing twice to heat-kill the bacteria.

Heat-killed bacteria were re-suspended in 1ml sodium bicarbonate buffer (8.4g $NaHCO_3$ in 100ml distilled water) before adding 10µl Alexa-fluor 488 dye (Invitrogen Life Technologies Corporation, excitation □480nm and emission □520nm) and rotating the bacteria overnight in a dark room, at room temperature. The bacteria were repeatedly washed in D-PBS to remove any unbound dye and re-suspended in D-PBS.

OD readings were taken at □600nm, and samples were diluted in D-PBS to achieve an OD of 1.5-1.7 for *H. influenzae* and 1.8-2.0 for *S. pneumoniae*. Previous work from Thomas, 2012 (PhD thesis) has shown that these OD readings correspond to a bacterial concentration of $5x10^9$ cfu/ml.

1ml aliquots of the fluorescently labelled, heat-killed bacteria were stored at -20°C, until further use.

3.11.2 Fluorescent beads

Fluorescently labelled carboxylate-modified polystyrene microspheres (2.0µm diameter, yellow-green fluorescence (excitation □505nm and emission □515nm, $4.5x10^9$ microspheres/ml)) (Invitrogen Life Technologies Corporation, UK) were stored at 4°C, protected from light, until use during the phagocytosis assay.

□

□ □□□

3.11.3 Dilution of bacteria and beads

Fluorescently labelled, heat-killed bacteria *H. influenzae* and *S. pneumoniae,* and the fluorescent beads were brought to room temperature and sonicated in a water bath for 2 minutes, to ensure an even suspension of bacteria and beads.

A 1:90.9 dilution of the fluorescent beads was carried out using RPMI (11µl/ml of RPMI) to achieve a final assay concentration of $50x10^6$ microspheres/ml. This concentration has previously been shown by our group to give the optimal readouts for bead phagocytosis (Taylor et al., 2010).

3.11.4 Phagocytosis of beads and bacteria

A 1:10 dilution of bacteria was made up using RPMI (100µl/ml of RPMI), to obtain a final assay concentration of $5x10^8$ cfu/ml. This concentration was based on the work from our group, which demonstrated that clinically relevant bacterial COPD exacerbations occur at $>10^7$ cfu/ml (Garcha et al., 2012).

100µl of RPMI alone (control) and the three prey; beads, *H. influenzae* and *S. pneumoniae* were added to wells containing $1x10^5$ MDM, in quadruplicate. The MDMs were incubated at 37°C and 5% CO_2 for 4 hours, after which the MDM supernatant was aspirated and stored at -80°C for subsequent measurement of cell supernatant cytokines. Any unbound preys were washed off using 100µl D-PBS. Autofluorescence of cells and extracellular particle fluorescence were quenched by the addition of 100µl of Trypan Blue (1% (v/v)) to each well for 1 minute. The Trypan Blue was then removed and fluorescence determined by fluorimetry (BMG Fluostar plate reader) at excitation □480nm and emission □520nm. □□□□□□□□□ shows

fluorescent microscopy of the phagocytosis of beads and bacteria by MDMs. A schematic of the phagocytosis assay is shown in ?????????.

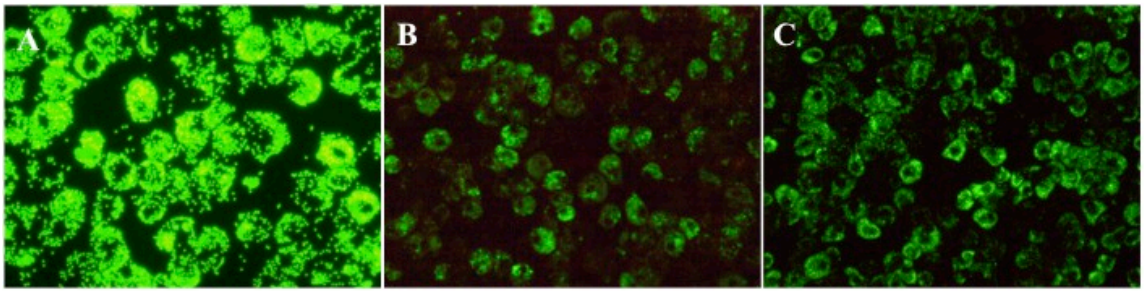


Figure 3.13. Fluorescent microscopy showing MDM phagocytosed (A) beads, (B) *H. influenzae* and (C) *S. pneumoniae*. Photographs taken at 40x magnification.

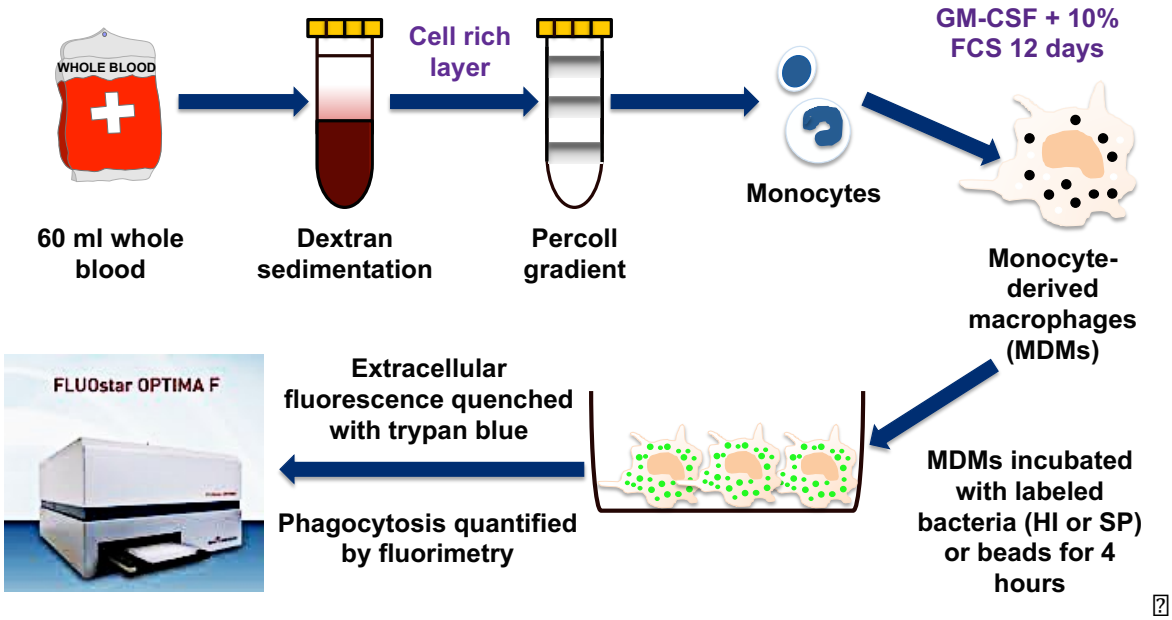


Figure 3.14. Schematic showing the steps involved in the culture of MDMs and the subsequent phagocytosis assay. HI=*H. influenzae*; SP=*S. pneumoniae*.

3.11.5 Cell viability Assay

Following the phagocytosis assay, a measurement of cell viability (using metabolic activity as a surrogate measure) was performed. 100µl of methylthiazoyldiphenyl-

tetrazolium bromide (MTT) solution (1mg/ml) was added to each well, and plates were incubated at 37^{o}C, 5% CO_2 for 30 minutes. The MTT was discarded and 100µl DMSO was added to each well to lyse the cells, resulting in the formation of a purple colour caused by the reduction of MTT to formazen if the cells were metabolically active. The plate was gently agitated to evenly distribute the colour, and absorbance was measured on a spectrophotometer at 570nm. Data was normalised to the viability of the control cells, which was given as 100%.

3.12 Measurement of cytokines from MDM cell supernatant

3.12.1 Measurement of TNFα

TNF ELISA antibodies were used according to the manufacturer's guidelines. Briefly, 96 well NUNC Maxisorp plates were coated with 2µg/ml (100µl/well) TNF monoclonal antibody diluted in sterile PBS. The plates were incubated at room temperature overnight. Capture antibody was removed and the plates were blocked using blocking buffer containing 1% (w/v) BSA, 5% (w/v) sucrose and 0.05% (w/v) sodium azide (150µl/well) for 2 hours at room temperature.

Blocking buffer was removed and TNF standards were added to the appropriate wells. MDM cell supernatants, stored after 4 hours phagocytosis with beads or bacteria, were diluted 1:40 using wash buffer (PBS containing 0.05% (v/v) Tween20) and added to the appropriate wells. The plates were covered and incubated for 2 hours at room temperature, before being washed three times in wash buffer. The detection antibody was added to all wells at a concentration of 0.1µg/ml and the plates were covered and incubated for a further 2 hours. Plates were washed three times, and 100µl streptavidin-horseradish peroxidase (HRP) diluted 1:200 from stock, was added

to each well. The plates were covered and incubated for 30 minutes at room temperature. The plates were washed and 100µl substrate solution was added to each well (equal volumes of Colour reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine)) mixed directly before addition to the plates. The reaction was stopped by the addition of 50µl/well 1M H_2SO_4.

The OD of each well was determined using a spectrophotometer read at 450nm while subtracting values at 650nm to correct for optical imperfections in the plate. Concentrations of cytokines were derived from extrapolation from standard curve graphs generated in GraphPad Prism v6.0 (□□□□□□□□□□). The lower limit of detection of the assay was 31.25 pg/ml.

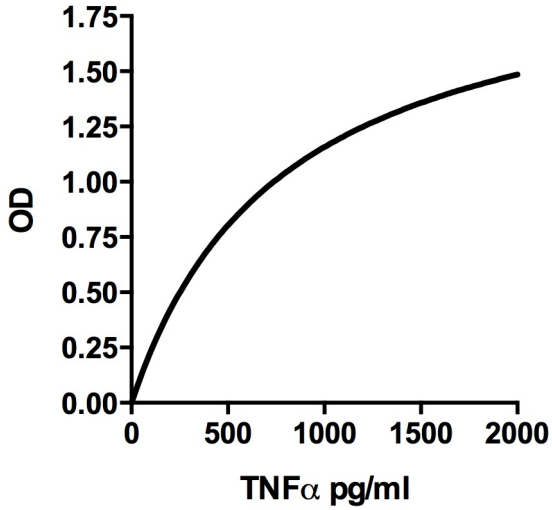


Figure 3.15. Standard curve generated in GraphPad Prism v6.0, used to determine unknown concentrations of TNFα in cell supernatants. The absorbance was read at □450nm.

3.12.2 Measurement of CXCL8

CXCL8 ELISA antibodies were used according to the manufacturer's instructions, and plates were captured with CXCL8 antibody and blocked before use, similarly to the TNF assay described above in section 3.12.1.

Blocking buffer was removed and CXCL8 standards were added to the appropriate wells. MDM cell supernatants, stored after 4 hours phagocytosis with beads or bacteria, were diluted 1:300 using wash buffer (PBS containing 0.05% (v/v) Tween20) and added to the appropriate wells. The remaining CXCL8 ELISA protocol was as described for TNF above.

The OD of each well and subsequent extrapolation from standard curves to calculate concentrations of CXCL8 was determined as described for TNF. The lower limit of detection of the assay was 6.3 ng/ml.

3.13 Statistical analysis

Data were analysed using GraphPad Prism v6.0 (GraphPad Software Inc., San Diego, CA, USA), PASW Statistics version 21 (SPSS Inc., Chicago, IL, USA) or STATA 8.2 (Stata Corporation, College Station, TX). The D'Agostino-Pearson omnibus normality test was used. Normally distributed data were expressed as mean and standard deviation (SD) and non-parametric data as median and interquartile range (IQR). Differences between groups were analysed by independent t-test, Mann-Whitney U Test, paired t-test, Wilcoxon-matched pairs, one-way ANOVA, Friedman test or Kruskal-Wallis analysis with multiple comparisons, depending on the sample population being investigated. Relationships between variables were investigated

using regression analysis. Categorical binary variables were analysed by χ^2-analysis. Survival analysis was analysed using the Kaplan-Meier method and a Gehan-Breslow-Wilcoxon test was applied for comparison of more than two survival curves. A probability of $p<0.05$ was considered to be statistically significant.

Chapter 4:

The relationship between airway bacteria and inflammation at stable and exacerbation states

4.1 Introduction

Historically, the tracheobronchial tree and lung parenchyma in healthy, non-smoking individuals were described as sterile, using traditional, culture-based techniques. However, in COPD patients, bacteria are frequently isolated from both sputum and bronchoscopic samples during periods of stability, termed lower airway bacterial colonisation (LABC) (Banerjee et al., 2004; Patel et al., 2002). *H. influenzae* is often the most commonly isolated PPM at both stable and exacerbation states, with *S. pneumoniae* and *M. catarrhalis* also frequently isolated (Rosell et al., 2005; Sethi and Murphy, 2008). LABC has been shown to have a detrimental influence on the natural history of COPD, with both increased airway and systemic inflammation (Banerjee et al., 2004; Hill et al., 2000; Marin et al., 2012; Sethi et al., 2006), increased exacerbation frequency (Patel et al., 2002), and an accelerated decline in lung function (Wilkinson et al., 2003).

Previous studies of LABC have focused on traditional, culture-based microbiological identification of airway bacteria. However, only 1% of all bacteria can be cultured using traditional methods (Staley and Konopka, 1985), and hence there has been increasing interest in the use of newer, culture-independent diagnostic techniques. Many of these newer techniques rely on the analysis of the bacterial 16S-rRNA gene, which is conserved amongst all bacteria. Studies using such techniques have demonstrated the presence of a wide range of bacterial species in healthy individuals, described as the core microbiome, which may become disrupted in disease states (Erb-Downward et al., 2011). However, these techniques are expensive and both time-consuming to perform and analyse, limiting their use to a small number of patient samples. The culture-independent qPCR technique targeting the commonly

isolated airway bacterial pathogens in COPD, namely *H. influenzae*, *S. pneumoniae* and *M. catarrhalis*, has been shown to be more discriminatory than qualitative culture in detecting a higher prevalence of airway bacteria at both stable and exacerbation states (Garcha et al., 2012). This technique is relatively inexpensive and can be used to examine a far larger number of samples than the more detailed microbiome analysis. However, the relationship between culture-independent bacterial loads and inflammation remains unclear.

Thus, the hypothesis is that qPCR would accurately detect airway pathogens and have a greater diagnostic yield than either qualitative or quantitative bacterial culture, and using qPCR, airway and systemic inflammation would be related to both airway bacterial presence and loads, which would be reflected in worse health status. In addition, as *H. influenzae* is the most frequently isolated bacteria at both stable and exacerbation states, the higher inflammatory response seen in colonised patients would be attributable to *H. influenzae*. Furthermore, the hypothesis is that at exacerbation, increases in airway inflammation would be related to changes in airway bacterial load, which would influence characteristics of the exacerbation itself.

Work from this chapter formed the basis of an original publication published in Respiratory Research:

Inflammatory thresholds and the species-specific effects of colonising bacteria in stable chronic obstructive pulmonary disease.

Singh R, Mackay AJ, Patel AR, Garcha DS, Kowlessar BS, Brill SE, Donnelly LE, Barnes PJ, Donaldson GC, Wedzicha JA.

Respir Res. 2014 Sep 14;15:114. doi: 10.1186/s12931-014-0114-1.

4.2 Methods

4.2.1 Patient recruitment

One hundred and twenty-two COPD patients enrolled in the London COPD cohort between January 2011 and July 2013 were included (section 3.1). The patients form part of a rolling cohort used to prospectively investigate the pathophysiology of COPD and its associated exacerbations (section 3.1.1 and 3.1.2).

4.2.2 Clinic visits

Patients completed daily diary cards for symptoms and were prospectively reviewed in clinic every three months when stable during the study period (section 3.3.1). At stable visits they were asked to perform spirometry, complete health assessment questionnaires and provide serum and sputum samples.

In a subset of patients, sampling at exacerbation was performed, with exacerbations defined according to daily symptom diary card criteria (section 3.3.2). Only exacerbations reported to the study team were sampled prior to the addition of any systemic therapy. Exacerbation duration and severity, as defined by symptom count at exacerbation onset, was determined using daily symptom diary cards (section 3.3.2). COPD exacerbation frequency was determined using diary card events or exacerbation recall (section 3.5).

4.2.3 Blood inflammatory analysis

Serum CRP quantification was performed using Modular Analytics E 170 Module (Roche, Burgess Hill, UK) and plasma fibrinogen measured using the Clauss method (IL ACL Top Coagulation Analyzer, Lexington, MA, USA) (section 3.10).

4.2.4 Sputum sampling, diagnostic microbiology and microbiological definitions

At stable and physician reported exacerbation visits, patients were asked to spontaneously expectorate sputum samples into a sterile pot. Patients unable to spontaneously expectorate sputum underwent sputum induction (section 3.6.2), and samples were colour graded using the BronkoTest® colour chart (section 3.6.3). Sputum samples were processed as soon as possible following collection to prevent RNA degradation (section 3.6.4).

On all sputum samples included, qPCR was carried out in the Centre for Clinical Microbiology, University College London, to detect and measure the load of the three common airway bacterial pathogens, *H. influenzae, S. pneumoniae* and *M. catarrhalis* as previously described (Garcha et al., 2012) (section 3.7.3). The lower limit of detection of the qPCR technique was 10^4 cfu/ml.

If sufficient sputum was obtained, an aliquot was sent for microbiological culture, either qualitative culture, carried out in the Department of Medical Microbiology, Royal Free Hospital (section 3.7.1) or quantitative culture, carried out in the Centre for Clinical Microbiology, University College London (section 3.7.2).

Patients were defined at colonised if one or more bacterial pathogen was detected by qPCR on sputum sample at recruitment. When two consecutive stable sputum samples were used to reflect the dynamics of bacterial colonisation, patients were described as never colonised, if both samples were negative for bacterial pathogen detection; persistently colonised, if both samples were positive; gaining colonisation, if the first sample was negative and the second positive; and losing colonisation if the first sample was positive and the second negative.

4.2.5 Airway biomarker analysis

Levels of interleukin (IL)-8, IL-1 and myeloperoxidase (MPO) in the PBS-processed supernatants were measured using high sensitivity enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Abingdon, UK) as described in section 3.9.1. The lower limit of detection in these assays were 3.5 $pg.ml^{-1}$, <1.0 $pg.ml^{-1}$ and 0.014 $ng.ml^{-1}$ for IL-8, IL-1 and MPO respectively.

4.2.3 Statistical Analysis

Data were analysed using GraphPad PRISM version 6.0 (GraphPad Software Inc., San Diego, CA, USA) and PASW Statistics version 21 (SPSS Inc., Chicago, IL, USA). Details of the statistical tests used are reported in section 3.13

4.2.4 Ethical considerations

Ethical approval for the study was granted from the Royal Free Hospital research ethics committee and all patients gave written informed consent.

4.3 Results

This study involved 122 patients providing 371 sputum samples between January 2011 and July 2013. All 122 patients provided at least one sputum sample for the analysis comparing diagnostic microbiology techniques, and 99 patients provided samples included in the analysis of airway bacteria and inflammation at stable and exacerbation states. [illegible] shows a consort diagram of patient and samples included in each sub-analyses. The clinical demographics of the 122 patients are included in [illegible].

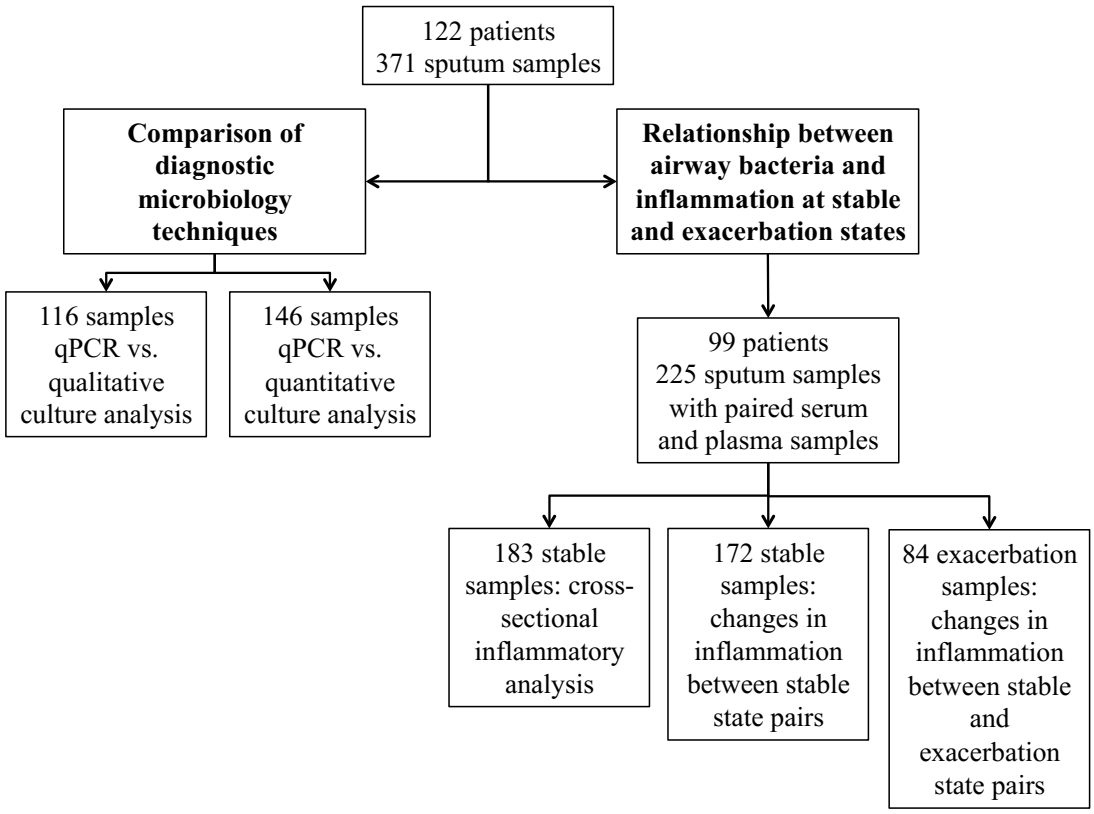


Figure 4.16. Consort diagram to show patient and sample numbers included in the comparison of diagnostic microbiology techniques and the relationship between airway bacteria and inflammation at stable and exacerbation states.

[illegible]
[illegible]
[illegible]

[illegible] [illegible]

	All patients n=122
Mean (SD) Age (years)	71.1 (9.93)
Mean (SD) FEV_1 (L)	1.31 (0.47)
Mean (SD) FEV_1 (% predicted)	51.4 (20.9)
Mean (SD) FVC (L)	2.77 (0.90)
Mean (SD) FEV_1 / FVC ratio (%)	47.9 (13.7)
Median [IQR] Exacerbation frequency	2.00 [1.00-3.00]
Median [IQR] Smoking pack years	47.0 [26.0-64.5]
Median [IQR] ICS dose (beclomethasone equivalent □g)	1500 [1000-2000]
N (%) Male gender	79 (65)
N (%) Current Smokers	45 (37)
N (%) Chronic Bronchitis	95 (78)

Table 4.12. Clinical demographics of the 122 COPD patients included. ICS: inhaled corticosteroids.

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4.3.1 Comparison of diagnostic microbiology techniques

To determine the relationship between culture-independent qPCR and traditional culture techniques for the detection of bacteria, 262 sputum samples were analysed by qPCR, of which 116 samples had concurrent analysis by qualitative culture for detection of bacteria only and 146 samples had concurrent analysis by quantitative culture (QC) enabling detection of both bacteria and load. Both stable and exacerbation samples were included in the dataset, as comparison was only being made on the detection of bacteria and the load.

In the 116 samples with both qPCR and qualitative culture performed, only 17/116 (11%) samples had bacteria identified on culture; 7 *H. influenzae*, 1 *M. catarrhalis*, 5 *S. pneumoniae*, 2 *Pseudomonas aeruginosa*, 1 *Staphylococcus aureus* and 1 *Proteus*

vulgaris, compared to 45/116 (39%) samples with bacteria detected by qPCR (χ^2, p<0.001).

Overall, 4/17 (24%) samples with bacteria detected by qualitative culture had a concurrent negative qPCR result for the detection of PPMs, of which only 1/17 (6%) was for *H. influenzae*. The remaining 3 culture positive but qPCR negative samples had the bacteria *Pseudomonas aeruginosa*, *Staphylococcus aureus* or *Proteus vulgaris,* which would not be identified by the qPCR technique used in this study.

In the 146 samples with both qPCR and QC analyses performed, 92/146 (63%) samples had bacteria detected by qPCR compared to 48/146 (33%) by QC (χ^2, p<0.001). [illegible] shows the number and frequency of detection of the three bacteria identified by qPCR and of the same bacteria by QC.

Bacteria	N (%) samples with bacteria detected		p-value
	qPCR (n=92)	QC (n=48)	
H. influenzae	57 (62)	29 (60)	0.857
S. pneumoniae	32 (35)	7 (15)	0.016
M. catarrhalis	22 (24)	13 (27)	0.686

Table 4.13. Number and frequency of the three bacteria detected by qPCR and of the same bacteria as detected by quantitative culture (QC) in the 146 samples with both qPCR and QC performed. Samples may have ≥1 bacteria detected.

H. influenzae was the most frequently isolated bacteria by both qPCR and QC methods. There was no significant difference between the frequency of *H. influenzae* (57/92 (62%) vs. 29/48 (60%), p=0.857) or *M. catarrhalis* (22/92 (24%) vs. 13/48

(27%), p=0.686) detected by the two techniques. However, the frequency of *S. pneumoniae* detection was significantly higher by qPCR than by quantitative culture (32/92 (35%) vs. 7/48 (15%), p=0.016). In 8 samples, bacteria other than the three tested by this qPCR technique were detected by quantitative culture: 3 *H. parainfluenzae*, 2 *Pseudomonas aeruginosa*, 1 *Klebsiella pneumoniae*, 1 *Bordetella bronchiseptica,* and 1 *Stenotrophomonas maltophilia.*

There were 42 sputum samples in which bacteria were detected by both qPCR and quantitative culture. The $\log_{10}$ total bacterial load measured by both methods (i.e. only *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* loads) was compared using a Bland-Altman plot (□□□□□□□□□□).

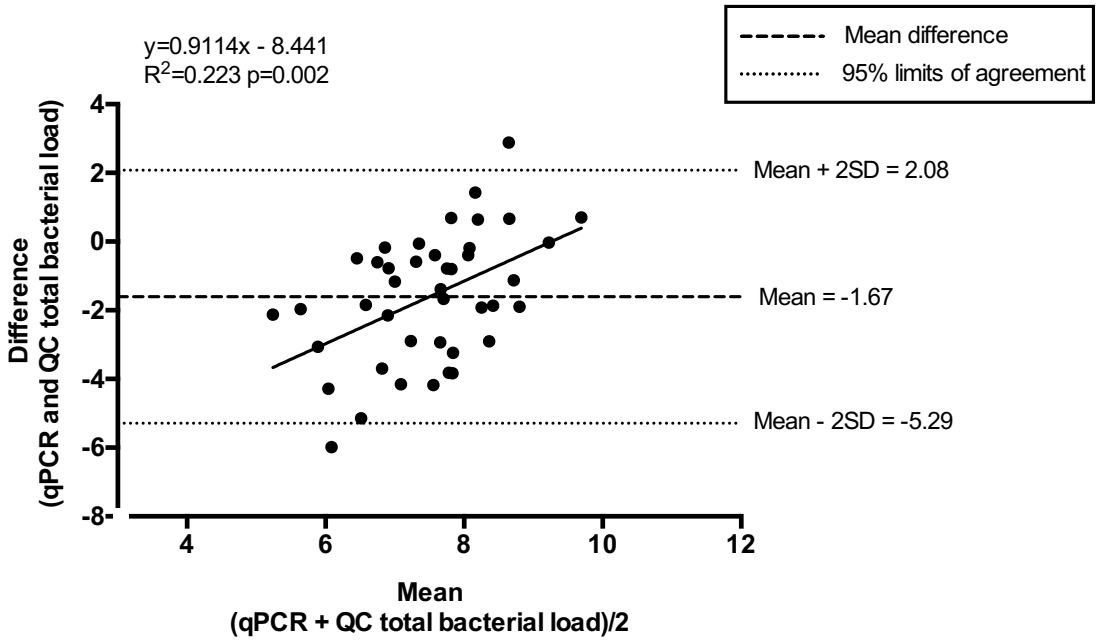


Figure 4.17. Bland-Altman plot showing the difference and average of total bacterial load measured by qPCR and quantitative culture (QC), n=42. As the qPCR technique only detects three bacteria (*H. influenzae*, *S. pneumoniae* and *M. catarrhalis*) the total load detected by QC included the same three bacteria for comparison.

Although agreement between the two methods is seen, the mean difference between qPCR and QC was -1.67 with limits of agreement of 2.08 to -5.29. Linear regression analysis demonstrated a significant regression line (p=0.002), suggesting the existence of proportional bias, with closer agreement between the two methods as the total bacterial load increases.

Due to the high diagnostic yield for the detection of bacteria by qPCR, all further analyses herein will use qPCR as the primary method to detect and measure airway bacteria.

4.3.2 Relationship between airway bacteria detected by qPCR and inflammation at stable and exacerbation states

Ninety-nine COPD patients provided 225 sputum samples for inflammatory analysis, of which 183 were stable samples and 42 were exacerbation samples. Their baseline characteristics are reported in □□□□□□□□□. Patients were sub-grouped into colonised (LABC) and non-LABC based on their sputum sample qPCR result at study recruitment. There were no significant differences in baseline characteristics between the two groups.

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	Overall n=99	Non-colonised n=64	Colonised n=35	p-value †
Mean (SD) Age (years)	72.1 (8.9)	72.0 (9.3)	72.0 (9.3)	0.947
Mean (SD) FEV_1 (L)	1.32 (0.54)	1.36 (0.59)	1.24 (0.44)	0.318
Mean (SD) FEV_1 (% predicted)	51.5 (21.6)	53.3 (21.7)	48.1 (21.4)	0.257
Mean (SD) FVC (L)	2.80 (0.86)	2.84 (0.93)	2.74 (0.73)	0.607
Mean (SD) FEV_1 / FVC ratio (%)	47.2 (13.0)	48.0 (13.4)	45.9 (12.3)	0.440
Median [IQR] Exacerbation frequency	2.00 [1.00-3.00]	1.97 [1.04-3.03]	2.04 [1.00-2.89]	0.511
Median [IQR] Smoking pack years	48.4 [24.4-67.5]	47.2 [25.4-64.7]	51.0 [25.4-105.6]	0.213
Median [IQR] ICS dose (beclomethasone equivalent □g)	1000 [500-2000]	1000 [500-2000]	1000 [500-2000]	0.224
N (%) Male gender	66 (67)	40 (63)	26 (74)	0.234
N (%) Current Smokers	34 (34)	23 (36)	11 (31)	0.651
N (%) Chronic Bronchitis	78 (79)	49 (77)	29 (83)	0.464

Table 4.14. Clinical characteristics of stable COPD patients, and by colonisation status at study onset. Bronchial colonisation defined as sputum positive for *H. influenzae*, *S. pneumoniae* and/or *M. catarrhalis* using qPCR. Parametric data displayed as mean (SD), non-parametric data displayed as median [IQR] and categorical data displayed as N (%). †p-value refers to unpaired t-test, Mann-Whitney U or χ^2 test between non-colonised and colonised samples. ICS: inhaled corticosteroids.

4.3.2.1 Stable state sputum bacterial isolates and loads

One or more bacteria were identified by qPCR in 64/183 (35%) of stable sputum samples and these samples were defined as colonised. A minimum of one colonised sample was obtained from 45/99 (45%) patients during the study period. Single *H.*

influenzae or *S. pneumoniae* isolation was equally prevalent, with each identified in 21/64 (33%) of all positive samples. Polymicrobial detection (mixed PPMs) was identified in 15/64 (23%) of samples, and *M. catarrhalis* detected in 7/64 (11%) (□□□□□□□□□A).

The mean bacterial load for all bacteria was $10^{7.1(SD1.7)}$ cfu.ml^{-1}. The bacterial loads detected for individual *H. influenzae*, *S. pneumoniae*, *M. catarrhalis*, and mixed isolates were significantly different ($10^{6.2(1.2)}$ vs. $10^{6.5(1.1)}$, vs. $10^{8.6(1.8)}$ and $10^{8.5(1.5)}$ cfu.ml^{-1} respectively, p<0.001, □□□□□□□□□B) and when each species load was compared, the *H. influenzae* and *S. pneumoniae* loads were similar to each other but were both significantly lower than the *M. catarrhalis* and mixed loads.

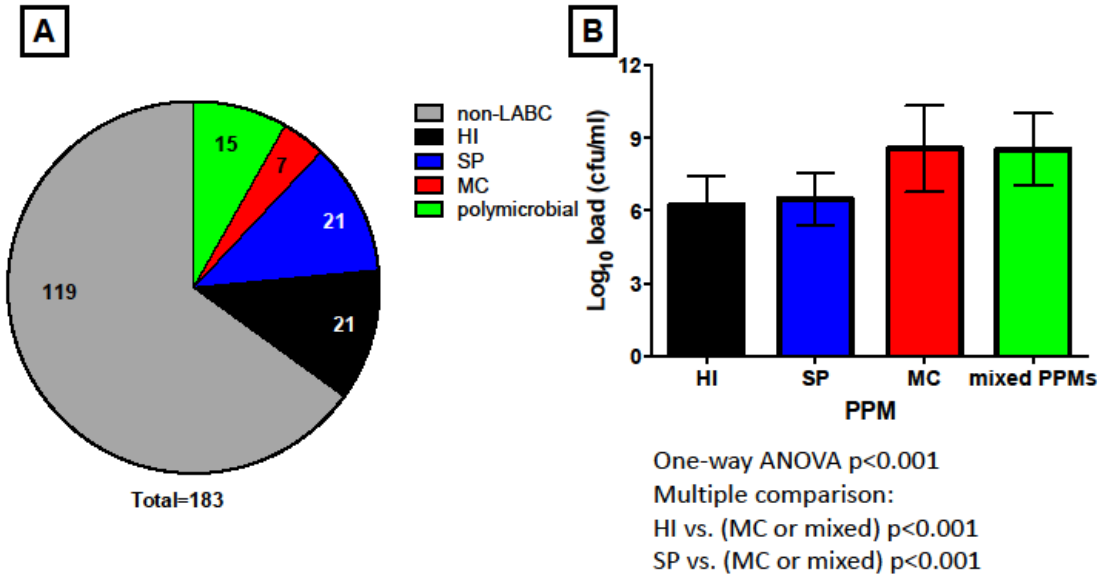


Figure 4.18. Distribution (A) and loads (B) of the PPMs identified by qPCR. There was a significant difference between bacterial loads for the individual *H. influenzae* (HI), *S. pneumoniae* (SP), *M. catarrhalis* (MC) and polymicrobial (mixed) isolates (p<0.001), and on multiple comparison both HI and SP loads were significantly lower than both MC and mixed samples (p<0.001). Data in (A) presented as absolute numbers and in (B) as mean (SD).

4.3.2.2 Bacterial colonisation status and BronkoTest® colour

172/183 (94%) of all stable samples had sputum colour recorded using the standardised BronkoTest® colour chart. The proportion of positive (LABC) sputum samples for any bacteria identified by qPCR was significantly higher with darker (higher BronkoTest® number) sputum (χ^2, p=0.001, [illegible]A).

57/64 (89%) LABC samples had sputum colour recorded. Increasing total bacterial load was significantly associated with a higher BronkoTest® colour (rho=0.39; p=0.003, [illegible]B), but no significant difference was seen between the BronkoTest® colour and the isolation of the different PPMs (p=0.817).

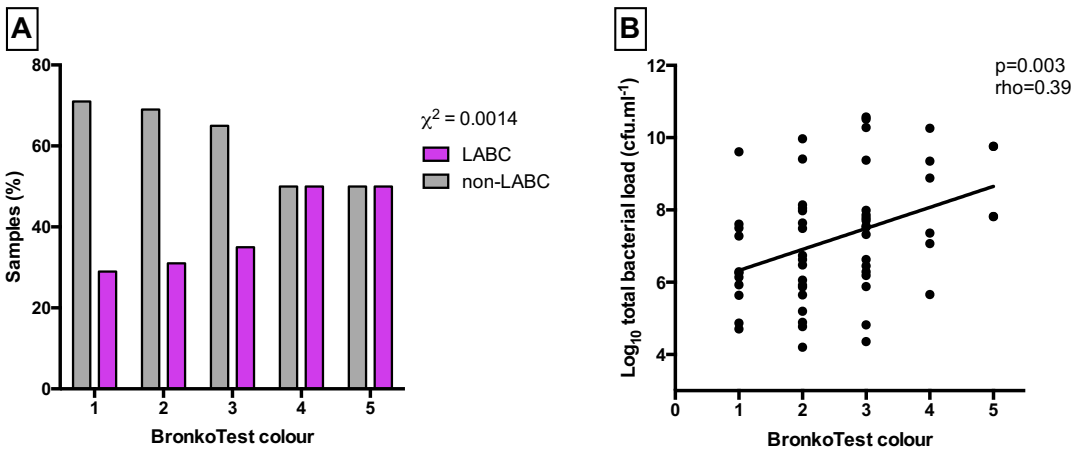


Figure 4.19. (A) Proportion of colonised (LABC) and non-LABC sputum samples according to BronkoTest® colour chart. (B) Relationship between BronkoTest® sputum colour and total bacterial load using qPCR. The proportion of LABC samples was significantly higher with darker sputum colour (p=0.0014, n=172). Increasing total bacterial load was significantly associated with darker sputum colour (p=0.003, n=57).

4.3.2.3 Bacterial colonisation and inflammation

Samples with LABC had significantly higher levels of sputum CXCL8, IL-1□ and MPO than non-LABC samples (p<0.001 for all measured cytokines, □□□□□□□□□).

Plasma Fibrinogen was significantly higher in LABC samples than in non-LABC samples (3.8 [3.3-4.4] vs. 3.5 [3.2-4.1] $g.l^{-1}$, p=0.049). However, there was no significant difference in serum CRP between LABC and non-LABC samples (2.0 [1.0-5.0] vs. 2.0 [1.0-8.0] $mg.l^{-1}$, p=0.261).

	Non-colonised (n=119)	Colonised (n=64)	p-value
Median [IQR] CXCL8 ($ng.ml^{-1}$)	83 [24-182]	162 [71-309]	<0.001
Median [IQR] IL-1□($ng.ml^{-1}$)	0.6 [0.2-1.9]	1.4 [0.7-5.9]	<0.001
Median [IQR] MPO (□$g.ml^{-1}$)	17.1 [7.6-30.6]	29.5 [16.2-40.9]	<0.001
Median [IQR] CRP ($mg.l^{-1}$)	2.0 [1.0-8.0]	2.0 [1.0-5.0]	0.261
Median [IQR] Fibrinogen ($g.l^{-1}$)	3.5 [3.2-4.1]	3.8 [3.3-4.4]	0.049

Table 4.15. Airway and systemic inflammation in colonised and non-colonised samples. n refers to the number of samples in each group.

□

Increasing total bacterial load was significantly associated with increasing CXCL8, IL-1□ and MPO (rho=0.44; p<0.001, rho=0.45; p<0.001 and rho=0.32; p=0.011 respectively, □□□□□□□□□). All measured airway cytokines significantly correlated with each other (for all 3 correlations, rho>0.60; p<0.001). There were no significant associations between the measured airway cytokines and clinical demographics including stable state FEV_1 % predicted, exacerbation frequency, smoking status or

pack year history (PYH) or inhaled corticosteroid use and dose (all p>0.05, [illegible]).

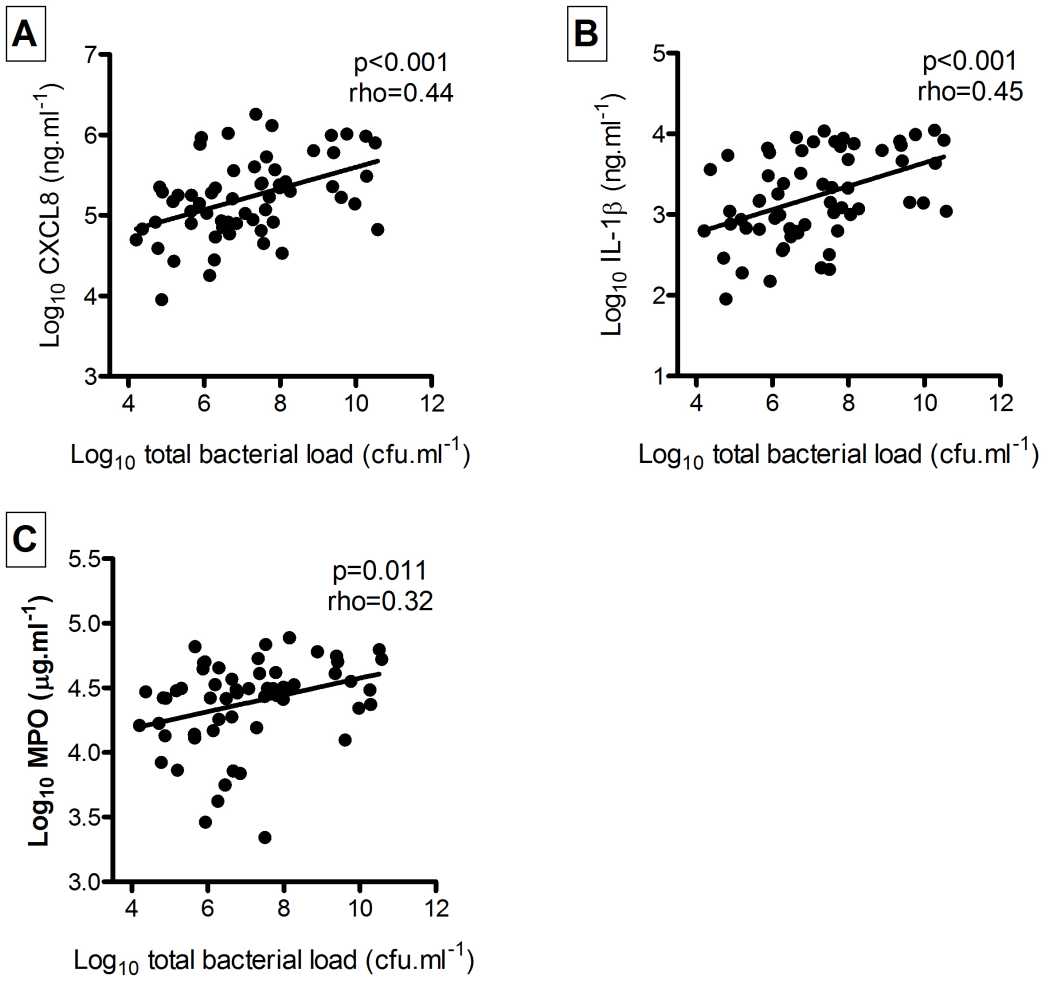


Figure 4.20. Relationship between total bacterial load as measured by qPCR and (A) CXCL8, (B) IL-1β and (C) MPO. There was a significant relationship between total bacterial load and all measured airway cytokines (p<0.001 for CXCL8 and IL-1□, and p=0.011 for MPO, n=64).

[illegible]

	p-value		
	CXCL8	IL-1□	MPO
FEV1 % predicted†	0.917	0.882	0.137
Exacerbation frequency†	0.172	0.211	0.658
Pack year history†	0.182	0.843	0.085
Smoking status§	0.205	0.337	0.267
ICS use*	0.543	0.217	0.756
ICS dose†	0.727	0.538	0.484

Table 4.16. Relationships between airway inflammation and clinical demographics from 99 stable COPD patients. Only the first sample was used from each patient to avoid complications with repeated measures. Definitions: ICS: inhaled corticosteroids. †p-value refers to Spearman's rank correlation between airway cytokines and clinical demographics. §p-value refers to Mann-Whitney U test between current smokers and ex-smokers. *p-value refers to Mann-Whitney U test between patients with concurrent ICS use and those without.

Unlike airway inflammation, increasing total bacterial load was not significantly associated with either an increase in serum CRP (rho=0.13; p=0.287) or plasma fibrinogen (rho=0.201; p=0.112), although CRP was significantly correlated with fibrinogen (rho=0.478; p<0.001). There were no significant associations between CRP or fibrinogen and clinical demographics (p>0.05 for all).

Using the mean bacterial load, LABC samples were sub-grouped into those with low total bacterial loads (□$10^{7.0}$ cfu.ml^{-1}, n=32) and those with high total bacterial loads (>$10^{7.0}$ cfu.ml^{-1}, n=32) and compared with non-LABC samples. There was a significant difference between the three sub-groups for all measured cytokines (p<0.001). When each group was compared with each other, the levels of the airway

cytokines were similar between the non-LABC and low total bacterial load groups, but the high total bacterial load had significantly higher levels of airway cytokines than both of these groups (□□□□□□□□□), suggesting a possible bacterial load threshold for increased airway inflammation.

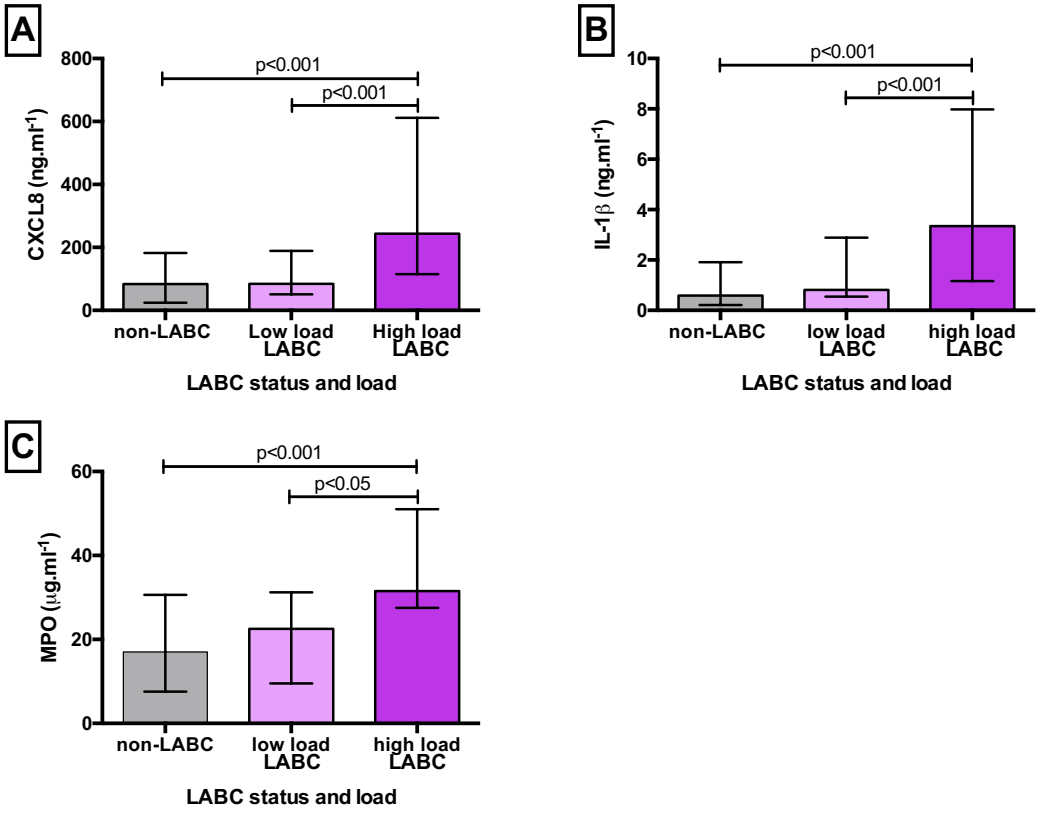


Figure 4.21. Inflammatory thresholds of (A) CXCL8, (B) IL-1β and (C) MPO. Low load was defined as a total bacterial load of □$10^{7.0}$ cfu.ml^{-1} (n=32) and high load as >$10^{7.0}$ cfu.ml^{-1} (n=32). There was a significant difference between the three subgroups for all measured cytokines (p<0.001), and on multiple comparison there was no significant difference between non-colonised (LABC) and low load LABC samples for all airway cytokines, but the high load LABC samples had significantly higher levels than both non-LABC and low load LABC groups. Data are presented as median and interquartile range.

Increasing *H. influenzae* load was significantly associated with higher CXCL8 and IL-1□response (rho=0.507; p=0.02, rho=0.635; p=0.002 respectively) and with a trend in MPO (p=0.07). Increasing *S. pneumoniae* load was significantly associated with higher CXCL8 and MPO response (rho=0.514; p=0.02, rho=0.503; p=0.03 respectively) and there was a trend with IL-1□(p=0.147). There was a trend towards an increase in the measured cytokines with higher *M. catarrhalis* loads, but this did not reach statistical significance due to the low numbers of samples positive for *M. catarrhalis* alone (7/64 (11%)).

4.3.2.4 Species-specific inflammatory responses

There was a significant difference in CXCL8, IL-1□and MPO response between the isolation of individual bacteria and mixed colonised samples (p=0.003, p<0.001 and p=0.011 respectively, □□□□□□□□).

	HI	SP	MC	Mixed	p-value †
Mean (SD) Bacterial load	$10^{6.2(1.2)}$	$10^{6.5(1.1)}$	$10^{8.6(1.8)}$	$10^{8.5(1.5)}$	<0.001
Median [IQR] CXCL8 ($ng.ml^{-1}$)	179 [95-453]	90 [50-194]	219 [140-309]	127 [67-677]	0.003
Median [IQR] IL-1□($ng.ml^{-1}$)	4.8 [0.8-7.8]	0.9 [0.6-1.5]	1.4 [0.4-7.2]	1.6 [0.9-6.7]	<0.001
Median [IQR] MPO (□$g.ml^{-1}$)	30.1 [26.4-38.9]	27.2 [13.3-37.6]	23.6 [18.1-35.6]	29.1 [11.1-51.0]	0.011

Table 4.17. Bacterial loads and associated airway inflammation in single and polymicrobial (mixed) PPM samples. †p-value refers to one-way ANOVA or Kruskal-Wallis between the single and polymicrobial PPM samples. Parametric data displayed as mean (SD), non-parametric data displayed as median [IQR]. HI: *H. influenzae*, SP: *S. pneumoniae*, MC: *M. catarrhalis*.

Despite similar bacterial loads in *H. influenzae*- and *S. pneumoniae*-colonised samples and higher bacterial loads in *M. catarrhalis*- and mixed samples, CXCL8 was significantly higher only in *H. influenzae*-colonised samples compared to non-LABC samples (179 [95-453] vs. 83 [24-182] ng.ml^{-}1, p<0.001, [illegible] [illegible]A). Furthermore, both IL-1□ and MPO levels were significantly higher in *H. influenzae*-colonised samples compared to non-LABC samples (4.8 [0.8-7.8] vs. 0.6 [0.2-1.9] ng.ml^{-1}, p<0.001 [illegible]B; 30.1 [26.4-38.9] vs. 17.1 [7.6-30.6] µg.ml^{-1}, p<0.05 [illegible]C).

Mixed samples also had a significantly higher IL-1□ level than non-LABC samples (1.6 [0.9-6.7] vs. 0.6 [0.2-1.9] ng.ml^{-1}, p<0.05, [illegible]B). However, despite a higher total bacterial load in mixed samples compared to *H. influenzae*-colonised samples, no significant augmentation of airway inflammatory responses was seen.

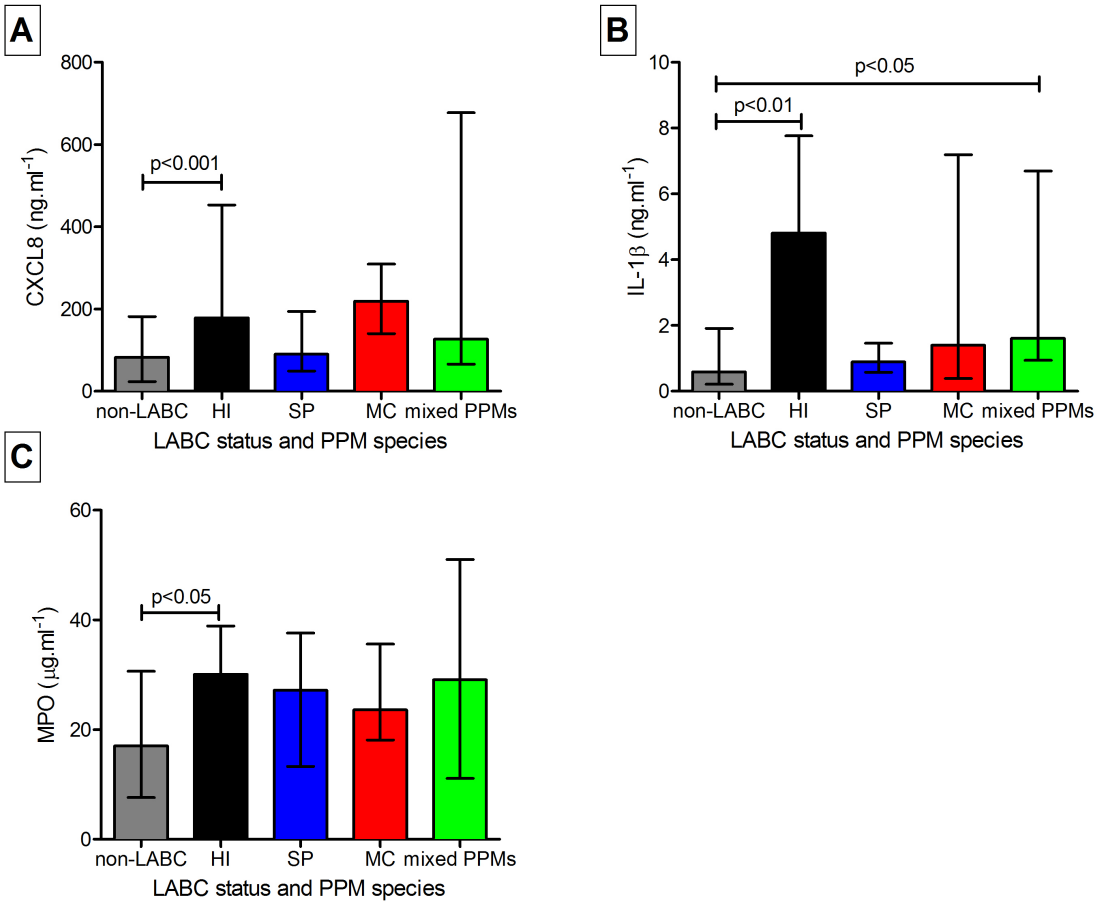


Figure 4.22. The species-specific effect of potentially pathogenic micro-organisms (PPMs) on (A) CXCL8, (B) IL-1β and (C) MPO. There was a significant difference in CXCL8, IL-1□and MPO response between non-colonised (LABC) samples and the isolation of individual PPMs (HI: *H. influenzae*, SP: *S. pneumoniae*, MC: *M. catarrhalis*) and mixed PPMs (p=0.003, p<0.001 and p=0.011 respectively). On multiple comparisons, only *H. influenzae* demonstrated significantly higher levels for all airway cytokines than non-LABC samples. In addition, mixed PPMs demonstrated higher IL-1□ response than non-LABC samples (p<0.05). Data are presented as median and interquartile range, n=183.

Adjusting for the loads of the pathogens, *H. influenzae*-colonised samples were associated with a significantly higher CXCL8, IL-1□ and MPO response than individual *S. pneumoniae*- or *M. catarrhalis*-colonised and the mixed-colonised samples on multiple regression analysis (p<0.001, p<0.001 and p=0.002 respectively,

□□□□□□□□□□). The rate of rise in the measured airway cytokines with increasing bacterial load was not significantly different between the different species (all cytokines, p>0.05).

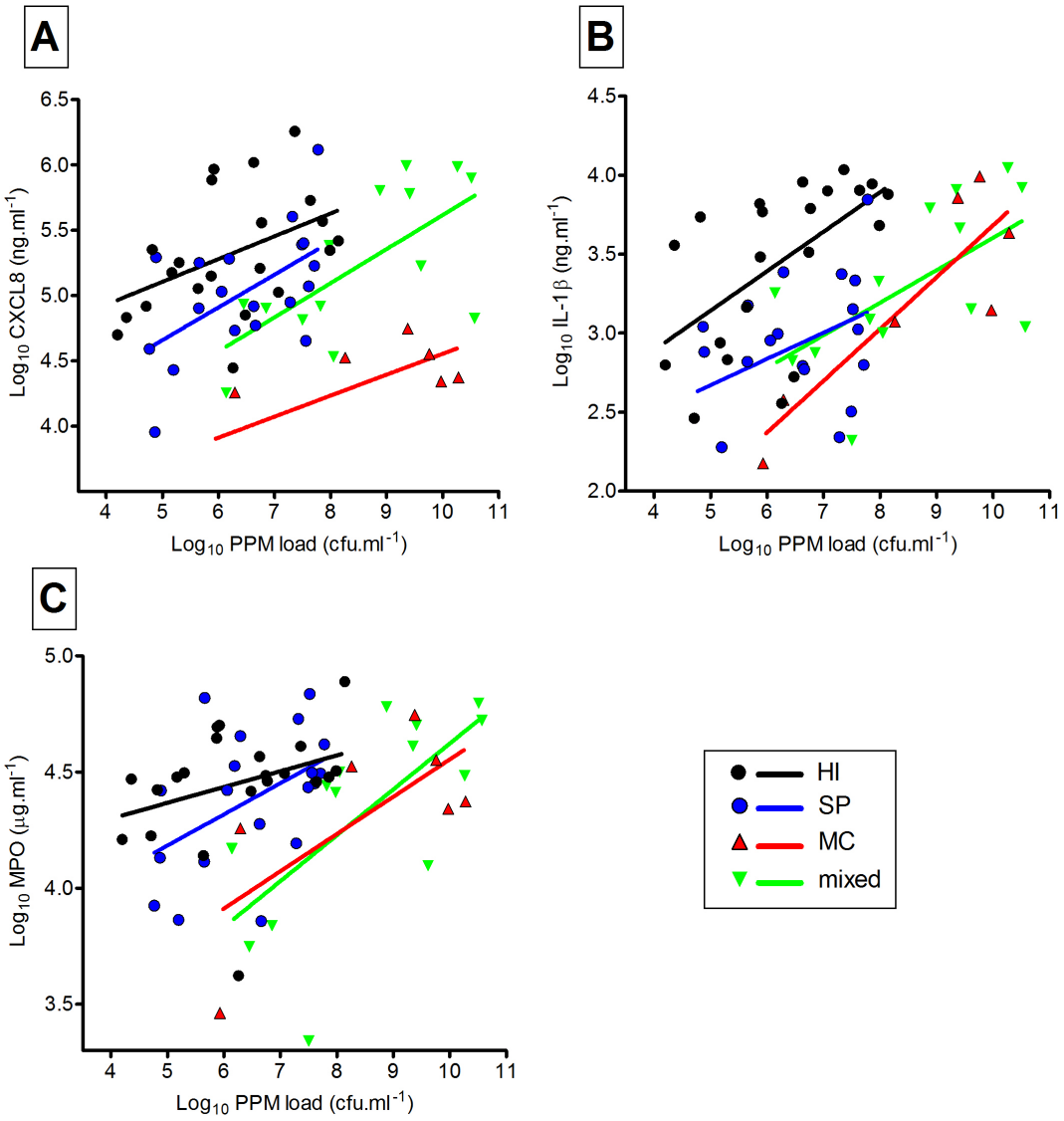


Figure 4.23. Multiple regression analysis showing change in (A) CXCL8, (B) IL-1β and (C) MPO in relation to the bacterial load of single isolate *H. influenzae* (HI), *S. pneumoniae* (SP) and *M. catarrhalis* (MC) and polymicrobial (mixed) samples. *H. influenzae*-colonised samples were associated with a significantly higher CXCL8, IL-1□ and MPO response than the individual *S. pneumoniae* or *M. catarrhalis*-colonised and mixed colonised samples (p<0.001, p<0.001 and p=0.002 respectively, n=64).

There were no species-specific differences in systemic inflammatory responses as measured by serum CRP or plasma fibrinogen (p=0.879 and p=0.247, respectively, [illegible]).

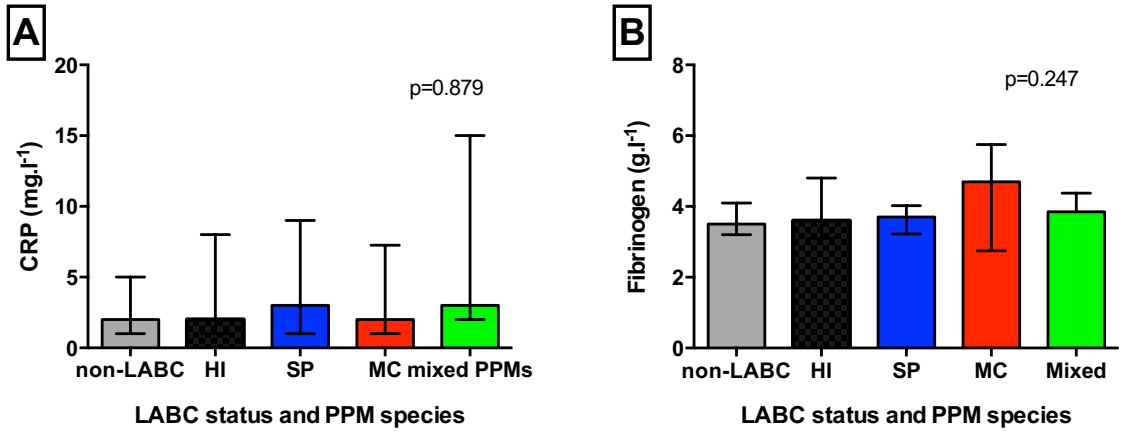


Figure 4.24. The species-specific effect of potentially pathogenic micro-organisms (PPMs) on systemic inflammation as measured by (A) C-reactive protein (CRP) and (B) Fibrinogen. Unlike airway inflammation, no species-significant effect of the different stable state PPMs was observed in either CRP or fibrinogen. p-value refers to Kruskal-Wallis test between the four groups.

4.3.2.5 Bacterial colonisation and health status

74 SGRQs and 113 CAT scores were available with paired sputum samples. Increasing CAT score was significantly associated with higher total SGRQ score (rho=0.502, p<0.001). Discordance was observed between inflammation and patient reported outcomes. In a univariate analysis, no significant association was observed between increasing SGRQ, or CAT scores with either airway inflammation ([illegible] and [illegible] respectively), or with systemic inflammation.

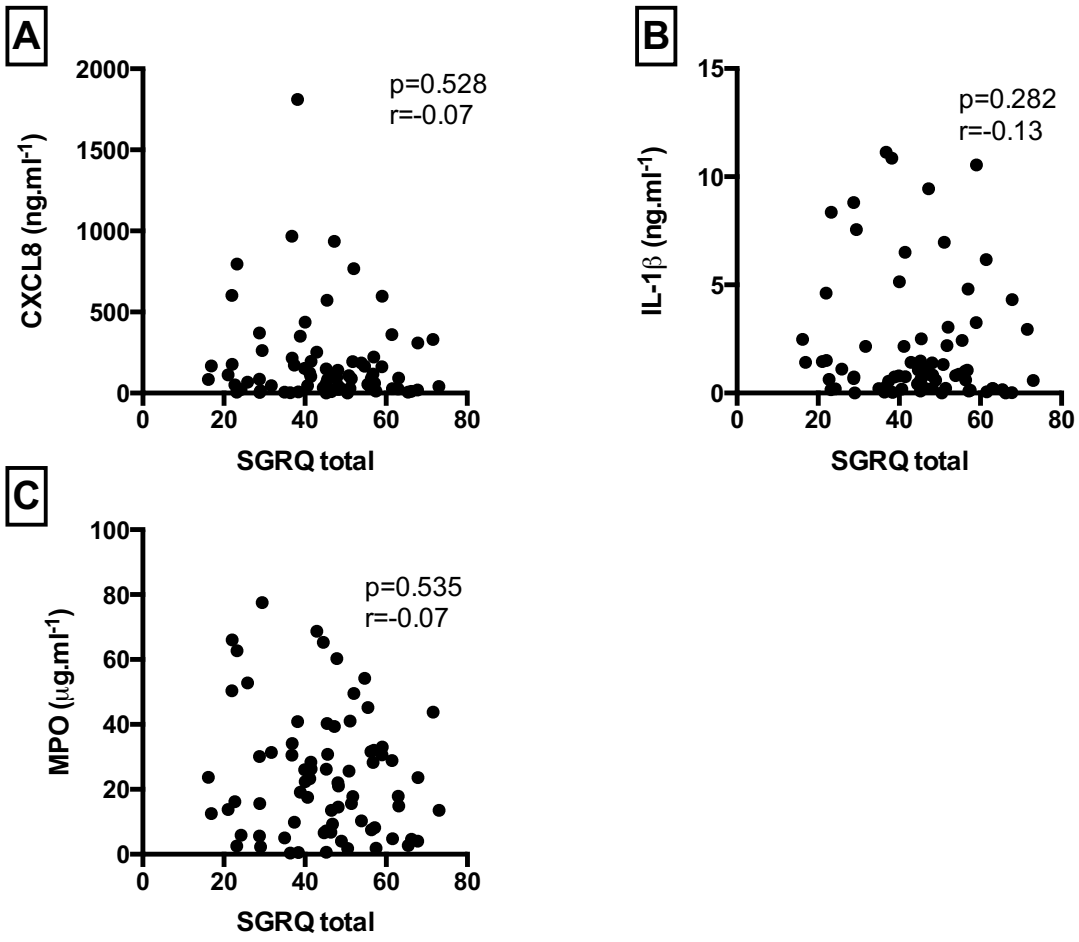


Figure 4.25. Relationship between St Georges' Respiratory Questionnaire (SGRQ) and airway inflammation as measured by (A) CXCL8, (B) IL-1β and (C) MPO. A lack of concordance was observed between the patient reported outcome and airway inflammation cytokines (n=74).

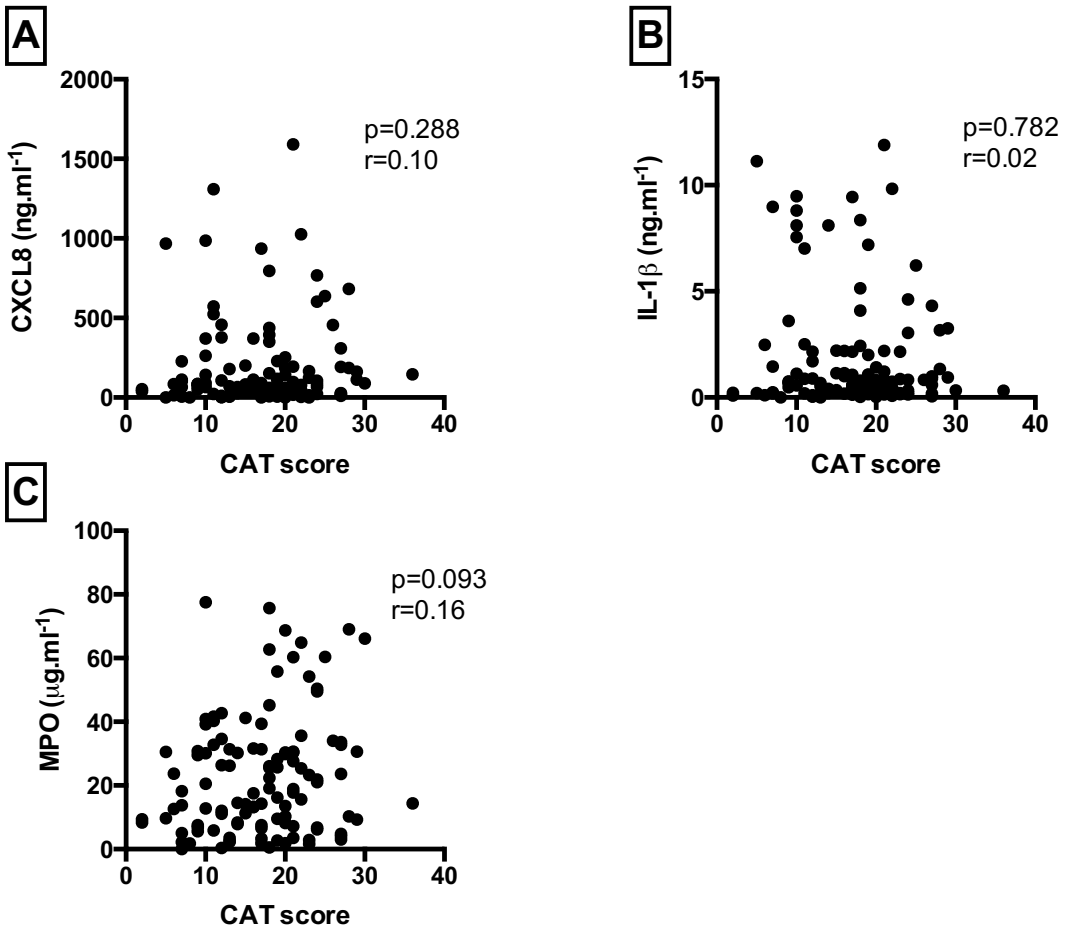


Figure 4.26. Relationship between COPD Assessment Test (CAT) and airway inflammation as measured by (A) CXCL8, (B) IL-1β and (C) MPO. Discordance was observed between the patient reported outcome and airway inflammation cytokines (n=113).

In addition there was no significant association between SGRQ or CAT score with either airway or systemic inflammation in a multivariate analysis taking into account FEV_1 % predicted and exacerbation frequency (all, $p>0.05$). No species-specific effects on either SGRQ or CAT scores were observed ($p>0.05$).

There was no significant difference between total SGRQ and CAT scores in non-colonised and colonised samples ($p>0.05$, [illegible]), although non-colonised

patients had statistically significantly higher SGRQ impact score than colonised patients.

	Non-colonised	Colonised	p-value
Mean CAT (SD)	17.1 (6.6)	16.2 (7.0)	0.556
Mean SGRQ (SD)			
Total	46.8 (12.6)	40.3 (15.0)	0.055
Symptoms	62.3 (17.5)	58.9 (14.5)	0.402
Activity	68.0 (16.6)	59.7 (23.7)	0.087
Impact	30.4 (11.6)	23.4 (13.9)	0.031

Table 4.18. CAT and SGRQ scores in non-colonised and colonised patients.

4.3.2.6 Changes in LABC status and the effect on inflammation

To determine whether changes in LABC status affected changes in either airway or systemic inflammation, consecutive paired stable samples from the same individual were analysed. Changes in the stable state bacteriology between the paired samples were described as never colonised, if both samples were negative for bacterial pathogen detection; persistently colonised, if both samples were positive; gaining colonisation, if the first sample was negative and the second positive; and losing colonisation if the first sample was positive and the second negative. Due to the numerous possibilities of changes between bacterial presence, species and load, only bacterial presence, irrespective of species or load changes was used to define colonisation.

Within the 183 stable sputum samples, 29 patients had evidence of paired never colonised samples, with a median time between sampling of 3.9 [3.0-5.2] months. There was no significant difference in airway or systemic inflammation between the two visits (□□□□□□□□□).

	LABC status at first stable visit	LABC status at second stable visit	
	Negative	Negative	p-value
Median [IQR] CXCL8 ($ng.ml^{-1}$)	105 [26-253]	79 [40-170]	0.721
Median [IQR] IL-1□ ($ng.ml^{-1}$)	0.7 [0.2-3.8]	1.3 [0.2-0.5]	0.286
Median [IQR] MPO ($\mu g.ml^{-1)}$)	15.6 [5.8-33.5]	17.5 [7.4-26.2]	0.843
Median [IQR] CRP ($mg.l^{-1)}$)	2.0 [1.0-4.0]	2.0 [1.0-4.0]	0.775
Median [IQR] Fibrinogen($g.l^{-1}$)	3.7 [3.2-4.2]	3.5 [3.1-4.1]	0.401

Table 4.19. Changes in inflammation in patients with never colonised sputum sample pairs. Never colonised samples were defined as both pairs being negative for the detection of the three potentially pathogenic bacteria detected by qPCR (n=29).

18 patients had persistently colonised samples, with a median time between sampling of 3.3 [3.0-7.5] months. Similarly to the never colonised samples, there was no significant difference in airway or systemic inflammation between the two visits (□□□□□□□□□).

	LABC status at first stable visit	LABC status at second stable visit	
	Positive	Positive	p-value
Median [IQR] CXCL8 ($ng.ml^{-1}$)	224 [103-553]	197 [77-573]	0.678
Median [IQR] IL-1□ ($ng.ml^{-1}$)	2.6 [0.5-7.1]	2.8 [0.6-6.4]	0.701
Median [IQR] MPO ($\mu g.ml^{-1)}$	28.0 [17.0-43.8]	28.7 [6.9-50.3]	0.339
Median [IQR] CRP ($mg.l^{-1)}$	2.0 [2.0-8.0]	2.0 [1.0-6.0]	0.781
Median [IQR] Fibrinogen($g.l^{-1}$)	3.8 [3.2-4.8]	3.7 [3.2-4.3]	0.701

Table 4.20. Changes in inflammation in patients with persistently colonised sputum sample pairs. Persistently colonised samples were defined as both pairs being positive for the detection of the three potentially pathogenic bacteria detected by qPCR (n=18).

22 patients gained colonisation status at the second stable visit, with a median time between visits of 3.9 [3.0-5.1] months. No significant change in airway inflammation was observed between the two visits. However, both CRP and fibrinogen significantly increased at the second stable visit following the detection of bacteria by qPCR (□□□□□□□□□□).

	LABC status at first stable visit	LABC status at second stable visit	
	Negative	Positive	p-value
Median [IQR] CXCL8 ($ng.ml^{-1}$)	92 [27-143]	113 [50-202]	0.167
Median [IQR] IL-1□ ($ng.ml^{-1}$)	0.9 [0.4-2.0]	0.8 [0.4-2.3]	0.865
Median [IQR] MPO ($\mu g.ml^{-1)}$	14.2 [9.3-27.1]	21.3 [12.2-30.4]	0.610
Median [IQR] CRP ($mg.l^{-1)}$	1.6 [1.0-6.3]	3.5 [3.1-5.0]	0.006
Median [IQR] Fibrinogen($g.l^{-1}$)	3.2 [2.5-3.8]	3.7 [3.1-5.0]	0.002

Table 4.21. Changes in inflammation in patients gaining colonisation between sputum sample pairs. Gaining colonisation was defined as the first sputum sample being negative for the detection of the three potentially pathogenic bacteria detected by qPCR and the second sample becoming positive (n=22).

17 patients lost their colonisation status at the second stable visit, with a median time between visits of 3.9 [3.0-5.6] months. There was a trend towards a reduction in airway inflammation at the second stable visit, although this did not reach statistical significance (□□□□□□□□□). No significant difference in systemic inflammation was seen between the two visits.

	LABC status at first stable visit	LABC status at second stable visit	
	Positive	Negative	p-value
Median [IQR] CXCL8 ($ng.ml^{-1}$)	152 [48-326]	91 [40-156]	0.305
Median [IQR] IL-1□ ($ng.ml^{-1}$)	1.1 [0.5-4.3]	0.8 [0.5-2.3]	0.685
Median [IQR] MPO ($\mu g.ml^{-1)}$	29.4 [18.8-32.0]	23.7 [10.5-35.3]	0.492
Median [IQR] CRP ($mg.l^{-1)}$	1.0 [1.0-13.0]	1.0 [1.0-2.0]	0.313
Median [IQR] Fibrinogen($g.l^{-1}$)	3.2 [2.6-4.5]	3.2 [2.3-3.7]	0.232

Table 4.22. Changes in inflammation in patients losing colonisation between sputum sample pairs. Losing colonisation was defined as the first sputum sample being positive for the detection of the three potentially pathogenic bacteria detected by qPCR and the second sample becoming negative (n=17).

4.3.2.7 Change in inflammation at exacerbation and relationship to bacterial presence at stable state

In 42 patients, paired stable state and subsequent exacerbation presentation sputum samples were available for bacteriology and airway cytokine analysis. All patients were treated with systemic therapy with antibiotics and corticosteroids following exacerbation presentation and once exacerbation samples had been collected. The median time between the stable and exacerbation visits was 3.7 [2.1-5.4] months.

The clinical demographics of these 42 patients included in this paired stable and exacerbation dataset are shown in □□□□□□□□. Compared to the overall 99 patients included in the stable only inflammatory analysis, there was a significantly higher exacerbation frequency in the patients with paired stable and exacerbation samples

compared to the 99 patients included in the stable only inflammatory analysis. However, no other significant differences in clinical demographics were noted.

	Stable patients n=99	Paired stable and exacerbation patients n=42	p-value†
Mean (SD) Age (years)	72.1 (8.9)	71.2 (8.5)	0.592
Mean (SD) FEV_1 (L)	1.32 (0.54)	1.31 (0.52)	0.924
Mean (SD) FEV_1 (% predicted)	51.5 (21.6)	54.1 (18.9)	0.504
Mean (SD) FVC (L)	2.80 (0.86)	2.74 (0.82)	0.702
Mean (SD) FEV_1 / FVC ratio (%)	47.2 (13.0)	48.5 (14.6)	0.616
Median [IQR] Exacerbation frequency	2.00 [1.00-3.00]	2.69 [1.82-4.07]	0.023
Median [IQR] Smoking pack years	48.4 [24.4-67.5]	50.0 [29.6-63.6]	0.743
Median [IQR] ICS dose (beclomethasone equivalent □g)	1000 [500-2000]	2000 [1000-2000]	0.328
N (%) Male gender	66 (67)	27 (64)	0.847
N (%) Current Smokers	34 (34)	12 (29)	0.560
N (%) Chronic Bronchitis	78 (79)	36 (86)	0.483

Table 4.23. Clinical demographics of the 42 COPD patients with paired stable and exacerbation samples and comparison to the 99 patients included in the stable inflammatory analysis. Parametric data displayed as mean (SD), non-parametric data displayed as median [IQR] and categorical data displayed as N (%). †p-value refers to unpaired t-test, Mann-Whitney U or χ^2 test between the patients with paired stable and exacerbation samples and the patients with stable only samples. ICS: inhaled corticosteroids.

□

When airway cytokines were compared between stable and exacerbation states, depending on the presence of bacteria, there was a significant difference between the concentrations of CXCL8, IL-1□ and MPO between the four groups (p=0.006, p<0.001, p<0.001 respectively, □□□□□□□□□□).

When each group was compared with each other, there were similar concentrations of both CXCL8 and IL-1□ between stable patients with airway bacteria detected and in exacerbating patients without bacteria detected, and no statistically significant differences were observed in CXCL8 and IL-1□ between stable and exacerbation patients either with or without airway bacteria at both visits (□□□□□□□□□A and □□□□□□□ □□□B, respectively). MPO concentrations were significantly higher at exacerbation compared to stable state, irrespective of the presence of bacteria (□□□□□□□ □□□C).

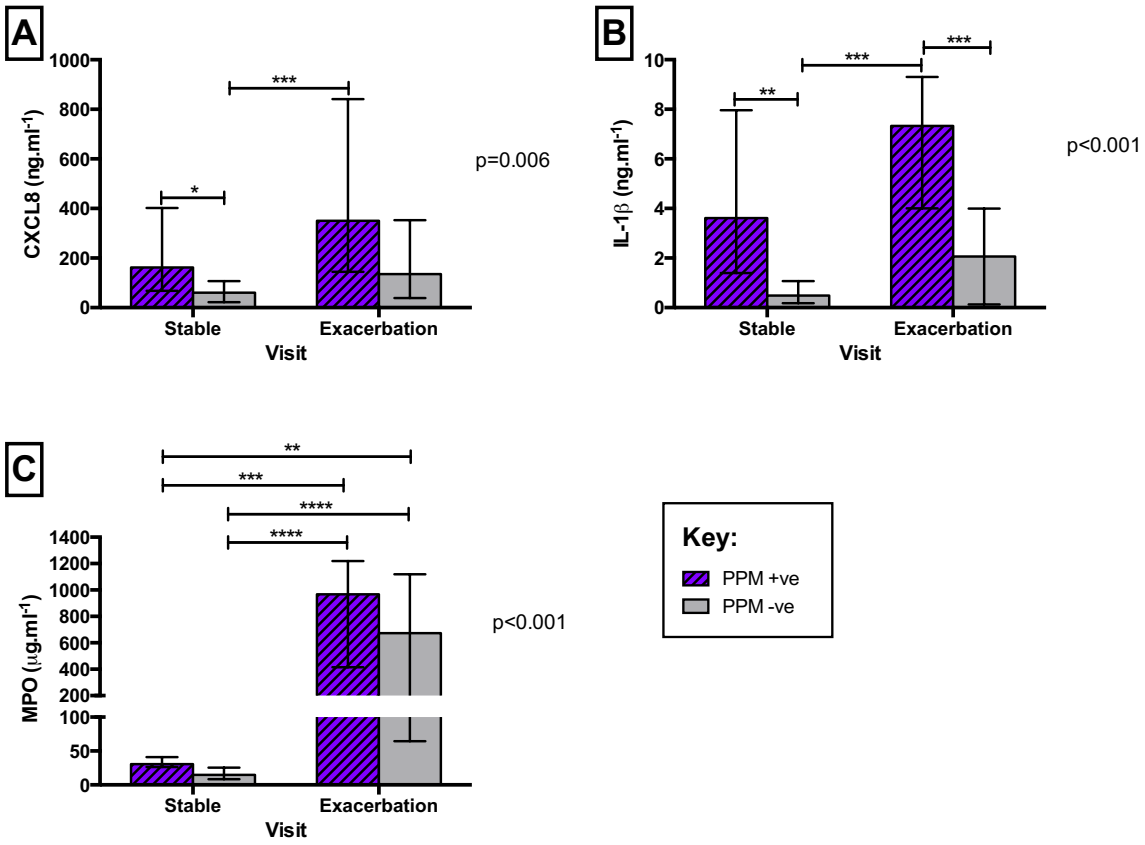


Figure 4.27. Comparison of airway (A) CXCL8, (B) IL-1β and (C) MPO by the presence of potentially pathogenic microorganisms (PPM) at both stable and exacerbation patient states. p-values refers to Kruskal-Wallis test between the four groups and * represent multiple comparison analysis with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n=42.

The change in airway total bacterial load between stable and exacerbation states was significantly associated with an increase in CXCL8 (rho=0.32; p=0.039, [illegible]A). There was a trend towards an increase in IL-1 with increasing total bacterial load between stable and exacerbation states, but this did not reach statistical significance (rho=0.25; p=0.098, [illegible]B). No significant change was observed between in changes in MPO with change in total bacterial load between stable and exacerbation states (rho=0.06; p=0.693, [illegible]C).

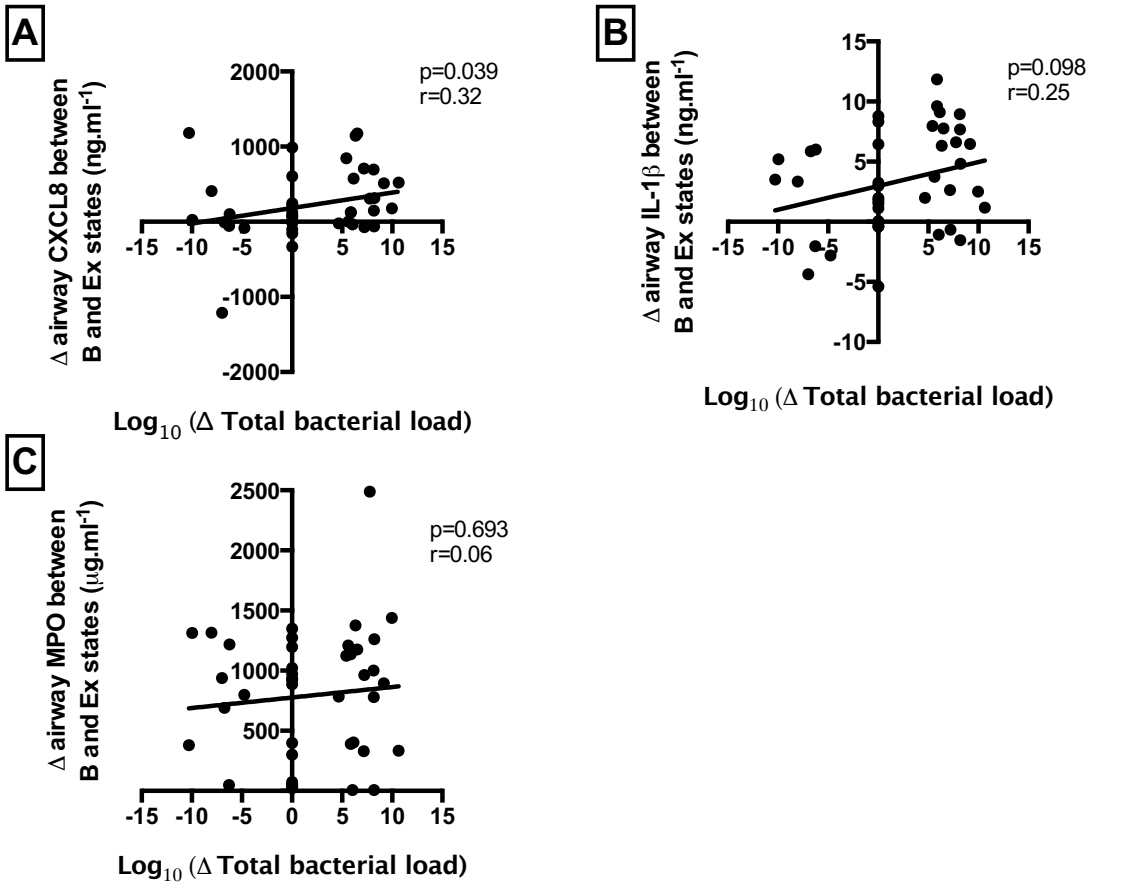


Figure 4.28. Relationship between change in airway inflammation and change in total bacterial load between stable (B) and exacerbation (Ex) states. (A) change in CXCL8, (B) change in IL-1 and (C) change in MPO (n=42).

The change in *H. influenzae* load between stable and exacerbation states significantly correlated with increasing CXCL8 and IL-1 (rho=0.39; p=0.011, rho=0.45; p=0.003, A and B, respectively), although there was no significant relationship between changes in MPO (rho=-0.01; p=0.988, C).

Unlike the above relationship changes in *H. Influenzae* load associated changes in airway inflammation between stable and exacerbation states, changes in either *S. pneumoniae* or *M. catarrhalis* load between stable and exacerbation states did not significantly correlate with changes in any of the three measured airway cytokines (Figure 4.30 and Figure 4.31, respectively).

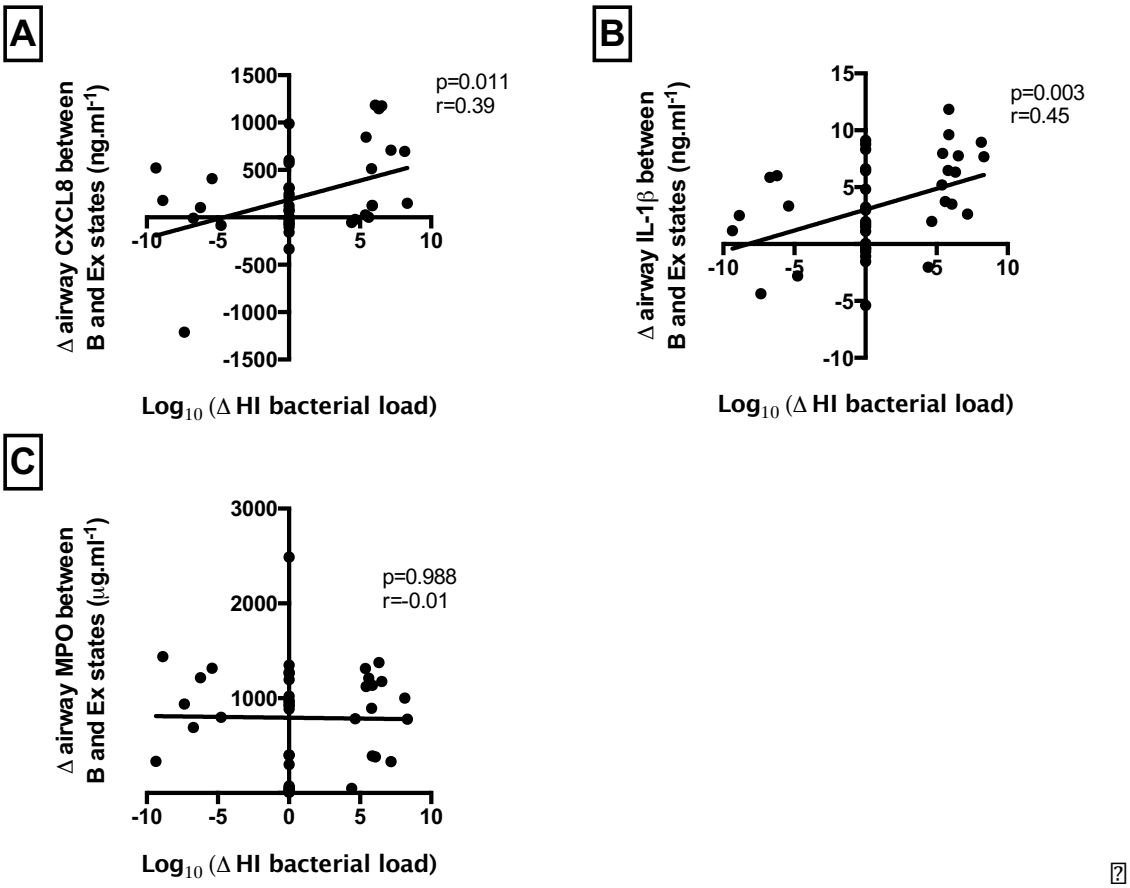


Figure 4.29. Relationship between changes in airway inflammation and change in *H. influenzae* (HI) load between stable (B) and exacerbation (Ex) states. (A) change in CXCL8, (B) change in IL-1 and (C) change in MPO (n=42).

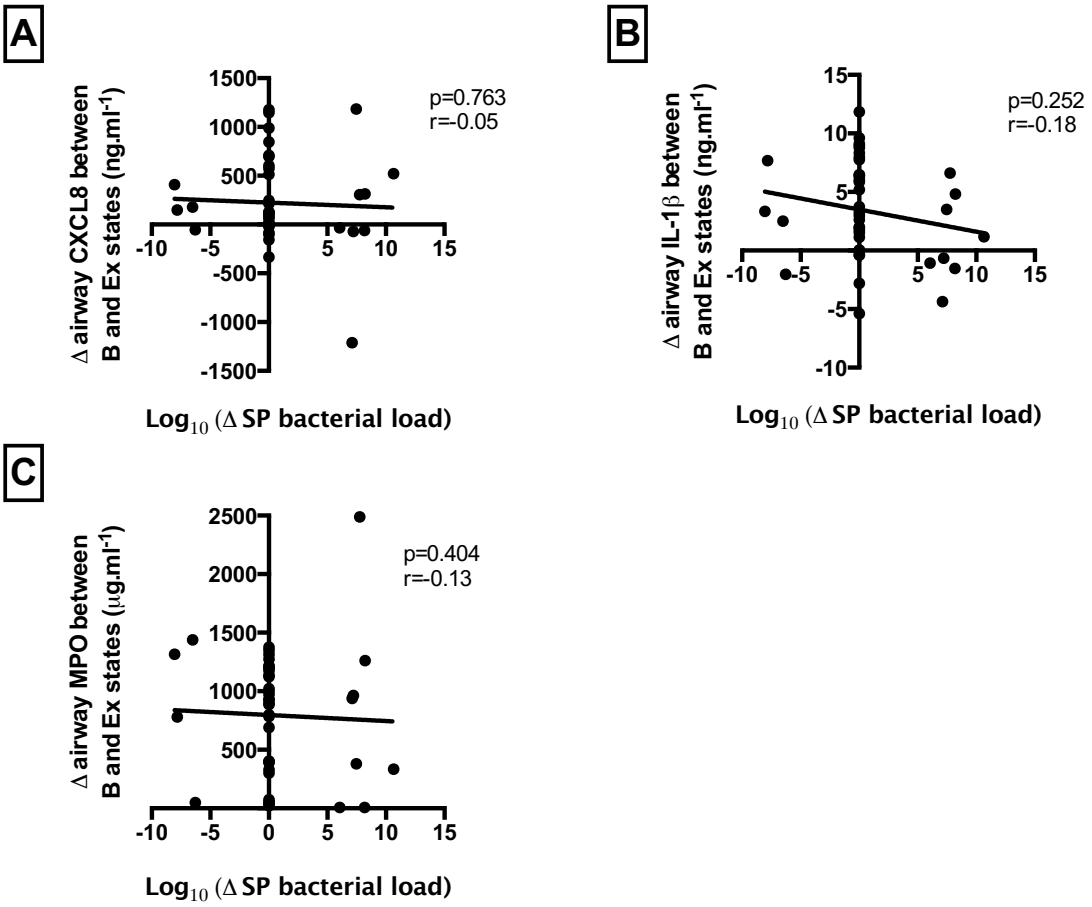


Figure 4.30. Relationship between change in airway inflammation and change in *S. pneumoniae* (SP) load between stable (B) and exacerbation (Ex) states. (A) change in CXCL8, (B) change in IL-1 and (C) change in MPO (n=42).

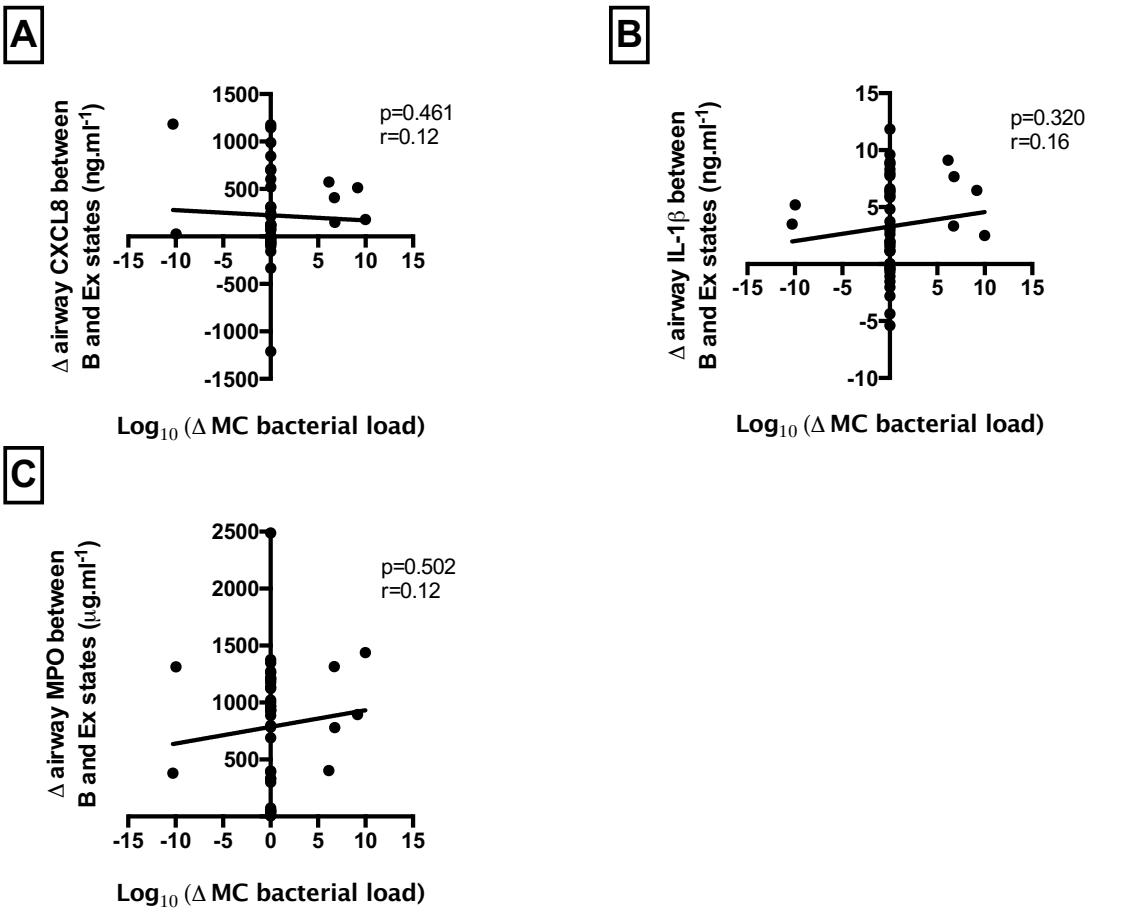


Figure 4.31. Relationship between change in airway inflammation and change in *M. catarrhalis* (MC) load between stable (B) and exacerbation (Ex) states. (A) change in CXCL8, (B) change in IL-1 and (C) change in MPO (n=42).

To determine whether there was any relationship between changes in airway inflammation and exacerbation characteristics, exacerbation duration and exacerbation symptom count at exacerbation onset were calculated from daily symptom diary cards. Increases in both IL-1 and MPO between stable and exacerbation states were associated with significantly longer exacerbation durations (rho=0.36; p=0.039, rho=0.38; p=0.026, and , respectively), although no similar relationship was observed with CXCL8 (rho=0.13; p=0.469,).

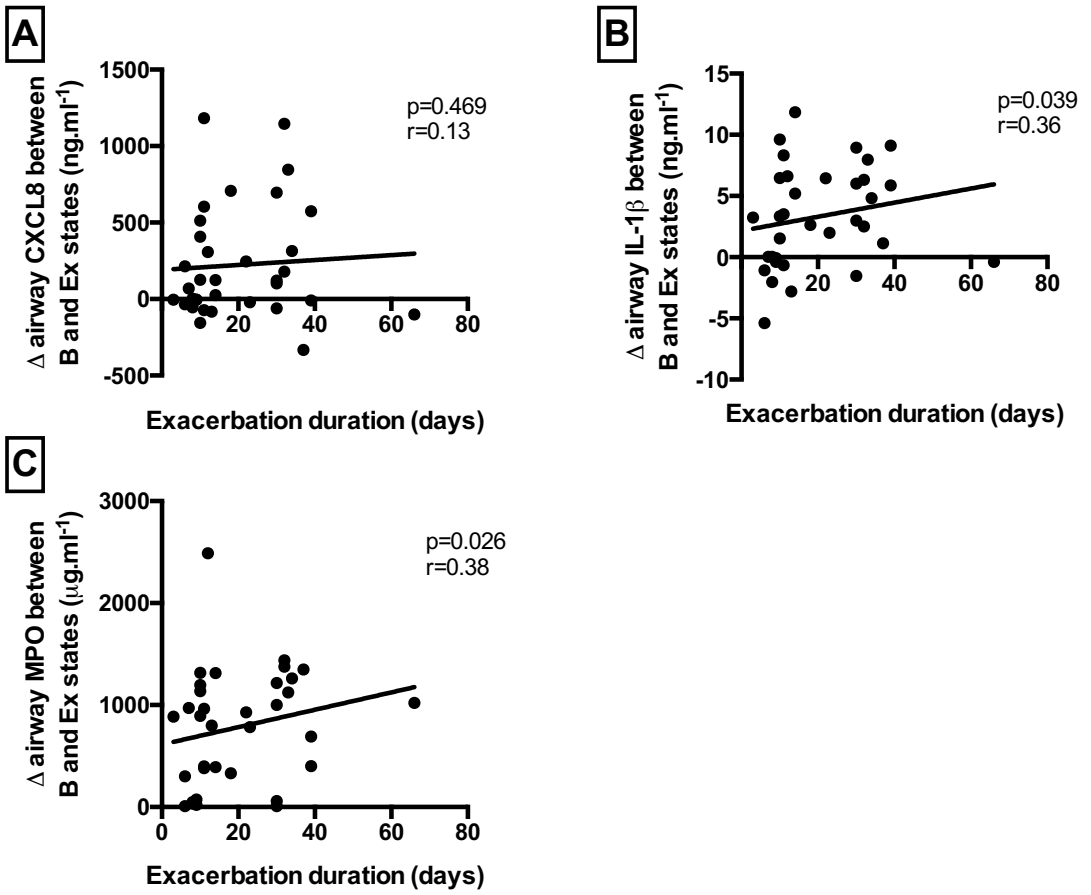


Figure 4.32. Relationship between change in airway inflammation and exacerbation duration. Exacerbation duration was calculated from daily diary cards (n=42).

However, only increases in MPO between stable and exacerbation states were significantly associated with a higher exacerbation symptom count (rho=0.40; p=0.009, [illegible]C).

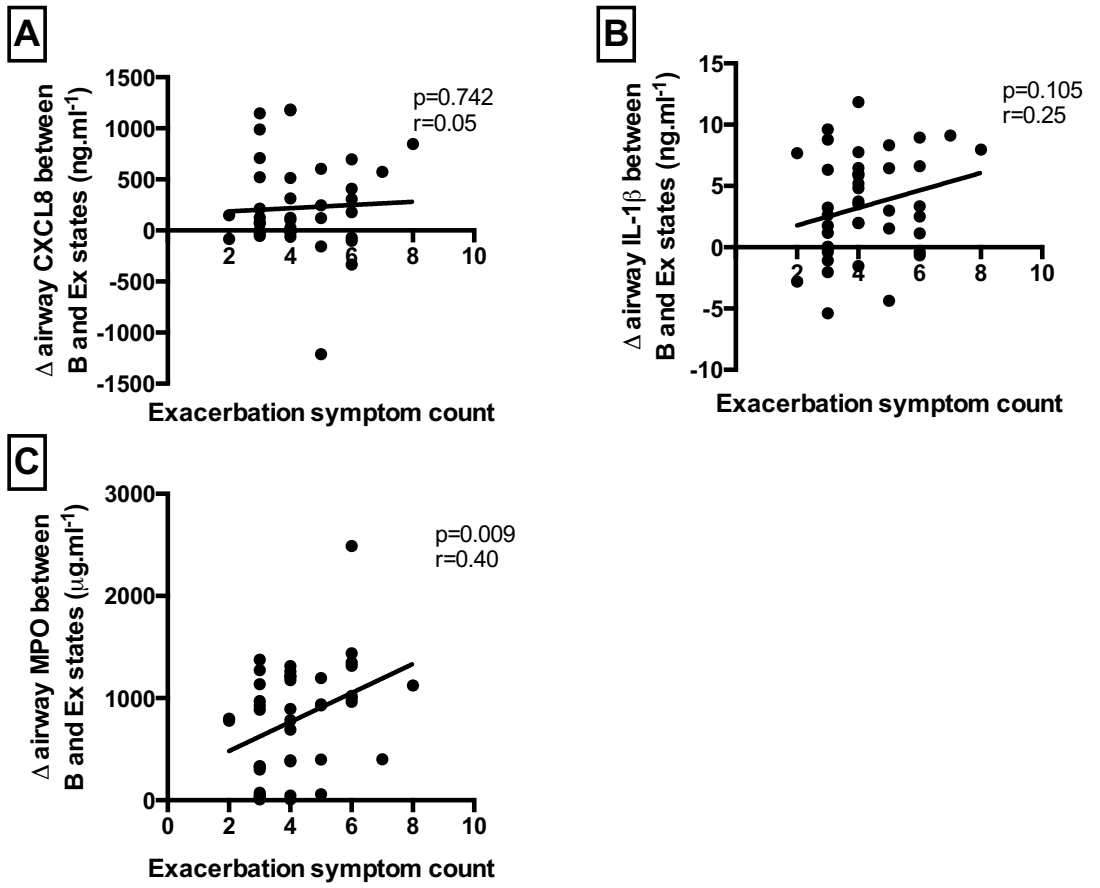


Figure 4.33. Relationship between change in airway inflammation and exacerbation symptom count. Exacerbation symptom count was calculated from the sum of symptoms recorded on daily diary cards on the day of exacerbation onset (n=42).

4.4 Discussion

This study directly compared the diagnostic abilities of the culture-independent qPCR technique with qualitative culture and quantitative culture, and subsequently validated the relationship between inflammation and airway bacterial presence, load and species-effects as determined using qPCR at both stable and exacerbation states.

qPCR demonstrated higher diagnostic yield than both culture techniques. The presence of airway bacteria was associated with both an increased airway inflammation and systemic inflammation as measured by fibrinogen, compared to samples without bacterial detection. Increasing total bacterial load was associated with higher levels of airway but not systemic inflammation and importantly an apparent inflammatory-bacterial load threshold may exist, where airway inflammation is significantly higher than in both samples without bacteria detection and those with lower-pathogen loads. In addition, a species-specific inflammatory response was observed, with *H. influenzae* presence being associated with a significantly higher airway inflammatory response for all given pathogen loads. However, there was discordance between health status and the airway inflammatory response. Airway inflammation did not significantly change over time in paired stable samples, irrespective of changes in bacterial presence, although systemic inflammation significantly increased following gaining bacterial colonisation. At exacerbation, changes in airway inflammation were positively associated with changes in airway bacterial load, likely driven by increases in *H. influenzae* load. A differential effect of increases in airway inflammation on exacerbation characteristics was seen, with some cytokines influencing exacerbation duration and others exacerbation severity.

qPCR detected a greater prevalence of airway bacteria than both qualitative and quantitative culture. Although qPCR detected bacteria in 63% of samples used in the comparison with quantitative culture, this high prevalence reflects that both stable and exacerbation samples were used in this sub-study, as samples were only being analysed for the presence and load of bacteria, irrespective of patient state. In this sub-study, the frequency of detection of both *H. influenzae* and *M. catarrhalis* using qPCR and quantitative culture was similar, although *S. pneumoniae* detection was greater using qPCR. This is likely due to the fact that *S. pneumoniae* is an autolytic organism (Martner et al., 2008), and thus naturally breaks down particularly during its stationary growth phase. Bacterial culture protocols for respiratory samples are usually designed to minimize this effect, particularly ensuring the sample is processed as soon as possible from time of collection. However, this remains a problem with *S. pneumoniae* culture, and thus the detection of its nucleic acid by culture-independent diagnostic microbiological techniques will remain higher than for traditional culture methods.

When the stable only samples were analysed for the sub-study examining the relationship between airway bacteria and inflammation, the prevalence rate for detection of the three typical bacteria in stable state samples was 35%, and at least one colonised sample was obtained from 45% of patients. Previous published literature reporting on prevalence of pathogen detection in stable samples varies widely, and can range between 30-50%, depending on the type of clinical samples analysed, the diagnostic microbiological technique used, disease severity and the proposed cut-off to define culture positivity (Banerjee et al., 2004; Rosell et al., 2005; Sethi et al., 2006). Bacterial presence in stable COPD is a dynamic process (Marin et al., 2010;

Murphy et al., 2004; Wilkinson et al., 2003) with samples often intermittently positive or negative at subsequent time points, which may contribute to the variability seen in published prevalence rates.

Increasing qPCR total bacterial load was strongly associated with an increase in airway but not systemic inflammation. This was also reflected by darker sputum colour, as measured by the BronkoTest®, which acts as a surrogate for MPO levels (Stockley et al., 2001a). Airway bacteria release potent pro-inflammatory mediators including lipopolysaccharide and outer membrane protein antigens, which have been shown to have similar dose-dependent effects *in vitro* (Berenson et al., 2005a) and in animal models (Starkhammar et al., 2012). It would be unlikely that such effects would not exist *in vivo,* supporting the hypothesis that the increasing bacterial load further perpetuates and heightens the underlying airway inflammatory response, rather than bacterial loads increase as a consequence of underlying increased airway inflammation. Further supporting evidence of this response comes from studies reporting on heightened airway inflammation after the acquisition of new airway bacterial pathogens (Parameswaran et al., 2009).

Importantly an apparent threshold of bacterial load was observed, where airway inflammation is significantly higher in samples without evidence of airway bacteria, or in those with lower loads. This is consistent with previous reports from culture-based studies in patients with stable chronic bronchitis where samples with bacterial loads between 10^6 to 10^7 cfu/ml had significantly higher airway inflammation than those samples with normal flora, depending on the airway inflammatory marker measured (Hill et al., 2000). In view of this the cut-off value, to define low- and high-

load, a total bacterial load of 10^7 cfu/ml was used, as this is similar to previously eluded inflammatory threshold using culture-based data (Hill et al., 2000). There has only been one recent study comparing bacterial loads between molecular and culture diagnostic microbiological techniques, and in this study 16S qPCR was used, rather than qPCR to the three common airway bacteria as presented in the current study (Brill et al., 2015). The total bacterial load by the qPCR technique used in this study was directly compared with that determined by quantitative culture, although only *H. influenzae, S. pneumoniae* and *M. catarrhalis* loads by culture were included to enable a direct comparison. As there was evidence of proportional bias in this analysis, an equivalent total bacterial load by qPCR to that suggested by previous inflammatory culture studies (Hill et al., 2000) could not be accurately determined, and thus the bacterial load threshold used in the present study was an arbitrary measure. However, in this study, the number of stable sample with airway bacteria detected may contribute to this dataset being underpowered the study this phenomenon in detail, especially with respect to any potential species-specific effects.

There has been a long-standing debate as to the meaning of the term ‘colonisation’ (Matkovic and Miravitlles, 2013). This terminology suggests a benign process and one without host-pathogen interactions and consequences, which are key to the definition of infection. Detailed microbiome studies in healthy, non-smoking individuals have demonstrated that a core pulmonary bacterial microbiome exists, which may include airway bacterial pathogens at low loads (Erb-Downward et al., 2011). Such lower loads of bacteria are more likely to be detected by molecular diagnostic microbiological techniques rather than culture, and hence the detection of airway bacteria alone may not be pathological.

The importance of using a bacterial load threshold which is associated with a greater inflammatory response is that this avoids using purely the detection of an airway bacterial pathogen to define colonisation, and thus enabling a more accurate definition which reflects that there are clear inflammatory consequences at higher bacterial loads. In this respect, it may be more appropriate to use the term ‘colonisation’ when airway bacteria are present, but in low loads and without inflammatory consequence, and ‘chronic airway infection’ used when there are higher loads associated with a greater inflammatory response.

Further support of using ‘chronic airway infection’ instead of colonisation is shown when airway inflammation was compared between stable and exacerbation states in patients with and without airway bacteria present. There was no significant differences in CXCL8 and IL-1□ between stable patients with airway bacteria and either exacerbation patients irrespective of the presence of airway bacteria, and this is similar to data previously reported (Desai et al., 2014). Exacerbations are typically inflammatory events, and while the aetiology of exacerbations are often multifactorial, an infectious trigger with increased bacterial and/or viral load are frequently seen (Bafadhel et al., 2011; Garcha et al., 2012; George et al., 2014; Wedzicha and Seemungal, 2007; Wedzicha et al., 2014). It is feasible that the exacerbation samples without any evidence of bacteria may have had either a bacteria present, which was not detected by this qPCR technique, or had a respiratory virus, which were not tested in these samples. Only MPO was significantly higher at exacerbation than stable state, irrespective of airway bacteria presence, and this is likely to be a consequence of the associated increased neutrophilic inflammation seen at exacerbation (Bhowmik et al., 1998).

Systemic inflammation, as measured by fibrinogen but not CRP, was significantly higher in the samples with airway bacteria detected than in those without, although the absolute difference was small. No relationship was observed between either total bacterial load or species-specific loads and systemic inflammation. The majority of previous studies investigating inflammatory response in stable COPD patients have focused on airway rather than systemic inflammation (Hill et al., 2000; Patel et al., 2002; Sethi et al., 2006; Wilkinson et al., 2003). When systemic inflammation has been measured in patients with stable airway bacterial detection, the evidence for raised fibrinogen and CRP has been conflicting, and no clear association has been found with bacterial loads (Banerjee et al., 2004; Marin et al., 2012). However, systemic inflammation and particularly raised fibrinogen has been suggested as a possible link between COPD and associated cardiovascular events (Fuschillo et al., 2012) and also as a potential biomarker to identify patients with a higher risk of mortality (Duvoix et al., 2013). Therefore, the role of fibrinogen in the pathogenesis and clinical outcomes in COPD with stable state airway bacterial presence is an important area for future studies.

Systemic inflammation is assumed to result from a 'spill-over' of airway inflammatory mediators (Barnes, 2010), although no clear correlations between airway and systemic mediators have been observed, suggestive that other mechanisms may be involved. In this dataset, a small number of serum samples were analysed by ELISA to the same airway cytokines, and almost all samples were below the level of assay detection. Therefore, paired airway and serum cytokines could not be measured in this study. Systemic inflammation in the stable state, even in patients with airway bacteria present, is likely to be far lower than at exacerbation, when bacterial loads are

significantly higher (Garcha et al., 2012), and viral acquisition may further increases levels of airway inflammation (Rohde et al., 2008; Wilkinson et al., 2006b). These higher pathogen loads are more likely to result in an acute-phase response, as seen by the significant rises in CRP and fibrinogen seen at COPD exacerbation rather than at stable state.

Greater airway inflammation for all measured cytokines appears to be attributable to *H. influenzae* rather than other detected airway bacteria. Species-specific effects on airway inflammation have been eluded to in an *in vitro* study of *M. catarrhalis* (N'Guessan et al., 2014), and in culture-based studies, with higher inflammatory responses seen with *Pseudomonas aeruginosa* detection compared to *H. influenzae*, which were in turn higher compared to *M. catarrhalis* in patients with chronic bronchitis (Hill et al., 2000). Higher airway inflammation was also observed in stable COPD patients with *H. influenzae* colonisation (Marin et al., 2012). However, importantly these previous studies did not take into account the different pathogen loads, which clearly influence the airway inflammatory response. In this study, the individual pathogens and polymicrobial samples had significantly different loads, and at all bacterial loads, airway IL-8, IL-1□ and MPO were significantly higher for *H. influenzae* than other PPMs. This species-specific inflammatory effect is suggestive of inflammation being driven by bacterial load, rather than the presence of airway bacteria being a result of increased airway inflammation.

H. influenzae is often the most prevalent airway bacterial pathogen cultured at stable state in COPD patients (Marin et al., 2012; Patel et al., 2002; Sethi et al., 2006; Wilkinson et al., 2003), and a key pathogenic mechanism is its ability to adhere to the

respiratory mucosa, evading mucosal immunity (King, 2012). Some studies have demonstrated that *H. influenzae* may reside between the epithelial and subepithelial tissues (Read et al., 1991), evading mucosal immunity and hence worsening the underlying airway inflammation seen in COPD.

An inflammatory threshold was not described for the individual airway bacteria, due to the small sample sizes with single isolate bacterial detection. However, in view of the species-specific effect described, it is likely that *H. influenzae* would have a lower load threshold, compared to other airway bacteria, where the airway inflammatory response is significantly greater than the underlying airway inflammation seen in non-colonised patients. However, further studies with larger number of samples are required to confirm this potential effect.

In this study, there was evidence of discordance between inflammation and health status as measured by the SGRQ and CAT scores, and therefore targeting inflammation alone may not necessarily improve health status. However, a recent longitudinal study by Desai and colleagues demonstrated that daily symptoms, as measured by the Breathlessness, Cough and Sputum Scale (BCSS) were significantly higher during periods of colonisation compared to periods without (Desai et al., 2014). Airway bacterial isolation in stable COPD has been shown to be associated with an increased exacerbation frequency (Patel et al., 2002) and faster decline in FEV_1 % predicted (Wilkinson et al., 2003), both of which contribute significantly to health status (Seemungal et al., 1998). Therefore, the lack of correlation between quality of life and airway inflammation may be explained by their daily monitoring of symptoms to be able to capture detailed changes in symptoms and the different health

questionnaire used. Therefore, further longitudinal studies are warranted to determine whether these important clinical outcomes are also species-specific, and thus whether targeting stable *H. influenzae* isolation by using long-term antibiotics, vaccination or perhaps specific anti-inflammatory agents would provide clinical benefit.

Airway bacterial presence during stable COPD appears to be a dynamic process, with patients changing their colonisation status as well as which species, strain and load of bacteria are isolated, likely to result in waxing and waning of airway inflammation (Marin et al., 2010; Murphy et al., 2004; Sethi et al., 2002; Wilkinson et al., 2003). The initial analyses between bacterial presence, species and load was limited to cross-sectional analysis, as the inflammatory responses to bacterial loads as measured by molecular techniques had not been previously reported prior to this study (Singh et al., 2014). Further studies have also noted similar findings, with increasing airway inflammation associated with increasing bacterial load in stable COPD patients, with *H. influenzae* associated with the strongest relationship with inflammation (Barker et al., 2014). There have been few studies examining the longitudinal relationship between airway bacterial presence and the associated inflammatory response. Desai and colleagues studied airway bacteria and inflammation in 41 patients every two-weeks in a longitudinal prospective study between October 2005 and January 2009 (Desai et al., 2014), and demonstrated doubling of IL-8 (CXCL8) during periods of colonisation, associated with worsening symptoms. However, detailed examination between changes in airway bacterial presence and inflammation was not carried out.

In the data presented herein, the relationship between airway bacterial presence and inflammation in paired consecutive stable sputum samples was investigated. Due to

the relatively small number of patients included, stable state microbiological definitions could only be defined by the presence or absence of bacteria at the two consecutive samples, rather than considering the numerous possible changes in species and/or load. Patients who were either never or always had airway bacteria at both stable visits, demonstrated no significant differences in either airway or systemic inflammation at consecutive visits. However, in those patients who gained airway bacteria after previously being negative, demonstrated no significant change in airway inflammation, but systemic inflammation significantly increased, whereas in those patients who lost the bacterial presence at the second stable visit, although there was a trend towards a reduction in airway inflammation, neither airway nor systemic inflammation were significantly different between the two visits. It is surprising that both CRP and fibrinogen showed a significant increase in patients who gained airway bacterial presence at the second stable visit, particularly as only fibrinogen was higher in colonised patients in the cross-sectional data, and neither CRP nor fibrinogen levels were correlated with bacterial load or species. However, it is possible that systemic inflammation is more responsive to change than airway inflammation, which demonstrates considerably more intra- and inter-patient variability (Pizzichini et al., 1996a; Pizzichini et al., 1996b; Sapey et al., 2008), and therefore to determine whether there are significant changes between longitudinal visits would require analyses in larger numbers, over a more prolonged prospective period.

As previously discussed, exacerbations are typically inflammatory events, frequently associated with an infectious aetiology (Bhowmik, 2000; Garcha et al., 2012; George et al., 2014; Hurst et al., 2006a; Hurst et al., 2006b; Wedzicha et al., 2000b). There have been limited studies examining the dynamic changes in both airway bacterial

load and inflammation between stable and exacerbation states, and therefore these relationships were explored this dataset. Increases in total bacterial load were significantly associated with increases in CXCL8, and there was a trend towards increasing IL-1□, although this just missed statistical significance. When the changes in the individual airway bacteria were considered, as with the cross-sectional, stable state inflammatory results, a species-specific response was observed with increases in *H. influenzae* load only between stable and exacerbation states associated with a significant increase in airway CXCL8 and IL-1□. This suggests that increases in bacterial load, and in particular *H. influenzae* load further amplifies airway inflammation seen at stable state, and further supporting the view that bacterial load drives airway inflammation rather than inflammation driving increases in bacterial load. In addition, changes in airway inflammation between stable and exacerbation state was related to clinically relevant exacerbation outcomes. Both increasing IL-1□ and MPO, but not CXCL8, were associated with longer exacerbation duration, and increasing MPO was associated with increased exacerbation severity, as measured by exacerbation symptom count (Seemungal et al., 2000). The significant relationship between MPO and exacerbation symptom count is likely to reflect the underlying heightened neutrophilic inflammation observed at exacerbation, as it has previously been demonstrated that increased neutrophil numbers correlate with a greater reduction in FEV_1 at exacerbation (Papi et al., 2006; Wilkinson et al., 2006a). However, these analyses between changes in patient states were carried out in a small number of patients, and therefore should be studied in investigated further.

Although qPCR may detect both viable and non-viable bacteria, a clear relationship between bacterial DNA load and airway inflammation is demonstrated. While this

qPCR technique does not detect bacteria other than *H. influenzae, S. pneumoniae*, or *M. catarrhalis*, the corresponding routine, qualitative culture data showed that only 2% of sputum samples had evidence of a bacteria other than those that are able to be detected by this technique. However, the London COPD cohort has low prevalence *Pseudomonas aeruginosa* (<1%) and therefore was not included in the set up of the qPCR (Garcha et al., 2012). In addition, routine bacterial culture only reports bacteria with a load greater than 10^5 cfu/ml, and therefore some patients without detectable airway bacterial pathogen on culture may indeed had bacteria present at stable state, which would contribute to the total bacterial load. Although total bacterial loads, as measured by quantitative bacterial culture and culture-independent techniques, such as 16S, are able to detect both recognised respiratory pathogens and commensal bacteria, these techniques also have limitations. Quantitative culture has the inherent inaccuracy of visual interpretation of colony counts, and the interaction between non-respiratory pathogens, included in the total bacterial load as measured by 16S and inflammation has not been well characterised. However, the high diagnostic yield of this qPCR technique highlights the strength of this technique and its potential utility in the clinical practice of microbiological study.

A further limitation in this study is that co-existing bronchiectasis was not assessed by CT scanning, although no patients had evidence of clinical bronchiectasis. Previous studies report that up to 50-60% of stable COPD patients do have radiological evidence of bronchiectasis, although these changes were generally mild (Martinez-Garcia et al., 2011; Patel et al., 2004). Secondary bronchiectasis is likely to be more common in COPD patients with airway bacterial presence and higher bacterial loads,

as a result of the vicious cycle of inflammation and infection. Thus the availability of CT scan results would be unlikely to alter the important findings from this study.

In conclusion, this work provides further evidence that this qPCR is a valuable diagnostic technique for the study if airway bacteria in stable COPD. Airway rather than systemic inflammation predominantly reflects the presence and load of airway bacteria, and this appears to have a pathogen-load threshold above which inflammation is considerably higher than the chronic inflammatory process already present in COPD. Airway inflammation both at stable state and also changes between stable and exacerbation is significantly higher with *H. influenzae* presence and increasing loads, although airway inflammation is poorly reflected in health questionnaires.

To further study the relationship of lower airway bacteria in the stable state and its effects on exacerbations, this will be examined in a larger dataset in the following chapter, with a focus on whether stable state airway bacteria would modulate exacerbation susceptibility and characteristics, including time to next exacerbation, the exacerbation severity and duration, as well as the prospective exacerbation frequency in a larger group of patients with moderate-severe COPD.

Chapter 5:

The relationship between lower airway bacterial colonisation and acute exacerbation in COPD

5.1 Introduction

COPD exacerbations are key events for patients, contributing to poor health status, disease progression and the significant morbidity and mortality associated with the disease (Donaldson et al., 2002; Seemungal et al., 1998; Wedzicha and Seemungal, 2007). These heterogeneous events are associated with increased airway and systemic inflammation as a result of complex interactions between the host, bacterial and viral pathogens as well as environmental factors (Wedzicha and Seemungal, 2007). The prevention of exacerbations is therefore a key target in both clinical guidelines and pharmacological studies.

The contribution of bacterial pathogens to the aetiology of exacerbations has been controversial, especially as lower airway bacterial colonisation (LABC) is observed in 30-50% of COPD patients (Banerjee et al., 2004; Patel et al., 2002; Rosell et al., 2005). The bacterial species that are frequently isolated in the stable state, most commonly *H. influenzae, S. pneumoniae* and *M. catarrhalis*, are also frequently isolated at exacerbation (Sethi and Murphy, 2008), and therefore their detection at exacerbation onset does not necessarily indicate causation. Indeed studies have shown conflicting results in bacterial prevalence at exacerbation, with some demonstrating an increase in prevalence (Hurst et al., 2006b; Rosell et al., 2005; Wilkinson et al., 2006b), while others do not (Bafadhel et al., 2011; Papi et al., 2006). Similarly, differing results have also been demonstrated with respect to changes in bacterial load at exacerbation, with some studies using both culture and culture-independent diagnostic microbiological techniques, demonstrating an increase in load (Garcha et al., 2012; Hurst et al., 2006b; Wilkinson et al., 2006b), whereas other studies fail to replicate these results (Sethi et al., 2007). In addition, changes in bacterial strain have

been shown to play an important role in some COPD exacerbations, increasing the risk of exacerbation, although not all strain changes were associated with exacerbations, or vice versa (Sethi et al., 2002).

There has been increasing interest in the use of long-term anti-microbial therapy, particularly macrolides, to reduce exacerbation susceptibility (Albert et al., 2011; Seemungal et al., 2008). The efficacy of these drugs is thought to be due to the anti-inflammatory rather than the anti-microbial effect, although this has not been consistently demonstrated (Seemungal et al., 2008) and it may be specific patient phenotypes, such as those with LABC, gain the most benefit from such long-term strategies.

I have previously described an inflammatory threshold and species-specific effect of colonising bacteria in Chapter 4 (Singh et al., 2014). Discordance was observed between stable state inflammation and health status and therefore targeting inflammation may not necessarily improve patients' quality of life. However, as previously mentioned acute exacerbations significantly contribute to the poor health status of COPD patients (Seemungal et al., 1998). Therefore, in this Chapter, the hypothesis is that both the species and the load of the stable colonising bacteria would modulate exacerbation susceptibility and characteristics, including time to next exacerbation, the exacerbation severity and duration, as well as the prospective exacerbation frequency in a larger group of patients with moderate-severe COPD. In addition, the hypothesis is that exacerbation characteristics would alter depending on changes in bacteriology between stable and exacerbation states.

5.2 Methods

5.2.1 Patient recruitment

Consecutive COPD patients enrolled in the London COPD cohort who provided a stable state sputum sample between January 2011 and December 2013 were included. The patients form part of a rolling cohort used to prospectively investigate the pathophysiology of COPD and its associated exacerbations (as described in section 3.1).

5.2.2 Stable State

Patients were prospectively reviewed in clinic every three months when stable (section 3.3.1) and asked to perform spirometry, complete health assessment questionnaires and provide serum and sputum samples. Serum was analysed for CRP as described in section 3.10.2.

5.2.3 Monitoring and definition of exacerbations

Patients were asked to prospectively complete daily symptom diary cards, recording daily PEFR, any increase in respiratory symptoms and additional changes to their usual treatment including systemic therapy (section 3.3). All exacerbations were symptom-defined using previously validated symptomatic criteria recorded on daily diary cards (Seemungal et al., 1998). Exacerbations were sub-classified in two ways; either ‘physician reported’ or ‘unreported’ (section 3.3.2), or by whether systemic treatment with antibiotics and/or oral corticosteroids was taken, either as a rescue pack, or following healthcare utilisation with either the study team or the patient’s

General Practitioner i.e. ‘treated’ or whether no systemic treatment was required i.e. ‘untreated’. Patients were reviewed by the clinical study team only at physician reported exacerbations, and were asked to perform spirometry, complete health assessment questionnaires and provide a sputum sample, enabling assessment of exacerbation bacteriology and change from stable state. The characteristics of the exacerbations were also defined into Type I, II and III, using the Anthonisen criteria (Anthonisen et al., 1987).

Exacerbation duration was calculated from the exacerbation onset to the end date as previously described (section 3.3.2). Exacerbation duration was deemed indeterminable if patients failed to record daily diary card symptoms or continuously recorded symptoms for more than 99 days after the exacerbation onset. Exacerbation symptoms were binary coded as present or absent, and the sum of these symptoms were defined as the symptom count which has been shown to be a validated marker of clinical severity (Seemungal et al., 2000), enabling clinical severity to be applied to unreported exacerbations.

Patients were followed up for a minimum of 12 months following study entry and prospective exacerbation frequency was calculated using this 12-month diary card data. Time to next exacerbation was analysed over a 180-day period from the stable sputum sample. All symptom-defined diary card documented exacerbations, as well as only treated exacerbations, were included for analysis. The effect of bacterial colonisation status, species and load on exacerbation characteristics was determined for paired stable and the next diary card documented exacerbation visit within a maximum of 1 year apart.

5.2.4 Sputum sampling and microbiological definitions

At stable and physician reported exacerbation visits, patients were asked to spontaneously expectorate sputum samples into a sterile pot. Patients unable to spontaneously expectorate sputum underwent sputum induction (section 3.6.2). Sputum samples were processed as soon as possible following collection to prevent RNA degradation (section 3.6.4).

qPCR to detect and measure the load of the three common airway bacterial pathogens, *H. influenzae, S. pneumoniae* and *M. catarrhalis* was performed as previously described (Garcha et al., 2012) (section 3.7.3). The lower limit of detection of the qPCR technique was 10^4 cfu/ml.

Patients were defined as colonised if one or more bacterial pathogen was detected by qPCR on sputum sample at recruitment. The colonised patients were sub-grouped by individual or mixed isolated pathogens, and into low bacterial load (□10^7 cfu/ml) and high bacterial load (>10^7 cfu/ml) based on the inflammatory threshold discussed in chapter 4. When two consecutive stable sputum samples were used to reflect the dynamics of bacterial colonisation, patients were described as never colonised, if both samples were negative for bacterial pathogen detection; persistently colonised, if both samples were positive; gaining colonisation, if the first sample was negative and the second positive; and losing colonisation if the first sample was positive and the second negative.

Changes in bacteriology between paired stable and exacerbation states were defined by acquisition of any pathogen at exacerbation, i.e. non colonised at preceding stable

visit, but one or more bacteria was present at exacerbation; loss of any pathogen at exacerbation, i.e. colonised at preceding stable visit but no bacteria detected at exacerbation; change in bacterial species detected between stable and exacerbation visit; same species at detected at both stable and exacerbation visit, and no pathogen at either state.

5.2.5 Statistical analysis

Data were analysed using GraphPad PRISM version 6.0 (GraphPad Software Inc., San Diego, CA, USA) (section 3.13). The time to next exacerbation was analysed by a survival analysis using the Kaplan-Meier method. A Gehan-Breslow-Wilcoxon test was applied to compare the survival curves from two groups and a Log-rank test applied for comparison of more than three survival curves. Patients were censored at 90- and 180-days from study entry for the time to next exacerbation analysis. A probability of $p \leq 0.05$ was considered to be statistically significant.

5.2.6 Ethical considerations

Ethical approval for the study was granted from the Royal Free Hospital research ethics committee and all patients gave written informed consent.

5.3 Results

5.3.1 Patient characteristics

172 consecutive stable COPD patients provided one stable sputum sample during the study period. Of these, 24/172 (14%) patients did not complete 12 months of diary card data and were excluded from analysis. 120/148 (81%) patients had at least one diary-card defined exacerbation during the one-year follow-up, of which 61 (41%) experienced their first exacerbation within 90 days from stable visit and 82 (68%) within 180 days. 84/148 (57%) patients had two consecutive stable sputum collected during the study of which 51/84 (61%) experienced an exacerbation within 180 days from the second stable visit. 70/148 (47%) patients provided 78 paired stable and reported exacerbation samples. The patients included in these analyses are shown in the consort diagram in [illegible].

The clinical demographics of the 148 included patients and the patient subgroups described in the analyses are shown in [illegible]. The patients had moderate-severe COPD with a mean FEV_1 of 51.2% predicted (SD 18.3) and a FEV_1/FVC of 49.2 (13.2). There were no clinically or statistically significant differences between the subgroups.

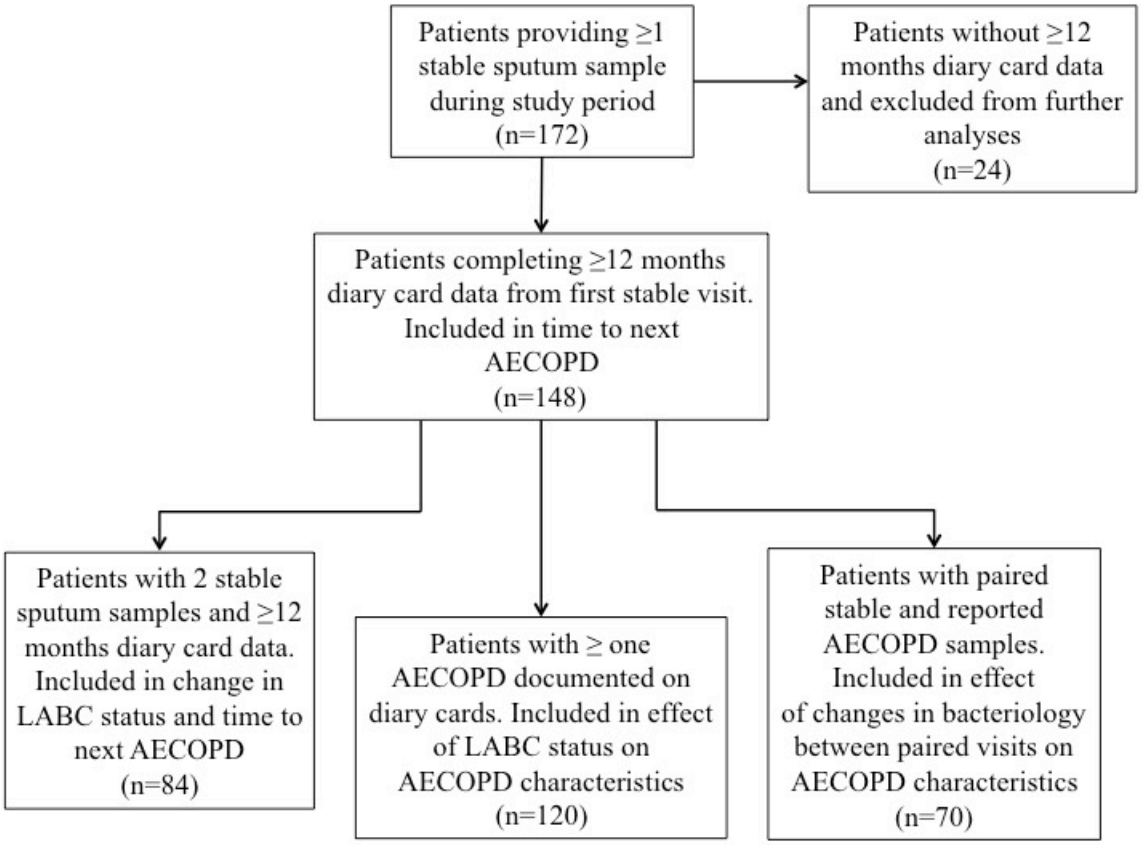


Figure 5.34. Consort diagram of the patients analysed throughout the study. LABC: lower airway bacterial colonisation. AECOPD: acute exacerbation of COPD.

	All patients n=148	Patients with 2 stable sputum samples n=84	Patients with □1 AECOPD during follow-up n=120	Paired stable and reported AECOPD n=70	p-value †
Mean (SD) Age (years)	71.4 (8.5)	72.2 (7.4)	71.5 (8.4)	72.8 (7.9)	0.659
Mean (SD) FEV_1 (L)	1.41 (0.58)	1.41 (0.57)	1.40 (0.57)	1.29 (0.51)	0.495
Mean (SD) FEV_1 (% predicted)	51.2 (18.3)	51.5 (18.5)	50.5 (17.8)	48.5 (16.9)	0.736
Mean (SD) FVC (L)	2.87 (0.93)	2.94 (0.95)	2.88 (0.92)	2.69 (0.86)	0.381
Mean (SD) FEV_1/FVC (%)	49.2 (13.2)	48.1 (12.6)	48.8 (13.3)	48.7 (13.6)	0.948
Median [IQR] Exacerbation Frequency	2.0 [1.5-3.0]	2.0 [1.0-2.5]	2.0 [1.0-3.0]	2.0 [1.0-3.0]	0.819
Median [IQR] smoking pack years	46.0 [28.5-60.0]	45.6 [26.5-58.0]	45.0 [27.0-58.0]	47.0 [28.5-60.0]	0.869
Median [IQR] ICS dose (beclomethasone equivalent μg)	2000 [400-2000]	2000 [600-2000]	2000 [400-2000]	2000 [800-2000]	0.854
N (%) Male sex	106 (72)	59 (70)	89 (74)	49 (70)	0.978
N (%) Current smokers	55 (37)	33 (39)	43 (36)	17 (24)	0.156
N (%) Chronic bronchitis	113 (76)	68 (81)	96 (80)	59 (84)	0.518

Table 5.24. Clinical demographics of the stable COPD patient subgroups. Parametric data displayed as mean (SD), non-parametric data displayed as median [IQR] and categorical data displayed as N (%). †Groups compared using one-way ANOVA, Kruskal-Wallis or $\square^2$, as appropriate. ICS: inhaled corticosteroids.

5.3.2 Stable microbiological characteristics at study entry

148 stable COPD patients were included in the analyses, of whom LABC was detected in 62 (42%) patients. In these patients, the mean total bacterial load was $10^{7.11(SD1.41)}$ cfu/ml.

Mono-microbial isolation with *H. influenzae* was identified in 22/62 (35%) of patients, with mono-microbial isolation of *S. pneumoniae* identified in 20/62 (32%). Only 5/62 (8%) patient had evidence of mono-microbial *M. catarrhalis* isolation. 15/62 (24%) patients had evidence of mixed detection; seven patients with *H. influenzae* and *M. catarrhalis* detected, four with both *H. influenzae* and *S. pneumoniae*, two with *S. pneumoniae* and *M. catarrhalis*, and two with all three bacteria detected (□□□□□□□□□). Using the proposed bacterial load-inflammatory threshold of 10^7 cfu/ml, as discussed in chapter 4, LABC patients were sub-grouped into low total bacterial load (□10^7 cfu/ml, n=29) and high total bacterial load (>10^7 cfu/ml, n=33).

PPM	Single Bacterial isolates (n)	Mixed isolates (n)	Bacterial load Log_{10} (SD) (cfu/ml)
H. influenzae	22	13	6.21 (1.10)
S. pneumoniae	20	8	7.08 (1.18)
M. catarrhalis	5	11	8.01 (1.58)

Table 5.25. Number and load of potentially pathogenic micro-organisms (PPMs) isolated from patients with lower airway bacterial colonisation using qPCR (n=62).

5.3.3 The effect of status, species and load of colonising bacteria on time to next exacerbation

61/148 (41%) patients experienced their next exacerbation, either physician reported or unreported, within 90 days of study entry. Of these, 47 (77%) were treated with antibiotics, either alone or together with oral corticosteroids. 82/148 (55%) patients experienced their first exacerbation within 180 days from study entry, of which 69 (84%) were treated with antibiotics.

The proportion of LABC and non-LABC patients experiencing an exacerbation within 90 days was not significantly different, irrespective of whether the exacerbation was treated (all exacerbations: 24/62 (39%) vs. 37/86 (43%), p=0.616; treated only exacerbations: 18/62 (29%) vs. 29/86 (34%), p=0.594). There was no significant difference in median time from stable visit to next exacerbation (either all or treated only) between the LABC and non-LABC patients (all: 49 [IQR 26-68] vs. 39 [22-63] days, p=0.600; treated only: 40 [20-71] vs. 41 [23-64] days, p=0.614).

Similarly, there were no significant differences between the proportion of LABC and non-LABC patients who experienced an exacerbation within 180 days, or had a treated exacerbation (all exacerbations: 33/62 (53%) vs. 49/86 (57%), p=0.737; treated only exacerbations: 27/62 (44%) vs. 42/86 (49%), p=0.662). There was no significant difference in the median time from stable visit to next exacerbation (either all or treated only) between the LABC and non-LABC patients (all: 56 [33-96] vs. 49 [26-90] days, p=0.737; treated only: 70 [29-101] vs. 63 [32-113] days, p=0.662).

In the 62 patients with evidence of LABC at study entry, 33 patients were defined as having high total bacterial load and 29 as low bacterial load. The proportion of high and low total bacterial load patients experiencing an exacerbation within 90 days was not significantly different, irrespective of whether all exacerbations or only treated exacerbations were considered, although there was a trend towards a higher proportion of any exacerbations in the high total bacterial load group (all exacerbations: 16/33 (48%) vs. 7/29 (24%), p=0.061; treated only exacerbations: 12/33 (36%) vs. 5/29 (17%), p=0.153). When all exacerbations were considered, there was a significantly shorter time from stable visit to next exacerbation in the high total bacterial load patients compared to the low load (40 [26-56] vs. 70 [45-74] days, p=0.030, □□□□□□□□□A). However, no significant difference was observed in the time to next treated exacerbation between the high and low total bacterial load groups (33 [21-58] vs. 70 [30-80] days, p=0.266).

There was no significant difference between the proportions of high and low load patients experiencing an exacerbation (either all or treated only) within the initial 180 days from stable visit (all: 19/33 (58%) vs. 13/29 (45%), p=0.445; treated only: 16/33 (48%) vs. 10/29 (34%), p=0.310). However, when considering all exacerbations, the median time to next exacerbation was significantly shorter in patients with high total bacterial load compared to those with low load (52 [29-74] vs. 85 [62-113] days, p=0.016, □□□□□□□□□B). When treated exacerbations only were considered, there was a trend towards a shorter time to treated exacerbations in the high total bacterial load group compared to the low load groups, but this did not reach statistical significance (48 [26-89] vs. 93 [64-114] days, p=0.118).

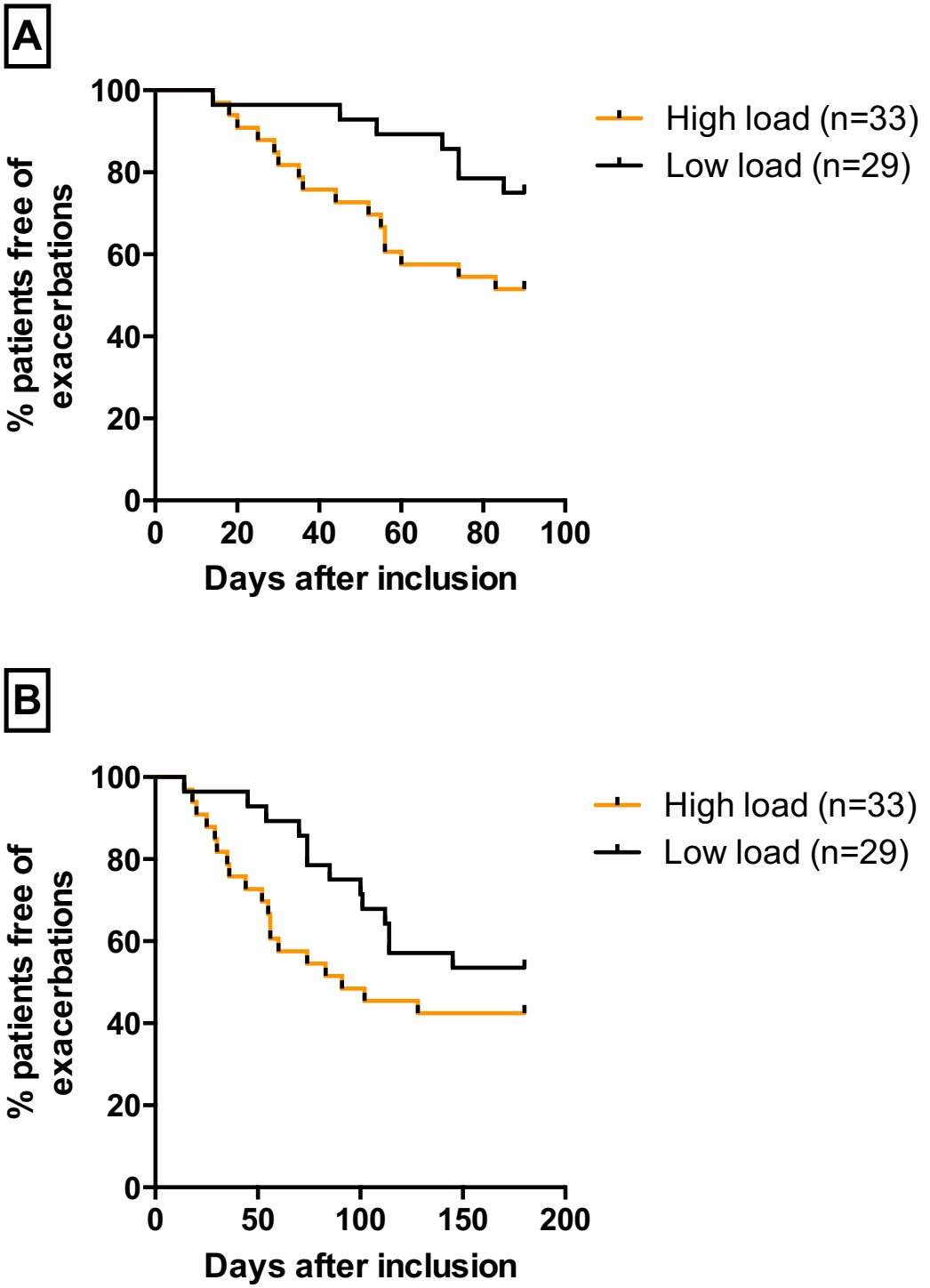


Figure 5.35. Kaplan-Meier survival analysis of exacerbation free interval to (A) 90-days and (B) 180-days by high and low total bacterial load in the 62 patients with evidence of lower airway bacterial colonisation at study entry. Low bacterial load was defined as □10^7 cfu/ml and high bacterial load as >10^7 cfu/ml.

The species of colonising bacteria, either *H. influenzae* (n=22), *S. pneumoniae* (n=20), *M. catarrhalis* (n=5), or mixed species (n=15), did not have a significant effect on the proportion of patients experiencing an exacerbation at 180 days (all: 10/22 (45%) vs. 12/20 (60%) vs. 3/5 (60%) vs. 8/15 (53%), for *H. influenzae, S. pneumoniae, M. catarrhalis* and mixed species respectively, p=0.803), although there was a trend towards a species-specific effect at 90 days (all: 5/22 (23%) vs. 12/20 (60%) vs. 2/5 (40%) vs. 5/15 (33%), for *H. influenzae, S. pneumoniae, M. catarrhalis* and mixed species respectively, p=0.066). These results were unchanged if only treated exacerbations were considered (p=0.198 and p=0.780 for 90- and 180-days follow-up, respectively).

There was a suggestion of a species-specific effect on time to next exacerbation within 90 days when all exacerbations were considered, but this did not reach statistical significance, possibly due to low patient numbers in the individual and mixed colonised groups (30 [14-60] vs. 53 [31-56], 28 [20-35] vs. 70 [43-79], for *H. influenzae, S. pneumoniae, M. catarrhalis* and mixed species respectively, p=0.099, [illegible]). When the same analysis was performed using a follow-up of 180 days, there was no significant difference or trend in the time to next exacerbation by colonising species (p=0.542).

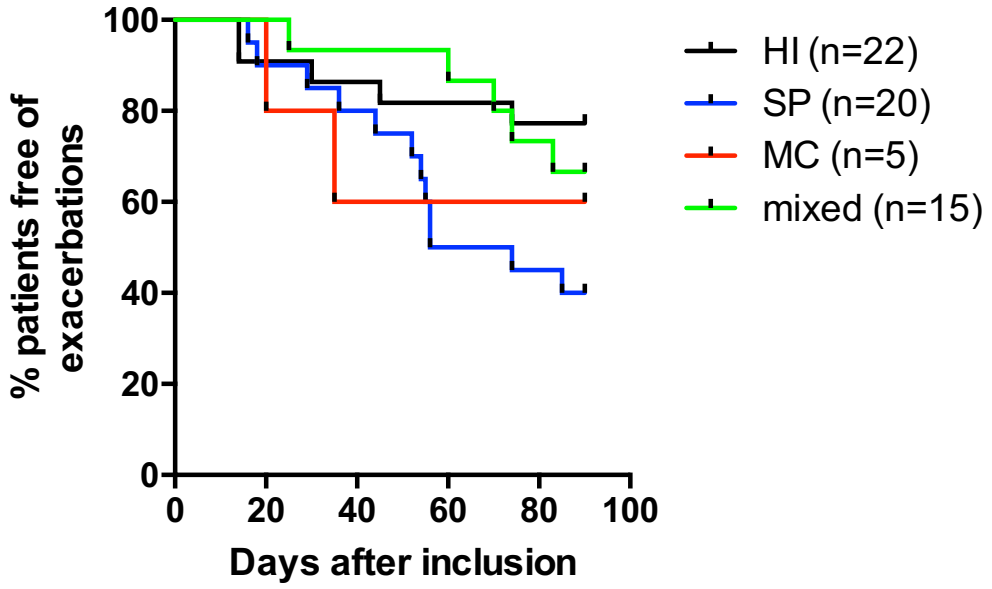


Figure 5.36. Kaplan-Meier survival analysis of exacerbation-free interval to 90-days by bacterial species in the 62 patients who had evidence of lower airway bacterial colonisation at study entry. HI: *H. influenzae*; SP: S. *pneumoniae*; MC: *M. catarrhalis.*

5.3.4 The effect of stable systemic inflammation on time to next exacerbation

The median serum CRP for the 148 patients included was 2 [1-6] mg/L. Similarly to the dataset in Chapter 4, there was no significant difference between serum CRP in the colonised and non-colonised patients (3.0 [1.0-6.0] vs. 2.5 [1.0-6.0], p=0.844) or between the different colonising species (3.0 [1.0-8.5] vs. 2.0 [1.0-5.7] vs. 2.0 [1.0-7.5] vs. 4.0 [2.0-6.0] for *H. influenzae, S. pneumoniae, M. catarrhalis* and mixed species respectively, p=0.654). In addition, there was no significant difference in serum CRP between the high load and low load colonised patients (4.0 [1.5-8.0] vs. 2.0 [1.0-6.0], p=0.263).

As there was no significant difference in systemic inflammation by colonising bacteria status, load or species, to determine whether systemic inflammation

influenced time to next exacerbation, the patients were sub-grouped into low systemic inflammation and high systemic inflammation using the median value of 2 mg/L as a cut-off. Time to next exacerbation was not significantly different between the low and high systemic inflammation groups, either with 90- or 180-days of follow-up (p=0.335 and p=0.261, respectively, □□□□□□□□□).

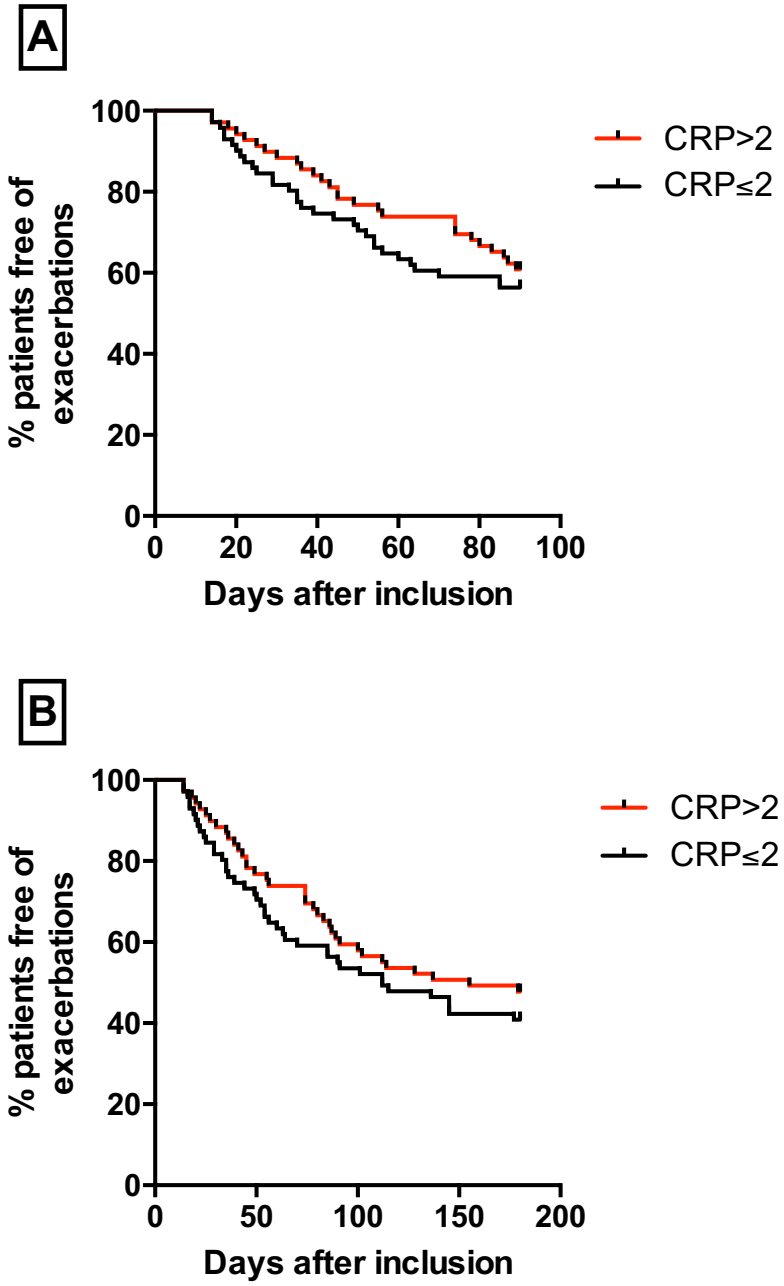


Figure 5.37. Kaplan-Meier survival analysis of exacerbation free interval during (A) 90-days and (B) 180-days based on stable systemic inflammation at study entry. Patients were sub-grouped into high and low systemic inflammation using the median CRP of 2 mg/L.

As systemic inflammation, as measured by CRP, in the stable state, is often at low levels, a higher cut-off value of 6 mg/L (the 75% percentile) was used to determine whether higher levels of CRP influenced time to next exacerbation. Again, no

significant difference was seen in time to next exacerbation between the low and high systemic inflammation groups at either 90- or 180-days of follow-up (p=0.287 and p=0.591, respectively).

5.3.5 The effect of changing colonisation status on time to next exacerbation

As bacterial colonisation is a dynamic process, repeat stable sputum samples were collected from 84 patients, with a median time of 137 [92-207] days between visits. All patients had 12 months of diary card data following the second stable sputum sample.

51/84 (61%) patients experienced an exacerbation, either physician reported or unreported, within 180 days from second stable visit. Of these, 40 (78%) were treated with antibiotics, either alone or together with oral corticosteroids. Change in bacterial colonisation status between the two visits was defined as either never or persistently colonised, or by gaining or losing colonisation status.

The proportion of never colonised patients who experienced a treated exacerbation within 180 days was significantly higher than the other changes in colonisation status (p=0.005,), but there was no significant difference between the proportions of patients experiencing any exacerbation in the different groups of changes in colonisation status (p=0.111). Change in colonisation status did not significantly affect the time to next exacerbation either when all exacerbations were considered (p=0.259) or only treated exacerbations (p=0.850).

	Change in colonisation status				p-value †
	Never n=38	Gain n=18	Lose n=15	Persistent n=18	
No (%) patients with **any** AECOPD	27 (71)	8 (44)	6 (40)	10 (56)	0.111
Median (IQR) days to next exacerbation	69 [43-123]	81 [57-133]	47 [17-79]	89 [76-130]	0.259
No (%) patients with **treated** AECOPD	25 (66)	7 (39)	3 (20)	5 (28)	0.005
Median (IQR) days to next **treated** exacerbation	69 [43-118]	76 [54-107]	74 [18-94]	90 [51-139]	0.850

Table 5.26. The effect of changing colonisation status on proportion of patients with an exacerbation and time to next exacerbation. Two stable sputum samples were used to reflect the dynamics of bacterial colonisation. Patients were described as never colonised, if both samples were negative for bacterial pathogen detection; persistently colonised, if both samples were positive; gaining colonisation, if the first sample was negative and the second positive; and losing colonisation if the first sample was positive and the second negative. All exacerbations were considered within 180 days from second stable sputum sample, and the data was reanalysed for treated exacerbations only. AECOPD: acute exacerbation of COPD. †p-value refers to χ^2 test or Kruskal-Wallis, as appropriate.

5.3.6 The effect of colonisation status, species and bacterial load on the clinical characteristics of the next exacerbation

120/148 (81%) patients included in this study had at least one exacerbation recorded on their daily diary cards within the 1-year follow-up period from first sputum sample. In these 120 stable patients, 52 (43%) had evidence of LABC; 19/52 (37%) had mono-microbial isolation with *H. influenzae*, 16/52 (31%) had mono-microbial

isolation of *S. pneumoniae* and 4/52 (8%) patients had evidence of mono-microbial *M. catarrhalis* isolation. Mixed species colonisation was detected in 13/62 (24%) patients.

There was no significant difference in the median exacerbation symptom score at the next exacerbation between LABC and non-LABC patients (4.0 [3.0-4.0] vs. 4.0 [3.0-5.0], p=0.439). There was a trend towards a higher symptom score at next exacerbation in patients with high total bacterial load compared to low total load, but this did not reach statistical significance (4.0 [3.0-4.0] vs. 3.0 [2.0-4.0], p=0.066). Similarly, there was a trend towards a species-specific effect on exacerbation symptom score, but this did not reach statistical significance (3.0 [2.0-4.0] vs. 4.0 [3.0-5.8] vs. 4.5 [3.3-6.5] vs. 4.0 [2.5-4.5] for *H. influenzae, S. pneumoniae, M. catarrhalis* and mixed species respectively, p=0.068).

Exacerbation duration was determinable from diary card data in 107/120 (89%) patients. There was no significant difference in the duration of the next exacerbation between LABC and non-LABC patients (11 [5-23] vs. 10 [7-24] days, p=0.713), or between patients with high and low bacterial loads (9 [5-21] vs. 10 [5-15] days, p=0.930). There was a trend towards the species of the colonising bacteria influencing the duration of the subsequent exacerbation, but this did not reach statistical significance (10 [5-18] vs. 18 [5-61] vs. 31 [24-37] vs. 8 [3-17] days, for *H. influenzae, S. pneumoniae, M. catarrhalis* and mixed species respectively, p=0.118).

The prospective median 12-month exacerbation frequency was unaffected by either the stable state colonisation status (2 [1-3] vs. 2 [1-3], LABC and non-LABC

respectively, p=0.331), the bacterial load threshold (2 [1-3] vs. 1 [1-3], high load and low load respectively, p=0.681) or by the colonising species (2 [1-3] vs. 2 [1-2] vs. 2 [1-4] vs. 2 [1-2], for *H. influenzae, S. pneumoniae, M. catarrhalis* and mixed species respectively, p=0.973).

5.3.7 The effect of change in bacteriology between paired stable and next exacerbation on exacerbation characteristics

78 sputum pairs from paired stable and physician reported, and hence sampled, exacerbation visits from 70 patients were available over this study period. Each individual stable and exacerbation pair represented a different sampling time-point, and therefore were considered as individual events when describing the relationship between changes in bacteriology between stable and exacerbation state and subsequent exacerbation characteristics. The median time between the stable and exacerbation visit was 76 [38-128] days. 31/78 (40%) of stable visits had one or more colonising bacteria detected whereas 49/78 (63%) of exacerbation visits had bacteria detected (p=0.006). The difference in prevalence of the different colonising species between stable and exacerbation states is shown in [illegible].

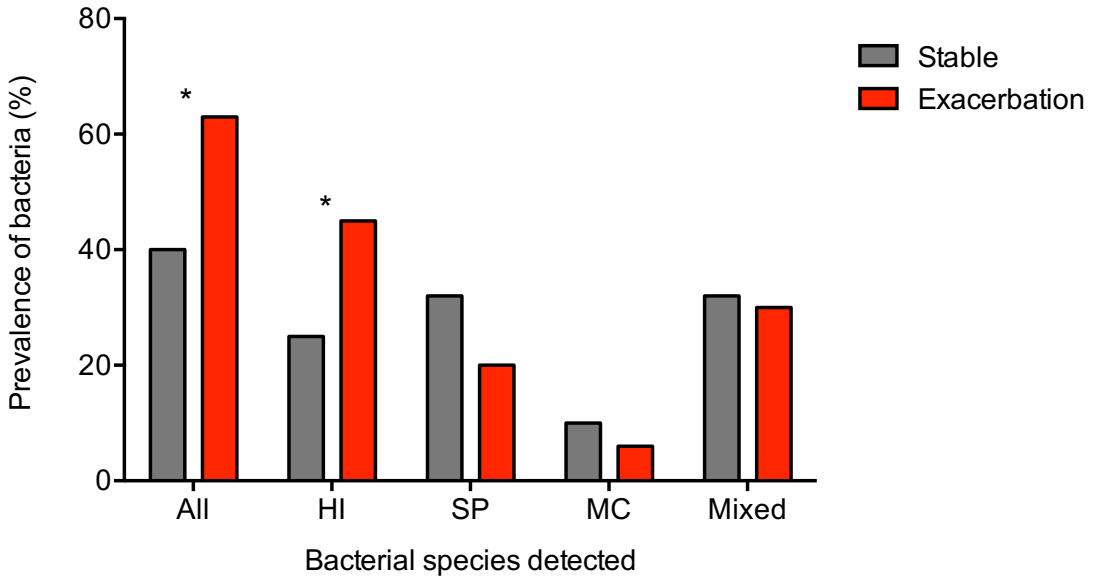


Figure 5.38. Prevalence of bacteria in paired stable and exacerbation samples (n=78). *p<0.05.

The changes in bacteriology between paired stable and exacerbation states were defined by 5 permutations; 26 (33%) of patients acquired any pathogen at exacerbation; 7 (9%) lost any pathogens at exacerbation from stable state; 15 (19%) changed species between stable and exacerbation visits; 9 (12%) had the same species at both stable and exacerbation visits and 21 (27%) had no bacterial pathogens at either state.

□□□□□□□□□ shows the effect of changes in bacteriology between stable and exacerbation states and exacerbation characteristics. There was no significant difference in either median symptom count or median exacerbation duration between the 5 permutations (all p>0.05). In addition, no significant difference was seen in the mean CAT score or the mean change (□) in CAT score (p>0.05), although there were limited paired CAT scores for both stable and exacerbation visits (29/78 (37%) pairs).

Exacerbation characteristics	Change in bacteriology between stable and exacerbation states					
	Acquisition of PPM n=26	Loss of PPM n=7	Change in PPM species n=15	Same PPM species n=9	No PPM at either n=21	p-value †
Median [IQR] symptom count	4.5 [4.0-6.0]	5.0 [4.0-6.0]	5.0 [3.0-6.0]	3.0 [2.5-5.0]	4.0 [3.0-5.0]	0.242
Mean (SD) CAT score	23.2 (5.5)	14.5 (3.5)	19.2 (8.0)	21.4 (8.7)	19.2 (7.3)	0.407
Mean (SD) □ CAT score	5.2 (4.0)	-0.5 (2.1)	2.2 (6.6)	1.8 (4.0)	3.7 (5.4)	0.502
Median [IQR)] exacerbation duration (days)	10 [7-27]	29 [7-63]	12 [9-18]	18 [13-32]	9 [7-19]	0.245

Table 5.27. The effect of changes in bacteriology presence and species between paired stable and exacerbation states on exacerbation characteristics (n=78). Changes in bacteriology between paired stable and exacerbation states were defined by acquisition of any pathogen at exacerbation, i.e. non colonised at preceding stable visit, but bacteria (any of the three detected by qPCR) present at exacerbation; loss of any pathogen at exacerbation, i.e. colonised at preceding stable visit but no bacteria detected at exacerbation; change in species detected between stable and exacerbation visit; same species at detected at both stable and exacerbation visit, and no pathogen at either state. PPM: potentially pathogenic bacteria; CAT: COPD Assessment Test. †p-value refers to one-way ANOVA or Kruskal-Wallis analysis as appropriate.

□
□
□

□ □□□

The total bacterial load increased from stable to exacerbation states in 35/78 (45%) of visits, decreased in 21/78 (27%) and did not change in 22/78 (28%). Changes in total bacterial load did not have a significant effect on the exacerbation symptom score (p=0.357), exacerbation CAT score (p=0.708), mean change in CAT score from stable to exacerbation state (p=0.423) or exacerbation duration (p=0.362) (□□□□□□□□).

	Change in total bacterial load between paired stable and exacerbation visits			p-value†
	Decrease n=21	No change n=22	Increase n=35	
Median [IQR] symptom count	5.0 [4.0-6.0]	4.0 [3.0-4.0]	4.0 [3.0-5.0]	0.357
Mean (SD) CAT score	20.7 (7.5)	19.2 (7.3)	21.3 (7.0)	0.708
Mean (SD) □ CAT score	1.4 (5.7)	3.7 (5.4)	4.2 (4.0)	0.423
Median [IQR)] exacerbation durations (days)	13 [8-32]	9 [7-19]	12 [7-29]	0.362

Table 5.28. The effect of change in total bacterial load between paired stable and exacerbation visits on exacerbation characteristics (n=78). CAT: COPD Assessment Test. †p-value refers to one-way ANOVA or Kruskal-Wallis analysis as appropriate.

44/78 (56%) of exacerbations fulfilled Anthonisen type I criteria, 30/78 (38%) type II and 4/78 (5%) type III. There was no significant difference in the mean change in total bacterial load at exacerbation by Anthonisen criteria (0.76 (6.53) vs. 1.93 (5.35) vs. 1.27 (6.54) $\log_{10}$ cfu/ml, for type I, II and III respectively, p=0.729). Similarly, the proportion of patients with increased total bacterial load at exacerbation was not

significantly different between Anthonisen type I, II or III exacerbations (19/44 (43%) vs. 14/30 (47%) vs. 2/4 (50%) respectively, p=0.132).

5.3.8 The effect of pathogen presence at preceding stable and exacerbation states on subsequent stable state bacterial presence

To determine how pathogens, both bacterial and viral at exacerbation and bacterial presence at preceding stable sample, may predict subsequent bacterial detection at stable state, 62 triplet sputum samples taken at three consecutive time-points; stable visit, subsequent exacerbation and repeat stable sample from 54 COPD patients were included. qPCR for bacteriology was performed at all three time points and qPCR for HRV was only performed at exacerbation. Each triplet sample was taken as a separate entity for analyses, even if from the same patient, as each sample was from a different date and therefore the pathogen detection is individual to that sample. At stable state, bacterial presence was defined as □1 pathogen detected by qPCR. At exacerbation state, bacterial presence was defined as an increase in total bacterial load from paired stable visit. Bacteria were detected at first stable visit in 25/62 (40%) samples and at second stable visit in 31/62 (50%) samples.

At exacerbation, in 25/62 (40%) samples an increase in total bacterial load was seen, whereas 36/62 (58%) had either no bacteria detected or had a decrease in total bacterial load. In one sample, changes in load could not be determined due to a problem with the qPCR result. There was no significant difference in bacterial presence at subsequent stable visit in those exacerbations samples either with or without bacteria detected (14/25 (56%) vs. 17/36 (47%), p=0.605), irrespective of HRV status.

23/62 (37%) exacerbation samples were positive for HRV, of which 11/23 (48%) were co-infected with bacteria. The presence of HRV at exacerbation, irrespective of co-infection, did not influence subsequent stable state bacterial presence (12/23 (52%) vs. 19/39 (49%), p=1.000).

There was no significant difference in the proportion of samples that had no pathogens detected, only bacterial pathogens, only HRV detected and those with co-infection at exacerbation that subsequently had evidence of stable state bacteria present (15/26 (58%) vs. 4/12 (25%) vs. 7/15 (47%) vs. 5/8 (63%), respectively p=0.475).

However, in the 25 first stable samples with bacteria detected, 17/25 (68%) remained positive on the second stable sample compared to 8/25 (32%), which were subsequently negative (p<0.001).

Changes in colonisation status between the two stable pairs were described as described in section 5.2.4, with 20 never colonised pairs, 17 gained colonisation paired, 6 losing colonisation pairs, and 19 persistently colonised pairs. Within the four different changes in colonisation groups, there was a significant difference in the proportion of patients that had increases in total bacterial load at exacerbation (1/20 (5%) vs. 17/17 (100%) vs. 0/6 (0%) vs. 7/19 (37%) in the never, gaining, losing and persistent colonised groups, p<0.001). Furthermore, there was a significant difference in the proportion of patients that had evidence of co-infection with HRV and bacteria at exacerbation and the proportion of patients changing colonisation (0/20 (0%) vs. 6/17 (35%) vs. 0/6 (0%) vs. 5/19 (26%) in the never, gaining, losing and persistent

colonised groups, p=0.017), suggesting that increased bacterial load and co-infection at exacerbation contributes to bacterial presence in the subsequent stable state. This effect was not influenced by the time, the number of exacerbations or courses of antibiotics between the two stable visits (□□□□□□□□□).

	Change in colonisation status				p-value †
	Never n=20	Gain n=17	Lose n=6	Persistent n=19	
Median [IQR] days between stable visits	190 [159-311]	214 [169-279]	192 [109-349]	253 [187-333]	0.481
Median [IQR] number all exacerbations	1.0 [1.0-2.0]	1.5 [1.0-3.0]	1.5 [1.0-2.0]	2.0 [1.0-3.0]	0.708
Median [IQR] number **treated** exacerbations	1.0 [1.0-3.0]	1.5 [1.0-3.0]	1.5 [1.0-2.5]	2.0 [1.0-4.0]	0.771

Table 5.29. The effect of time and exacerbation frequency and treatment on changing colonisation status. Two stable sputum samples were used to reflect the dynamics of bacterial colonisation. Patients were described as never colonised, if both samples were negative for bacterial pathogen detection; persistently colonised, if both samples were positive; gaining colonisation, if the first sample was negative and the second positive; and losing colonisation if the first sample was positive and the second negative. †refers to Kruskal-Wallis analysis between the four groups.

5.4 Discussion

This study examined in detail the temporal relationships between stable airway bacterial colonisation in COPD and the susceptibility and characteristics of subsequent exacerbations, including the influence of changes in bacteriology between stable and exacerbation states. For the first time, this study has demonstrated that high stable airway bacterial load rather than colonisation status alone significantly decreases the time to next exacerbation during the subsequent 90- and 180-days from stable visit, although systemic inflammation and changing colonisation status did not have a similar effect. Furthermore, while stable bacterial load and species appeared to influence the characteristics of the subsequent exacerbation, changes in bacteriology, as measured by bacterial prevalence, load or species, between stable and exacerbation states did not significantly effect subsequent exacerbation characteristics. Increases in bacterial load or co-infection at exacerbation were associated with a higher proportion of patients gaining colonisation or remaining persistently colonised between two stable samples, and furthermore, a single sputum sample positive for bacteria was a good predictor of subsequent positivity in stable COPD patients.

Exacerbations are significant clinical adverse events and are a major cause of morbidity, mortality and financial burden to our healthcare system (Donaldson et al., 2002; Seemungal et al., 1998; Wedzicha and Seemungal, 2007; Wedzicha et al., 2014). Thus, they are key targets for intervention, both for therapeutic clinical trials and in management guidelines, and the appropriate patients need to be targeted to maximise the potential effects on reducing exacerbation risk. LABC has been shown to alter the natural history of COPD, increasing airway and systemic inflammation (Banerjee et al., 2004; Patel et al., 2002; Singh et al., 2014) and worsening lung

function decline (Wilkinson et al., 2003). As exacerbation symptoms can be attributed to the pathophysiological changes associated with increased airway inflammation (Wedzicha and Seemungal, 2007), it is therefore unsurprising that bacterial colonisation has been shown to be associated with increased exacerbation frequency (Patel et al., 2002).

In the study by Patel and colleagues, 29 COPD patients with severe disease (mean FEV_1 predicted 38.7%) were followed up over 18 months (Patel et al., 2002). LABC was evident in 51.7% of patients and the presence of LABC was associated with a significantly higher exacerbation frequency. In addition, their study demonstrated that LABC with *H. influenzae* altered the clinical features of the subsequent exacerbation, albeit in a small number of patients, with increased total symptom count and sputum purulence compared to patients with non-specific growth. In a subsequent retrospective pooled study, a dose-response relationship was observed between airway bacterial load detected during stable disease and risk of exacerbation, with increased exacerbation risk with higher bacterial loads after adjusting for covariates (Rosell et al., 2005).

In the current study, the total bacterial load was measured using the qPCR technique for the three most prevalent pathogens due to its high diagnostic yield, particularly in stable state (Garcha et al., 2012). Stable COPD patients with a high bacterial load, defined as $>10^7$ cfu/ml using the airway inflammatory results described in Chapter 4 (Singh et al., 2014), had a significantly shorter time to next exacerbation during both 90 and 180 days of follow-up when all exacerbations were considered, although this effect was not observed when only those exacerbations treated with antibiotics (either

alone or combined with oral corticosteroids) were analysed. This may be due to the lower number of treated only exacerbations, although by 180 days 84% of the next exacerbations were treated. The underlying pathophysiological characteristics of exacerbations differ between patients and may be inflammatory eosinophilic, bacterial, viral or pauci-inflammatory in nature (Bafadhel et al., 2011), and it is unlikely that LABC only predisposes to one type of exacerbation whereby antibiotics are more likely to be prescribed. In addition, the 13 non-treated exacerbations occurring within 180 days from study entry were all unreported, and may have been suitable for antibiotic therapy if reviewed by the study team. Previous studies have demonstrated the clinical importance of these unreported events (Wilkinson et al., 2004) and therefore all exacerbations should be considered for determination of exacerbation risk.

There was a trend towards increased symptomatic severity at the subsequent exacerbation in patients with high stable bacterial loads, as measured by exacerbation symptom count. Exacerbation symptom count was used as a validated marker of severity (Seemungal et al., 2000), as the subsequent exacerbation following the stable visit may not have been reported to the study team when the patient-reported outcome CAT questionnaire would have been performed, which has also been shown to be a validated marker of exacerbation severity (Mackay et al., 2012). These results add weight to the existing data that stable bacterial load is important for stratifying exacerbation risk as well as the symptomatic burden experienced by the patients at exacerbation.

Airway bacterial load has been shown to be closely related to airway inflammation (Hill et al., 2000; Patel et al., 2002), and at bacterial loads $>10^7$ cfu/ml, airway inflammation has been demonstrated to be significantly higher than in patients without evidence of airway bacteria or those with lower loads (Chapter 4) (Singh et al., 2014). Patients with frequent exacerbations have been shown to have significantly higher levels of airway IL-6 and CXCL8 in the stable state (Bhowmik, 2000), and IL-6 has been shown to increase over time, with a faster rise in patients with frequent exacerbations (Donaldson et al., 2005). Thus a heightened airway inflammatory response is certainly a feature of patients with frequent exacerbations, and while it is plausible that this may increase the risk of subsequent exacerbations and shorten the time to the next event, further work is needed to clarify whether this is a causative effect or a consequence of the other pathophysiological mechanisms.

There has been considerable interest in the role of long-term antibiotics, both macrolides and non-macrolides, in the management of stable COPD to modify exacerbation risk (Albert et al., 2011; Seemungal et al., 2008; Sethi et al., 2010). However, long-term antibiotic therapy is not without potentially serious adverse events, including increased risk of cardiovascular complications (Ray et al., 2012), hearing loss and importantly bacterial resistance (Albert et al., 2011) and therefore the suitability of long-term antibiotics for COPD patients must take into account both the potential benefits and risks. Currently, there is little information on the phenotype of the patient who should be considered for long-term antibiotic therapy and what biomarkers will aid this decision.

In the PULSE study, a *post-hoc* analysis demonstrated that a larger reduction in exacerbation risk was seen in patients with purulent/mucopurulent sputum at baseline (Sethi et al., 2010). Sputum purulence and colour has been shown to be related to both bacterial presence and load (Miravitlles et al., 2010; Singh et al., 2014) and stable airway bacterial load has also been demonstrated to be associated to disease severity and inhaled corticosteroid use (Garcha et al., 2012). COPD patients with severe disease and a history of frequent exacerbations are more likely to be treated with an inhaled corticosteroid (Vestbo et al., 2013), and this may have the undesirable effect of increasing stable bacterial load and further increasing exacerbation susceptibility. Therefore, patients with high stable airway bacterial loads may gain the most benefit from long-term antibiotic strategies, and potentially even withdrawal or dose reduction of inhaled corticosteroids (Magnussen et al., 2014). The data presented herein will help to inform on the selection of patients for such future clinical trials targeting those patients with high stable bacterial loads. However, rapid microbiological diagnostics will need to be developed to enable measurement of airway bacterial load quickly and hence the timely identification of this susceptible patient group. It is unlikely that sputum colour alone will be sufficiently accurate to use for initial clinical trials, although using this surrogate marker for bacterial load would be potential alternative in subsequent real-world studies.

Systemic biomarkers, such as leucocyte count, CRP or fibrinogen, are much easier to sample from patients by peripheral venepuncture, and do not rely on the patients providing a valid sputum sample which is one of the limitations of airway biomarkers. COPD is increasingly recognised as a systemic inflammatory condition, and increased systemic inflammation has been shown to be associated with poor clinical outcomes,

including higher mortality and exacerbation frequency (Agusti et al., 2012), as well as simultaneously elevated CRP, fibrinogen and leucocyte count being associated with an increased risk of moderate-severe exacerbations (Thomsen et al., 2013). In patients with LABC, the relationship between stable systemic biomarkers and airway bacterial load remains controversial with studies demonstrating conflicting results (Banerjee et al., 2004; Marin et al., 2012). In this study, similarly to the results in chapter 4, no significant difference in CRP was seen between those patients with or without LABC and also between the different species. Therefore, the effect of systemic inflammation on time to next exacerbation was determined using two different cut-offs to define patients as having low and high systemic inflammation; the median CRP value and the 75% percentile. Despite using two different cut-offs, no significant difference was seen in the time to next exacerbation at either 90- or 180-days. The range of systemic inflammation in stable COPD is likely to be much narrower than that of airway inflammation and previous studies demonstrating a significant relationship between systemic inflammation and exacerbation risk and frequency were conducted in much larger cohorts of patients from the ECLIPSE, Copenhagen City Heart and Copenhagen General Population Studies (Agusti et al., 2012; Thomsen et al., 2013), and thus it is likely that this study is underpowered to detect any significant difference. In addition, a combined biomarker approach using more than one biomarker, as used in the study by Thomsen and colleagues (Thomsen et al., 2013), may enable a more definite relationship between systemic inflammation and exacerbation risk to be established and this warrants further study.

As bacterial colonisation is not a constant state, but one that fluctuates over time, the effects of variability in colonisation status on subsequent exacerbation risk was

examined. In this study, change in colonisation status did not significantly change the risk of subsequent exacerbation at either 90- or 180-days. Previous work by Sethi and colleagues showed that the presence of new strains of bacteria at stable state was associated with an increased risk of exacerbation, although not all patients with new strains had an exacerbation (Sethi et al., 2002). The authors suggested that could be due to either the virulence of the new strain and its ability to worsen the underlying airway inflammation or due to patient not reporting exacerbation symptoms to their study team. There is evidence of a species-specific effect of colonising bacteria on airway inflammation, particularly with *H. influenzae* (Chapter 4) (Marin et al., 2012; Singh et al., 2014), and in the data presented herein, there was a trend towards a species-specific effect on time to next exacerbation. Therefore changing strains to a less virulent pathogen may alter exacerbation susceptibility. In this study, only 16 of the 84 patients studied to determine the effect of changes in colonisation status on time to next exacerbation remained persistently colonised, and therefore the number was too small to examine this in further detail. However, this warrants further investigation in larger, prospective studies.

Paired bacteriological analysis from stable and reported exacerbation visits were analysed to determine how changes in bacteriology between these two states affect the subsequent exacerbation characteristics. Similar to the study by Garcha and colleagues, the prevalence of bacterial pathogens at exacerbation in this study was significantly higher at exacerbation (Garcha et al., 2012). Garcha and colleagues also demonstrated that the total airway bacterial load significantly increased at exacerbation in paired samples, but did not consider how bacterial species may also change between the two states. Therefore, in this current study, 5 different

permutations were used to represent the changes in bacteriology between stable and exacerbation states; acquisition or loss of bacterial pathogen, change in species, same species or no pathogen detected at both visits. Using these groups, no significant difference was seen in exacerbation severity, measured either by symptom count or by using the absolute or change in CAT score, and there was no significant difference in exacerbation duration. This paired data analysis was only available in 70 patients, and as 5 sub-groups were considered, this considerably decreases the number of patients in each group, and so any effect of change in bacteriology may not be seen due to the analysis being underpowered. The mean change in CAT score was higher at 5.2, albeit not statistically significantly, in patients who acquired a pathogen at exacerbation. CAT score has been shown to be a reliable score of exacerbation severity (Mackay et al., 2012), although a minimally important clinical difference has not been established. However, a recent prospective analysis has suggested that the most reliable estimate of a minimally important difference is 2 points (Kon et al., 2014), and therefore further work in a much larger prospective cohort is required to examine the effects on changes in bacteriology on subsequent exacerbation characteristics in more detail.

The presence of bacteria during stable and exacerbated disease is a dynamic process with different species, strains and load of bacteria isolated at different time-points, and it is likely that this is influenced by both patients' characteristics and the use of systemic treatment with antibiotics. There are very few studies that have investigated this extremely complex longitudinal relationship. An early study by Wilkinson and colleagues demonstrated that there was no significant change in the percentage of stable patients with bacteria detected in their sputum between the start and the end of

the study, 1-year later (Wilkinson et al., 2003). However, 50% of their study participants changed which bacterial species was cultured after the follow-up period, and in those patients changing species, a subsequently higher bacterial load was observed. A further study demonstrated that the proportion of patients with bacteria cultured at stable state was similar at the start and end of a 9-month follow-up study, with *H. influenzae* being the most common bacteria isolated, although in the successive positive culture, molecular genotyping demonstrated that this was due to acquisition of new bacteria (Marin et al., 2010). Furthermore, a recent study with 2-weekly sputum sampling showed that periods of clinical stability with associated airway bacterial presence were associated with elevated airway inflammation and symptoms (Desai et al., 2014). However, none of these studies investigated the influence of exacerbations on the dynamics of airway bacteria during stability.

In the current study, there was no significant effect of rises in bacterial load, HRV presence or co-infection at exacerbation on subsequent bacterial presence during stability. Nevertheless, in patients who gained or were persistently colonised between the two stable samples, there was a higher proportion of patients with either an increased total bacterial load or co-infection with both bacteria and HRV at exacerbation visit between the two stable samples, demonstrating that exacerbations themselves may drive bacterial colonisation. Furthermore, prior sputum positive for bacteria was shown to be a good predictor of subsequent positivity in stable COPD patients, which is an important aspect for a stable clinical phenotype. However, the due to the small numbers, stable state bacteriology was only considered as a binary phenomenon, despite it being clear that this relationship is far from simple, with both load and species, not solely presence of a bacteria contributing to the dynamics of

colonisation. Thus, to fully explore and understand the complex nature of these interactions involved in the dynamics of bacterial colonisation, further work is needed in much larger datasets.

In this study, the qPCR technique that detects the three most prevalent bacteria in stable and exacerbated states was used (Sethi and Murphy, 2008), rather than high-throughput genomic techniques. This qPCR technique has a high diagnostic yield compared to microbiological culture and demonstrates a good relationship between total bacterial load and physiological parameters including disease severity (Garcha et al., 2012) and airway inflammation (chapter 4) (Singh et al., 2014). Although other pathogens may be present in the airway, including *Pseudomonas* species, its detection is more common in hospitalized patients (Garcia-Vidal et al., 2009). This current study was confined to the outpatient management of patients in the London COPD cohort, where its culture detection rate is very low (<1%) (Garcha et al., 2012). Although there is a move towards using high throughput sequencing to measure the airway microbiome, it is unlikely to be used in clinical practice in the foreseeable future, whereas limited qPCR techniques are easier and considerably cheaper to perform to accurately measure airway bacterial load. However, future research is warranted to assess whether exacerbation susceptibility relates changes in airway microbiome.

Spontaneous sputum collection rather than induced samples was used to determine airway bacteriology, as this is more relevant to usual clinical practice. Although cell viability has been shown to be higher in induced samples, no difference in airway inflammation has been shown between these two methods (Bhowmik et al., 1998). No

direct comparison between these two sampling methods has been made with respect to the measurement of bacterial loads. In practice, samples collected following sputum induction may have an additional dilution effect, which would be unaccounted for when the total bacterial load is measured. As qPCR detects both viable and non-viable bacteria, induced samples would not have had a significant benefit over spontaneously produced samples. Airway inflammation was not measured in this study, as the aim was to determine the influence of bacterial colonisation on exacerbation susceptibility and characteristics. While the relationship between airway inflammation and stable bacterial load is well established (Hill et al., 2000; Patel et al., 2002; Singh et al., 2014), there is considerable variability in airway cytokines (Sapey et al., 2008) and therefore these are not ideal biomarkers to identify suitable patients to be recruited for clinical studies.

In conclusion, in stable COPD patients, high airway bacterial load is associated with an increased susceptibility to exacerbations and likely worsening symptoms, but stable systemic inflammation or change in colonisation status did not have a similar effect. These results may help to define which patient phenotypes should be targeted for future clinical trials of long-term antibiotic strategies in stable COPD patients.

Chapter 6:

Profiling systemic and airway biomarkers and airway pathogens during stable, exacerbation and repeat stable states

6.1 Introduction

The hallmark of COPD is an abnormal inflammatory response to inhaled particles and gases, most commonly from cigarette smoke (Vestbo et al., 2013). Chronic inflammation is evident within the small airways and lung parenchyma and involves both the innate and adaptive inflammatory and immune system, linked through the activation of dendritic cells. A similar pattern of inflammation is observed in healthy smokers, but in COPD, this inflammatory response is amplified and persistent even once exposure to these particles and gases is removed, suggesting the initial insult results in a self-perpetuating cycle of inflammation resulting in lung injury (Hogg and Timens, 2009; MacNee, 2005).

The underlying inflammatory response has been shown to alter the natural history of the disease. Over time, airway and systemic inflammation has been shown to increase, and in those patients with high levels of inflammation, a faster decline in lung function has been observed (Donaldson et al., 2005). Patients with frequent exacerbations have increased airway and systemic inflammation, even in the stable state (Bhowmik, 2000; Fu et al., 2015) and this may modulate the inflammatory pathways further, increasing subsequent exacerbation risk (Fu et al., 2015). In addition, the heightened inflammation, particularly systemic inflammation, has been associated with clinical worsening of co-morbidities (MacNee, 2013). Variations in the inflammatory profiles between individuals with COPD may represent different clinical and phenotypes with distinctive clinical courses, and variable morbidity and mortality (Agusti et al., 2012; Rennard et al., 2015).

During episodes of acute exacerbation, the abnormal inflammatory response is further heightened (Bhowmik, 2000; Hurst et al., 2006a; Hurst et al., 2006b; Wedzicha et al., 2000b). Failure of this rise in inflammation to return to baseline during exacerbation recovery has been shown to be associated with a shorter time to the next exacerbation (Perera et al., 2007) and may explain the phenomenon of clustering of exacerbations (Hurst et al., 2009). Furthermore, recent studies have investigated whether inflammatory biomarkers can identify specific exacerbation phenotypes, such as those associated with bacterial or viral infections (Bafadhel et al., 2011; Clark et al., 2015), so as to use a potentially more targeted approach to their management.

Therefore, this chapter aims to investigate the airway and systemic biomarkers during three consecutive time-points; stable visit, subsequent exacerbation and repeat stable sample, and relate this to visit, airway bacterial and viral presence, and changes in airway bacterial load between stable and exacerbated disease, as well as determining whether a species-specific effect of airway bacteria at exacerbation exists. In addition, the inflammatory 'spill-over' hypothesis will be examined in paired airway and serum biomarkers measured at both stable and exacerbated states.

6.2 Methods

6.2.1 Patient recruitment

Forty-five COPD patients enrolled in the London COPD cohort (section 3.1) between March 2011 and December 2013 were included in the biomarkers analysis, and all patients were seen at three different time-points providing both serum and sputum samples at all visits.

6.2.2 First stable state visit

Patients were recruited during a stable visit, which occurred on a three-monthly basis as part of the rolling cohort, and asked to perform spirometry, complete health assessment questionnaires and provide serum and sputum samples (section 3.2). Stable disease was defined as no evidence of symptom-defined exacerbations in the preceding 4 weeks and the subsequent 2 weeks post-clinic visit.

6.2.3 Monitoring and assessment of exacerbation visit

Patients were asked to prospectively complete daily symptom diary cards, recording daily PEFR, any increase in respiratory symptoms and additional changes to their usual treatment including systemic therapy (section 3.2.3). All exacerbations were symptom-defined using previously validated symptomatic criteria recorded on daily diary cards (Seemungal et al., 1998). Patients were reviewed by the clinical study team at exacerbation presentation and were asked to perform spirometry, complete health assessment questionnaires and provide serum and sputum samples. Only patients presenting within 7 days of exacerbation symptoms onset, and without prior use of systemic therapy such as antibiotics and corticosteroids, were included.

6.2.4 Second stable state visit

Following the exacerbation visit, patients were seen at a subsequent stable visit, and asked to repeat spirometry, complete health assessment questionnaires and provide serum and sputum samples. All patients must have recovered from their preceding exacerbation, or from any recurrent exacerbations following their initial sampled exacerbation within 4 weeks of the stable visit. Similar to the first stable visit, patients must have had no symptom-defined exacerbations in the 2 weeks post- the second stable clinic visit.

6.2.5 Sample processing and microbiological definitions

At all visits, a 10-ml sample of venous blood was collected into a sterile Vacutainer®, before centrifugation and the subsequent serum stored at -80°C for later analysis of inflammatory biomarkers.

At both stable and exacerbation visits, patients were asked to spontaneously expectorate sputum samples into a sterile pot. Patients unable to spontaneously expectorate sputum underwent sputum induction (section 3.6.2). Sputum samples were processed as soon as possible following collection to prevent RNA degradation (section 3.6.4).

On all sputum samples included, qPCR was carried out in the Centre for Clinical Microbiology, University College London, to detect and measure the load of the three common airway bacterial pathogens, *H. influenzae, S. pneumoniae* and *M. catarrhalis* as previously described (Garcha et al., 2012) (section 3.7.3). The lower limit of detection of the qPCR technique was 10^4 cfu/ml. At exacerbation visits only, qPCR to

detect and measure the load of HRV was carried out (section 3.8). The lower limit of detection was 10.23 pfu/ml.

Patients were defined at colonised if one or more bacterial pathogen was detected by qPCR on the sputum sample collected at the first stable visit. An increase in either total bacterial load, or species-specific load from baseline at exacerbation was considered positive for a bacterial-associated exacerbation. As the prevalence of HRV during stability is low, a HRV load above zero at exacerbation was considered positive for a viral-associated exacerbation.

6.2.6 Inflammatory mediator selection and measurement

Inflammatory biomarkers were measured in stored sputum supernatant and serum samples using the V-PLEX Human Biomarker 40-PLEX Meso Scale Discovery platform (MSD, Rockville, Maryland, USA). The MSD is a quantitative sandwich immunoassay, using 96-well plates with pre-coated capture antibodies on independent and well-defined spots, as described in detail in section 3.9.1. The assays in the V-PLEX Human Biomarker 40-PLEX kit are provided in 5 multiplex panels; chemokine, cytokine, pro-inflammatory, vascular injury and angiogenesis (□□□□□□□ □□□), allowing 40 analytes to be measured in either serum or sputum supernatants samples. All biomarkers were measured in both type of samples, to enable comparison between airway and systemic inflammation.

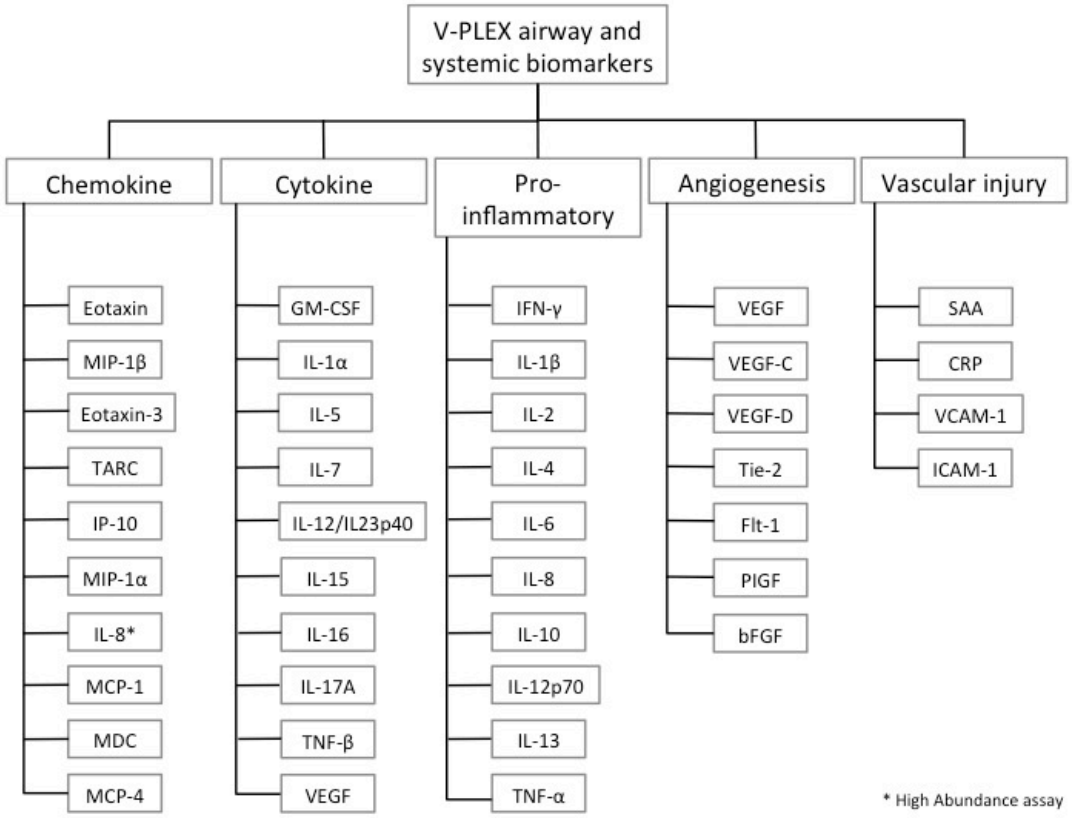


Figure 6.39. The V-PLEX Human Biomarker kit showing the 5 multiplex panel biomarkers. Definitions of abbreviations: MIP: macrophage inflammatory protein. TARC: thymus and activation regulated chemokine. IP: interferon gamma-induced protein. IL: interleukin. MCP: monocyte chemoattractant protein. MDC: macrophage-derived chemokine. GM-CSF: granulocyte-macrophage colony-stimulating factor. TNF: tumour necrosis factor. VEGF: vascular endothelial growth factor. IFN: interferon. Tie: PIGF: placental growth factor. bFGF: basic fibroblast growth factor. SAA: serum amyloid A. CRP: C-reactive protein. VCAM: vascular cell adhesion protein. ICAM: intercellular adhesion molecule.

6.2.7 Statistical analysis

Data were analysed using GraphPad PRISM version 6.0 (GraphPad Software Inc., San Diego, CA, USA) and STATA-13 (StataCorp, Texas, USA). Details of the majority of statistical tests used are reported in section 3.13. Tests used specifically in this chapter include comparison of 3 groups of unpaired or paired parametric or non-parametric variables by one-way ANOVA, RM one-way ANOVA, Kruskal-Wallis or Friedman analysis with multiple comparisons, depending on the sample population being investigated. Multiple linear regressions were used to assess the species-specific effect of bacterial and viral pathogens on the change in inflammation between stable and exacerbated states. A probability of $p \leq 0.05$ was considered to be statistically significant. A full Bonferroni correction would be highly conservative for exploratory biomarker analysis, which may potentially miss real differences. Therefore, for the multiple linear regression, a $p<0.01$ was considered statistically significant, balancing type I and type II errors.

6.2.8 Ethical considerations

Ethical approval for the study was granted from the Royal Free Hospital research ethics committee and all patients gave written informed consent.

6.3 Results

6.3.1 Patient characteristics

Forty-five COPD patients were included in the biomarker analyses. The stable clinical demographics are shown in [illegible].

	All patients n=45
Mean (SD) FEV_1 (L)	1.28 (0.52)
Mean (SD) FEV_1 (% predicted)	53.0 (19.1)
Mean (SD) FVC (L)	2.79 (0.87)
Mean (SD) FEV_1/FVC (%)	0.46 (0.14)
Median [IQR] HCU* Exacerbation Frequency	2.0 [2.0-3.0]
Median [IQR] smoking pack years	47.0 [25.1-60.0]
Median [IQR] ICS dose (beclomethasone equivalent μg)	2000 [1000-2000]
N (%) Male sex	34 (76)
N (%) Current smokers	11 (24)
N (%) Chronic bronchitis	45 (100)

Table 6.30. Clinical demographics of the stable COPD patients. Parametric data displayed as mean (SD), non-parametric data displayed as median (IQR) and categorical data displayed as N (%). *HCU: health care utilisation for exacerbations, where systemic therapy was given either by the study team when participants were seen in the research clinic at exacerbation presentation, or by another health-care provider, such as their General Practitioner.

6.3.2 Characteristics of the exacerbations

All exacerbations included showed similar characteristics in their time-course as previously described (Seemungal et al., 2000) ([illegible]).

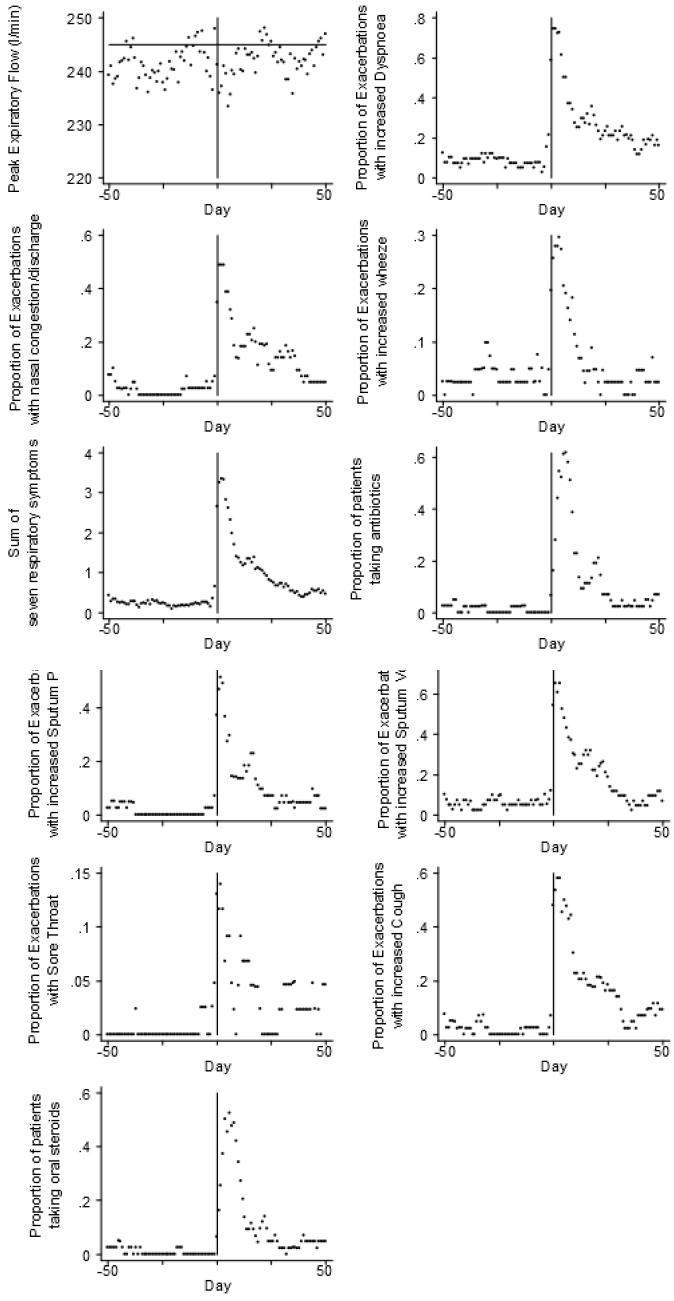


Figure 6.40. Time-course of PEFR, respiratory symptoms and treatment during the 45 exacerbations from 45 patients included in the biomarker analyses.

On the day of onset of exacerbation, symptoms reported sharply increased, with 78% reporting dyspnoea, 52% increased sputum purulence and 65% increased sputum volume. Minor symptoms also increased at exacerbation onset, with 50% reporting

upper respiratory congestion, 30% increased wheeze, 14% sore throat and 59% reporting increased cough.

The median time between the first stable visit and the exacerbation visit was 65 [IQR 30-116] days, and the median time from exacerbation onset to the exacerbation presentation visit, and thus sampling, was 3 [2-4] days. All patients were treated with systemic treatment using either, or both, antibiotics and oral corticosteroids, of which 44/45 (98%) of patients received antibiotics and 42/45 (93%) of patients received with oral corticosteroids.

2 patients were unable to perform paired exacerbation presentation spirometry due to severe dyspnoea and a fractured sternum. In the 43 patients with paired spirometry, the mean FEV_1 at significantly decreased at exacerbation from first stable visit (1.23 (SD0.47) L vs. 1.29 (0.52) L, p=0.013). CAT scores were available in 30/45 (67%) of first stable visits and 23/45 (51%) of exacerbation visits. CAT scores were significantly higher at exacerbation than at the preceding first stable visit (20.9 (6.8) vs. 16.7 (5.8), p=0.030). Exacerbation duration was available in 41/45 (91%) of patients and the median duration was 11 [7-29] days.

The pathogens detected only at the exacerbation visit are shown in [illegible]A. 11/45 (24%) patients had no airway bacterial pathogens or HRV detected, 19/45 (42%) patients only bacterial pathogens isolated, 9/45 (20%) patients had only HRV isolated and 6/45 (13%) had co-infection with both HRV and bacterial pathogens detected. When an increase in total bacterial load only was considered as ‘positive’ for bacterial-associated exacerbations, there was a higher proportion of patients

defined as having no organism at exacerbation and a reduction in the proportion in patients defined as having a bacterial pathogen detected alone, although this was not statistically significant (17/45 (38%) vs. 11/45 (24%), p=0.255 and 13/45 (29%) vs. 19/45 (42%), p=0.237, respectively, [illegible]B).

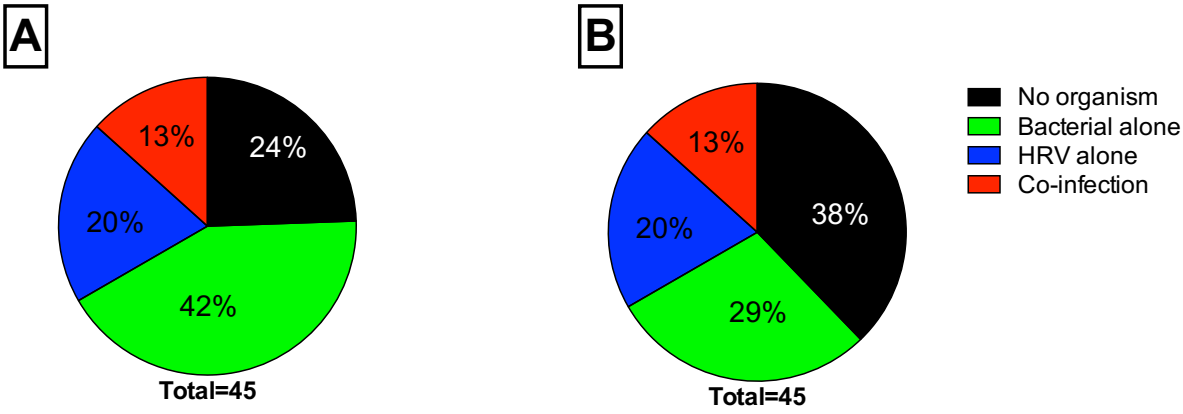


Figure 6.41. Proportion of airway pathogens at exacerbation. The presence of bacterial pathogens was measured as (A) presence at exacerbation irrespective of stable state bacteriology and (B) an increase in total bacterial load at exacerbation compared to preceding stable state, n=45. HRV: human rhinovirus. Co-infection defined as presence of both HRV and [illegible]1 bacterial pathogens detected by qPCR.

Following exacerbation recovery, patients were sampled at a repeat stable visit, with a median time of 113 [90-185] days. The time between first and second stable visits was 205 [156-298] days.

[illegible]

[illegible]

[illegible] [illegible]

6.3.3 Changes in airway biomarkers between first stable, exacerbation and subsequent stable visits

The concentrations of the airway biomarkers for the 5 different multiplex panels at the first stable, exacerbation presentation and subsequent (second) stable visits are reported in [illegible] to [illegible]. Differences between the three states were analysed by Friedman test with Dunn's multiple comparison. For those biomarkers with significant differences between the three states, the corresponding graphs show the significant multiple comparison results.

6.3.3.1 Airway chemokine biomarkers

Marker	First stable visit median [IQR] (pg/ml)	Exacerbation visit median [IQR] (pg/ml)	Second stable visit median [IQR] (pg/ml)	p-value
Eotaxin	88.7 [33.7-205.7]	11.6 [2.9-73.7]	81.8 [32.1-145.5]	**0.004**
MIP-1□	457.6 [85.9-1097.0]	276.2 [34.5-1383.0]	552.7 [171.7-1241.0]	0.920
Eotaxin-3	77.4 [16.6-221.1]	20.8 [5.7-73.1]	68.8 [10.0-302.5]	**0.005**
TARC	78.2 [29.4-176.7]	13.1 [2.1-63.1]	88.6 [23.1-161.7]	**0.002**
IP-10	2078 [375-15166]	248 [7-2937]	2167 [243-6743]	0.072
MIP-1□	143.4 [30.8-391.0]	147.3 [11.4-681.6]	143.6 [44.9-670.7]	0.706
IL-8*	16758 [7909-34602]	32158 [14798-50507]	22335 [9597-46007]	0.266
MCP-1	389.0 [235.5-934.5]	234.9 [110.0-884.4]	330.0 [218.3-660.0]	0.088
MDC	635.2 [183.7-1492.0]	250.6 [51.2-943.3]	601.1 [209.5-1213.0]	**0.005**
MCP-4	16.9 [6.45-32.1]	9.9 [4.5-19.3]	15.9 [8.4-30.0]	0.051

Table 6.31. Differences in airway chemokine biomarkers between first stable, exacerbation presentation and second stable visit in the 45 patients included. *High abundance assay.

There were significant differences in Eotaxin, Eotaxin-3, TARC and MDC in the chemokine panel. The multiple comparisons for these airway biomarkers are shown in [illegible]. Only comparisons between the exacerbation state with either stable state was statistically significant (all p<0.01). There were no significant differences in the airway chemokine concentrations between the first and second stable state visits.

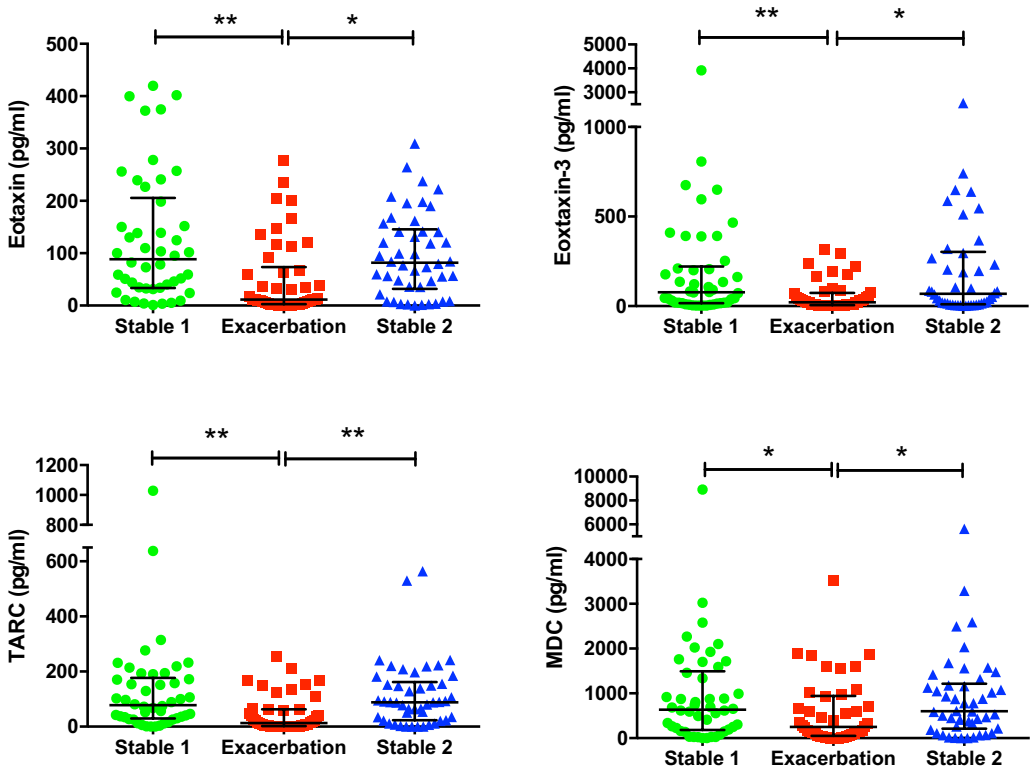


Figure 6.42. Airway chemokines with significantly different concentrations between the first stable, exacerbation presentation and second stable visits (n=45). *p<0.01, **p<0.001. Error bars represent the median and interquartile range.

[illegible]

[illegible]

[illegible] [illegible]

6.3.3.2 Airway cytokine biomarkers

Biomarker	First stable visit median [IQR] (pg/ml)	Exacerbation visit median [IQR] (pg/ml)	Second stable visit median [IQR] (pg/ml)	p-value
GM-CSF	0.7 [0.3-1.3]	1.2 [0.6-3.1]	0.7 [0.4-1.8]	**0.002**
IL-1□	58.1 [13.8-187.6]	163.3 [54.8-386.8]	61.9 [26.6-207.5]	**0.030**
IL-5	1.2 [0.4-8.6]	1.1 [0.7-4.5]	2.0 [0.5-9.3]	0.205
IL-7	17.0 [7.4-28.1]	8.8 [3.7-12.3]	13.6 [7.4-22.2]	**0.030**
IL-12/ IL23p40	7.9 [2.9-24.5]	15.7 [5.6-30.1]	7.2 [2.5-14.7]	**0.003**
IL-15	1.2 [0.6-2.4]	1.6 [1.1-3.1]	1.3 [0.8-1.8]	0.138
IL-16	313.0 [132.9-695.1]	163.8 [5.6-798.3]	345.1 [132.5-575.9]	0.226
IL-17A	2.0 [0.9-9.8]	5.8 [2.5-25.7]	2.0 [1.2-5.2]	**<0.001**
TNF-□	0.15 [0.06-0.26]	0.50 [0.20-1.07]	0.13 [0.08-0.18]	**<0.001**
VEGF	1467 [907-2068]	1921 [1514-2969]	1482 [997-1879]	**0.005**

Table 6.32. Differences in airway cytokine biomarkers between first stable, exacerbation presentation and second stable visit in the 45 patients included.

There were significant differences in GM-CSF, IL-1□, IL-7, IL-12/IL23p40, IL17A, TNF-□ and VEGF in the cytokine panel. The multiple comparisons for these airway biomarkers are shown in [illegible]. Only comparisons between the exacerbation state with either stable state was statistically significant (all $p<0.01$). There were no significant differences in the airway chemokine concentrations between the first and second stable state visits.

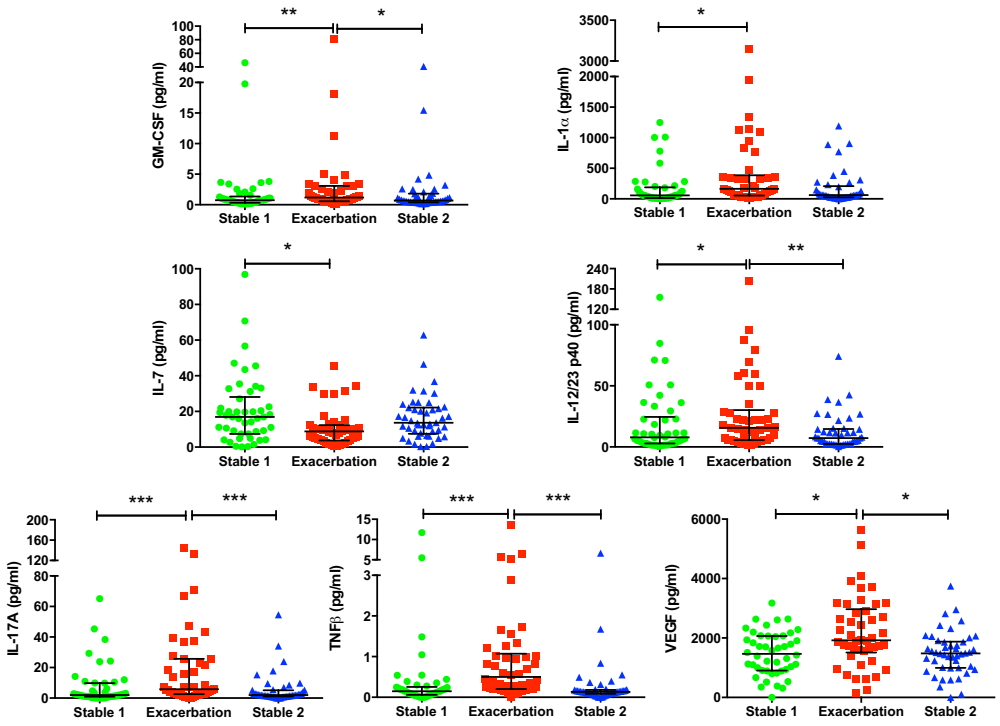


Figure 6.43. Airway cytokines with significantly different concentrations between the first stable, exacerbation presentation and second stable visits (n=45). *p<0.01, **p<0.001, ***p<0.0001. Error bars represent the median and interquartile range.

6.3.3.3 Airway pro-inflammatory biomarkers

Biomarker	First stable visit median [IQR] (pg/ml)	Exacerbation visit median [IQR] (pg/ml)	Second stable visit median [IQR] (pg/ml)	p-value
IFN-□	7.2 [5.1-17.0]	42.8 [17.8-315.8]	8.0 [4.7-12.8]	**<0.001**
IL-1□	59.3 [23.4-203.9]	614.9 [196.3-1815.0]	64.6 [25.3-203.4]	**<0.001**
IL-2	2.3 [1.7-3.2]	4.0 [2.5-5.4]	2.1 [1.5-3.4]	**<0.001**
IL-4	0.4 [0.2-0.6]	0.8 [0.5-1.7]	0.5 [0.3-0.7]	**<0.001**
IL-6	32.5 [11.0-61.6]	63.3 [23.0-199.9]	29.2 [13.0-67.0]	**0.002**
IL-10	3.1 [2.0-4.0]	13.2 [5.2-24.2]	2.9 [2.2-4.2]	**<0.001**
IL-12p70	2.0 [1.5-2.8]	2.8 [2.2-4.2]	2.0 [1.2-2.6]	**0.022**
IL-13	9.6 [6.2-14.5]	12.7 [9.7-17.2]	9.4 [6.9-12.4]	**0.020**
TNF-□	5.9 [3.2-25.0]	142.7 [15.7-309.5]	10.3 [3.5-36.0]	**<0.001**

Table 6.33. Differences in airway pro-inflammatory biomarkers between first stable, exacerbation presentation and second stable visit in the 45 patients included.

IL-8 was not included in this panel, as the airway concentrations were significantly higher than the standard curve and hence for measurement of this biomarker was included in the chemokine panel using high abundance assay. A significant difference between all the pro-inflammatory biomarkers was seen between the three visits. The multiple comparisons for these biomarkers are shown in [illegible].

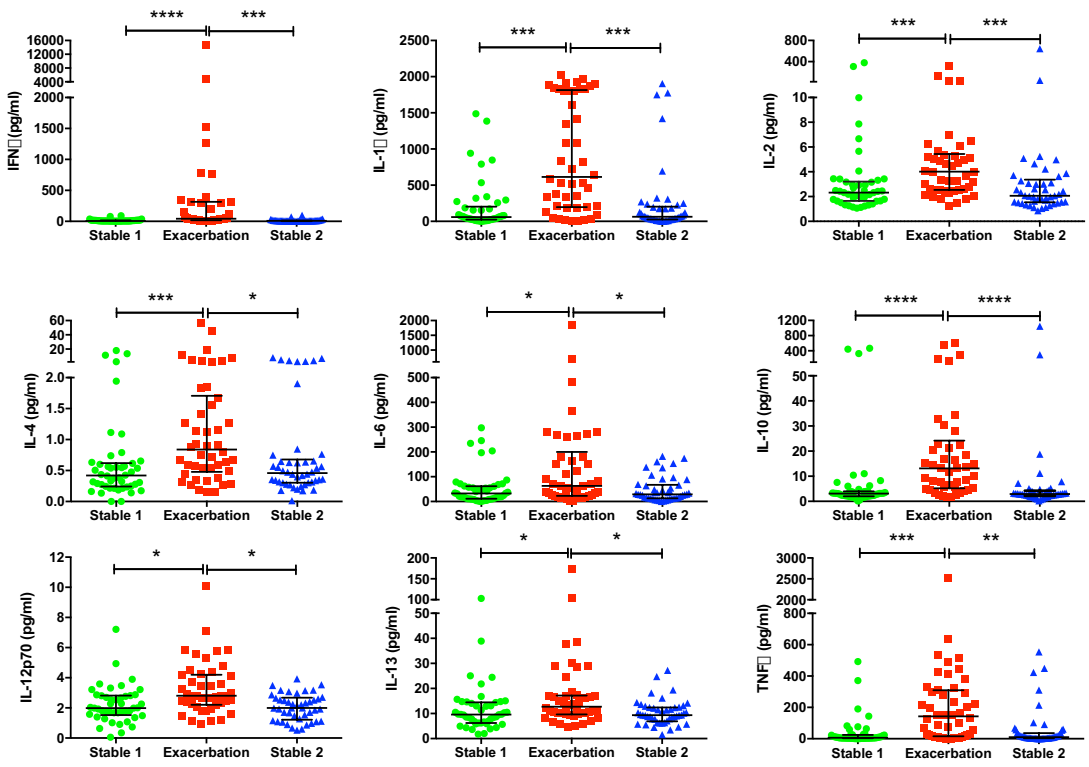


Figure 6.44. Airway pro-inflammatory biomarkers with significantly different concentrations between the first stable, exacerbation presentation and second stable visits (n=45). *p<0.01, **p<0.001, ***p<0.0001. Error bars represent the median and interquartile range.

6.3.3.4 Airway angiogenesis biomarkers

Biomarker	First stable visit median [IQR] (pg/ml)	Exacerbation visit median [IQR] (pg/ml)	Second stable visit median [IQR] (pg/ml)	p-value
VEGF	4870 [2741-5783]	5699 [3906-7446]	4687 [3189-5998]	0.075
VEGF-C	260.1 [170.8-371.8]	169.2 [68.0-270.3]	250.2 [163.5-377.3]	0.053
VEGF-D	113.9 [51.0-435.5]	68.1 [33.1-226.7]	78.0 [37.3-151.0]	0.135
Tie-2	175.3 [89.0-309.9]	159.8 [102.8-364.9]	168.9 [117.4-381.9]	0.556
Flt-1	163.8 [93.4-294.0]	114.7 [49.4-259.0]	176.1 [107.7-449.0]	0.161
PIGF	49.5 [26.0-66.1]	24.7 [5.5-52.1]	41.6 [25.3-60.7]	**0.007**
bFGF	2.1 [0.8-3.3]	2.1 [0.8-5.1]	2.3 [1.2-5.0]	0.633

Table 6.34. Differences in airway angiogenesis biomarkers between first stable, exacerbation presentation and second stable visit in the 45 patients included.

Only PIGF was significantly different between the three visits and the multiple comparison between the visits is shown in [illegible].

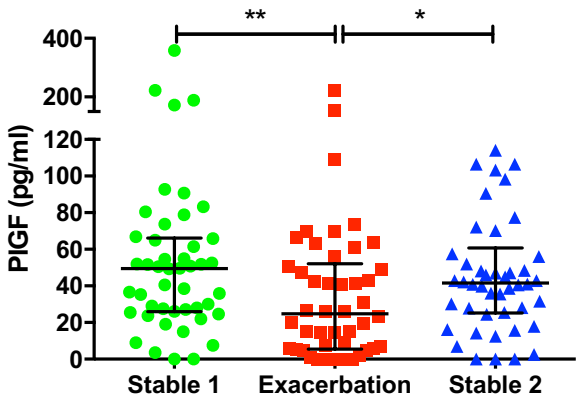


Figure 6.45. Airway PIGF between the first stable, exacerbation presentation and second stable visits (n=45). *p<0.01, **p<0.001. Error bars represent the median and interquartile range.

6.3.3.5 Airway vascular injury biomarkers

Marker	First stable visit median [IQR] (pg/ml)	Exacerbation visit median [IQR] (pg/ml)	Second stable visit median [IQR] (pg/ml)	p-value
SAA	902 [376-1929]	850 [356-3457]	1185 [411-2405]	0.937
CRP	559 [74-1957]	4969 [106-80125]	922 [171-2488]	**0.002**
VCAM-1	182 [91-611]	127 [44-700]	140 [61-365]	0.223
ICAM-1	6737 [1721-13186]	6591 [2401-23403]	5975 [2263-18870]	0.859

Table 6.35. Differences in airway vascular injury biomarkers between first stable, exacerbation presentation and second stable visit in the 45 patients included.

Only CRP showed significantly different concentrations between the three visits. The multiple comparisons are showed in [illegible]. As for all other airway biomarkers, there was only a significant difference between either stable visits with the exacerbation visits but no difference was seen between the two stable visits.

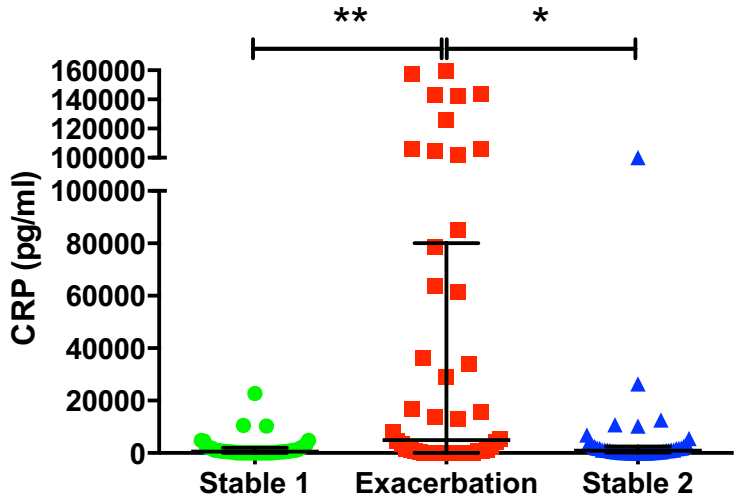


Figure 6.46. Airway CRP between the first stable, exacerbation presentation and second stable visits (n=45). *p<0.01, **p<0.001. Error bars represent the median and interquartile range.

6.3.4 Changes in serum biomarkers between first stable, exacerbation and subsequent stable visits

Similarly for the airway biomarkers, the concentrations of the serum biomarkers for the 5 different multiplex panels at the first stable, exacerbation presentation and subsequent (second) stable visits are reported in [illegible] to [illegible], with multiple comparisons for significant changes shown in [illegible] to [illegible].

6.3.4.1 Serum chemokine biomarkers

Biomarker	First stable visit median [IQR] (pg/ml)	Exacerbation visit median [IQR] (pg/ml)	Second stable visit median [IQR] (pg/ml)	p-value
Eotaxin	211.6 [154.4-255.4]	153.2 [116.0-221.3]	180.5 [142.3-241.5]	**0.003**
MIP-1□	116.1 [88.1-153.7]	119.7 [81.3-138.6]	113.9 [89.4-139.4]	0.979
Eotaxin-3	8.1 [3.2-12.6]	6.4 [2.2-12.1]	8.9 [4.5-12.7]	0.863
TARC	345.7 [192.9-492.2]	286.9 [191.6-446.4]	300.8 [201.0-504.3]	0.064
IP-10	503.7 [329.6-616.3]	586.1 [340.0-882.4]	473.5 [372.9-653.3]	0.649
MIP-1□	15.6 [12.5-21.3]	13.7 [10.6-18.8]	15.2 [10.9-17.3]	0.051
MCP-1	412.7 [358.7-464.5]	362.0 [282.8-434.7]	395.4 [320.1-486.3]	0.057
MDC	1035 [858-1563]	1003 [816-1476]	1136 [891-1380]	0.165
MCP-4	226.1 [161.4-333.0]	192.0 [142.1-275.5]	191.7 [163.9-295.2]	**0.023**

Table 6.36. Differences in serum chemokine biomarkers between first stable, exacerbation presentation and second stable visit in the 45 patients included.

The concentration of serum IL-8 was too low to be used in the high abundance assay present in this panel and so is included in the latter pro-inflammatory panel. Only

Eotaxin and MCP-4 were significantly different between the three visits. The multiple comparisons are shown in [illegible]. A significant difference ($p<0.01$) was only observed between the first stable visit and exacerbation presentation for both chemokines.

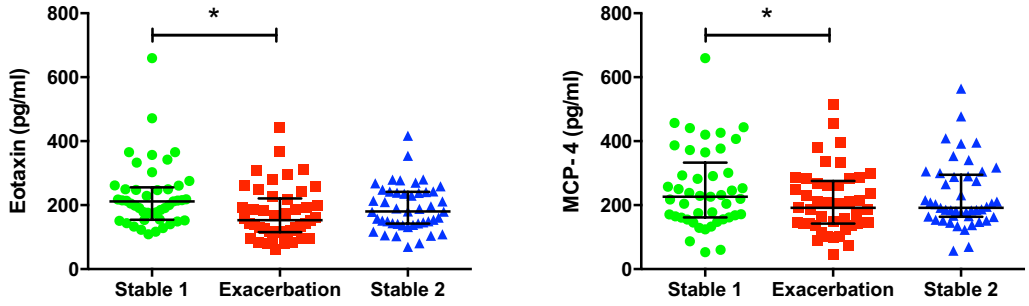


Figure 6.47. Serum chemokines with significantly different concentrations between the first stable, exacerbation presentation and second stable visits (n=45). *$p<0.01$. Error bars represent the median and interquartile range.

6.3.4.2 Serum cytokine biomarkers

Marker	First stable visit median [IQR] (pg/ml)	Exacerbation visit median [IQR] (pg/ml)	Second stable visit median [IQR] (pg/ml)	p-value
GM-CSF	0.05 [0.01-0.16]	0.08 [0.00-0.18]	0.06 [0.00-0.16]	0.840
IL-1□	0.00 [0.00-0.02]	0.00 [0.00-0.00]	0.00 [0.00-0.03]	0.324
IL-5	0.23 [0.06-0.55]	0.25 [0.04-0.50]	0.20 [0.05-0.52]	0.649
IL-7	19.7 [14.7-27.7]	22.4 [15.3-30.7]	21.72 [16.0-30.3]	**0.005**
IL-12/ IL23p40	98.1 [70.3-167.6]	103.0 [76.3-167.2]	103.2 [59.4-163.7]	0.662
IL-15	2.1 [1.9-2.6]	2.5 [2.1-3.0]	2.2 [2.0-2.6]	**<0.001**
IL-16	298.2 [234.9-393.5]	256.0 [191.7-355.9]	281.7 [198.8-345.2]	0.345
IL-17A	0.84 [0.36-1.90]	1.23 [0.73-2.67]	0.91 [0.32-1.67]	0.218
TNF-□	0.18 [0.09-0.22]	0.13 [0.06-0.20]	0.16 [0.10-0.23]	0.059
VEGF	149.9 [79.7-223.4]	127.6 [67.3-253.0]	118.8 [73.9-238.3]	0.754

Table 6.37. Differences in serum cytokine biomarkers between first stable, exacerbation presentation and second stable visit in the 45 patients included.

Significant differences in cytokines between the three different visits were only observed for serum IL-7 and IL-15. The multiple comparisons are shown in [illegible].

There was a significantly higher IL-7 at both exacerbation and second stable visit compared to the first stable visit. IL-15 was significantly higher at exacerbation than both stable visits but there was no significant difference in concentration between the two stable visits.

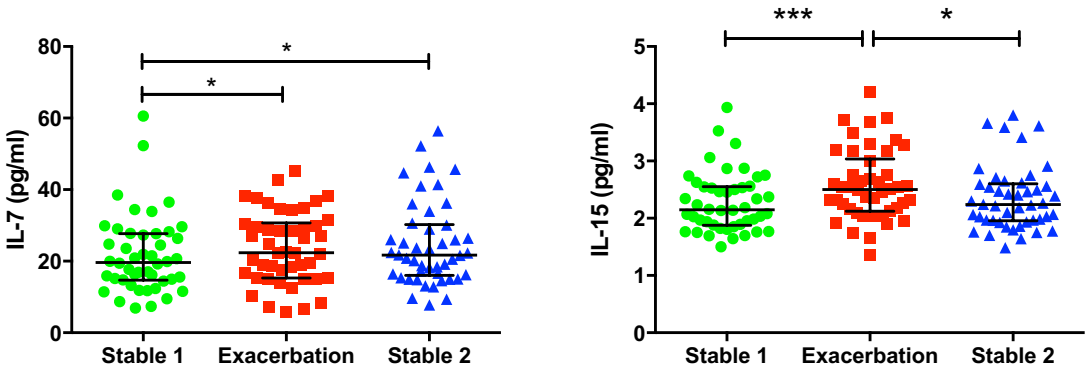


Figure 6.48. Serum cytokine biomarkers with significantly different concentrations between the first stable, exacerbation presentation and second stable visits (n=45). *p<0.01, **p<0.001, ***p<0.0001. Error bars represent the median and interquartile range.

6.3.4.3 Serum pro-inflammatory biomarkers

Biomarker	First stable visit median [IQR] (pg/ml)	Exacerbation visit median [IQR] (pg/ml)	Second stable visit median [IQR] (pg/ml)	p-value
IFN-□	5.7 [4.0-9.2]	9.7 [4.6-32.3]	6.6 [4.8-12.1]	0.544
IL-1□	0.15 [0.02-0.19]	0.12 [0.02-0.19]	0.13 [0.01-0.21]	0.959
IL-2	0.44 [0.35-0.63]	0.52 [0.38-0.62]	0.57 [0.35-0.76]	0.494
IL-4	0.00 [0.00-0.02]	0.01 [0.00-0.03]	0.00 [0.00-0.04]	0.433
IL-6	1.1 [0.8-3.9]	1.7 [1.0-4.4]	1.3 [0.7-1.9]	**<0.001**
IL-10	0.38 [0.23-0.50]	0.46 [0.26-0.97]	0.38 [0.26-0.58]	**0.023**
IL-12p70	0.25 [0.14-0.32]	0.19 [0.13-0.33]	0.21 [0.08-0.34]	0.306
IL-13	0.75 [0.00-1.77]	0.96 [0.00-1.62]	1.39 [0.00-2.56]	0.144
TNF-□	2.9 [5.3-3.5]	3.0 [2.5-3.7]	3.0 [2.2-3.6]	0.64
IL-8	15.4 [12.1-19.9]	14.5 [11.1-19.5]	14.4 [9.9-21.4]	0.282

Table 6.38. Differences in serum pro-inflammatory biomarkers between first stable, exacerbation presentation and second stable visit in the 45 patients included.

Unlike in the airway samples where all pro-inflammatory biomarkers were significantly different between the three visits, in the serum, only IL-6 and IL-10 showed any significant differences. The multiple comparisons are shown in □□□□□□□□□□. There was a significant difference between the exacerbation concentration of IL-6 with both the first and second stable visit, but there was no significant difference between the two stable visits. For IL-10, there was only a significant difference in the serum concentrations between the first stable visit and the exacerbation visit.

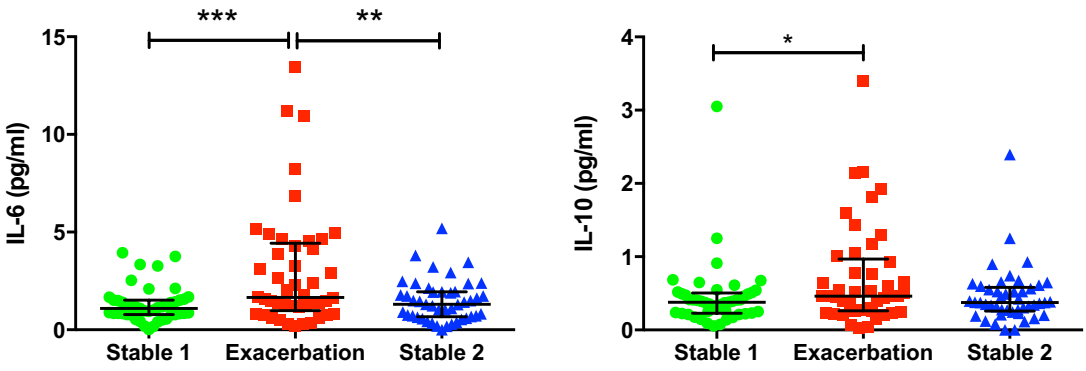


Figure 6.49. Serum pro-inflammatory biomarkers with significantly different concentrations between the first stable, exacerbation presentation and second stable visits (n=45). *p<0.01, **p<0.001, ***p<0.0001. Error bars represent the median and interquartile range.

6.3.4.4 Serum angiogenesis biomarkers

Biomarker	First stable visit median [IQR] (pg/ml)	Exacerbation visit median [IQR] (pg/ml)	Second stable visit median [IQR] (pg/ml)	p-value
VEGF	861 [419-1372]	755 [394-1545]	832 [431-1330]	0.918
VEGF-C	560.5 [396.8-778.5]	535.2 [380.9-645.9]	535.3 [418.3-669.8]	**0.013**
VEGF-D	1197 [961-1563]	1123 [933-1361]	1207 [987-1456]	**0.005**
Tie-2	5972 [5327-6691]	5508 [4884-6041]	6015 [5344-6787]	**0.002**
Flt-1	109.7 [86.6-144.5]	114.6 [92.0-152.0]	124.9 [95.5-153.1]	0.544
PIGF	44.6 [31.1-51.6]	42.2 [31.1-51.1]	41.3 [34.3-51.1]	0.859
bFGF	9.4 [3.7-18.8]	7.4 [2.1-14.3]	4.6 [2.6-8.6]	**0.014**

Table 6.39. Differences in serum angiogenesis biomarkers between first stable, exacerbation presentation and second stable visit in the 45 patients included.

VEGF-C, VEGF-D, Tie-2 and bFGF showed significant differences between the three visits. The multiple comparisons are shown in . All serum angiogenesis biomarkers except bFGF showed significant differences between the first stable visit and the exacerbation visit. Only VEGF-D and Tie-2 also demonstrated a significant difference between the exacerbation and second stable visits. bFGF was the only serum angiogenesis biomarker which showed a significant difference between the two stable visits.

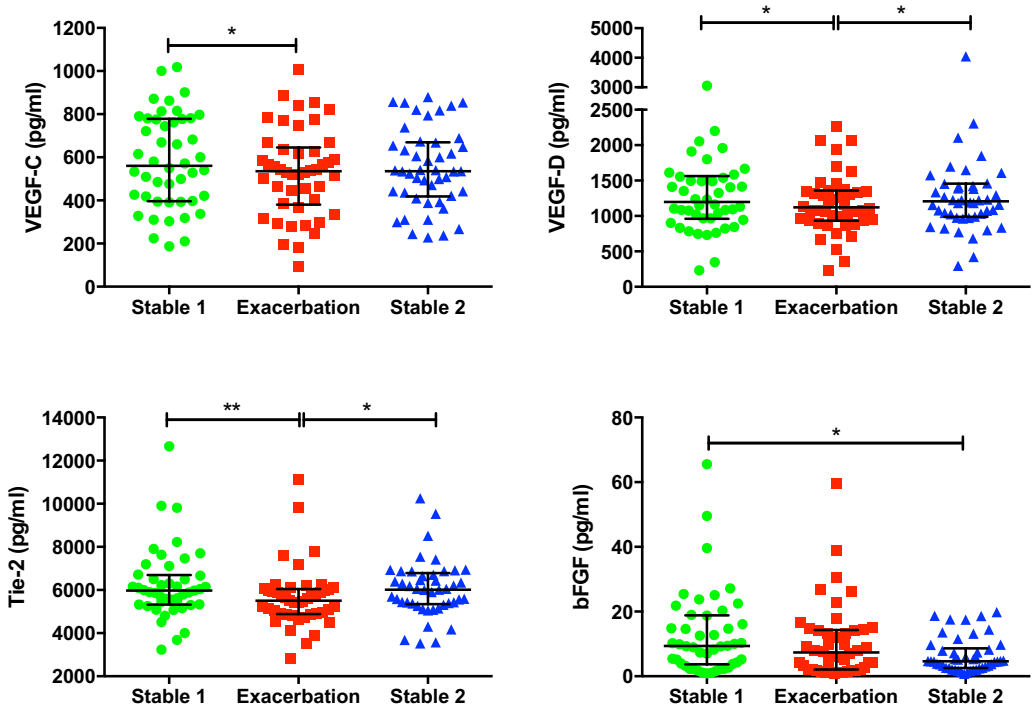


Figure 6.50. Serum angiogenesis biomarkers with significantly different concentrations between the first stable, exacerbation presentation and second stable visits (n=45). *p<0.01, **p<0.001. Error bars represent the median and interquartile range.

6.3.4.5 Serum vascular injury biomarkers

Biomarker	First stable visit median [IQR] *(g/ml) (pg/ml)	Exacerbation visit median [IQR] *(g/ml) (pg/ml)	Second stable visit median [IQR] *(g/ml) (pg/ml)	p-value
SAA*	9.2 [3.8-22.9]	54.7 [7.5-323.3]	11.6 [3.1-59.1]	**<0.001**
CRP*	7.8 [0.8-23.5]	40.1 [3.1-155.0]	7.3 [0.9-30.4]	**<0.001**
VCAM-1	182.0 [90.5-611.2]	126.9 [43.8-700.1]	139.5 [60.7-365.2]	**<0.001**
ICAM-1	6737 [1721-13186]	6591 [2401-23403]	5975 [2263-18870]	**<0.001**

Table 6.40. Differences in serum vascular injury biomarkers between first stable, exacerbation presentation and second stable visit in the 45 patients included.

All vascular injury biomarkers were significantly different between the three visits. The multiple comparisons are shown in [illegible].

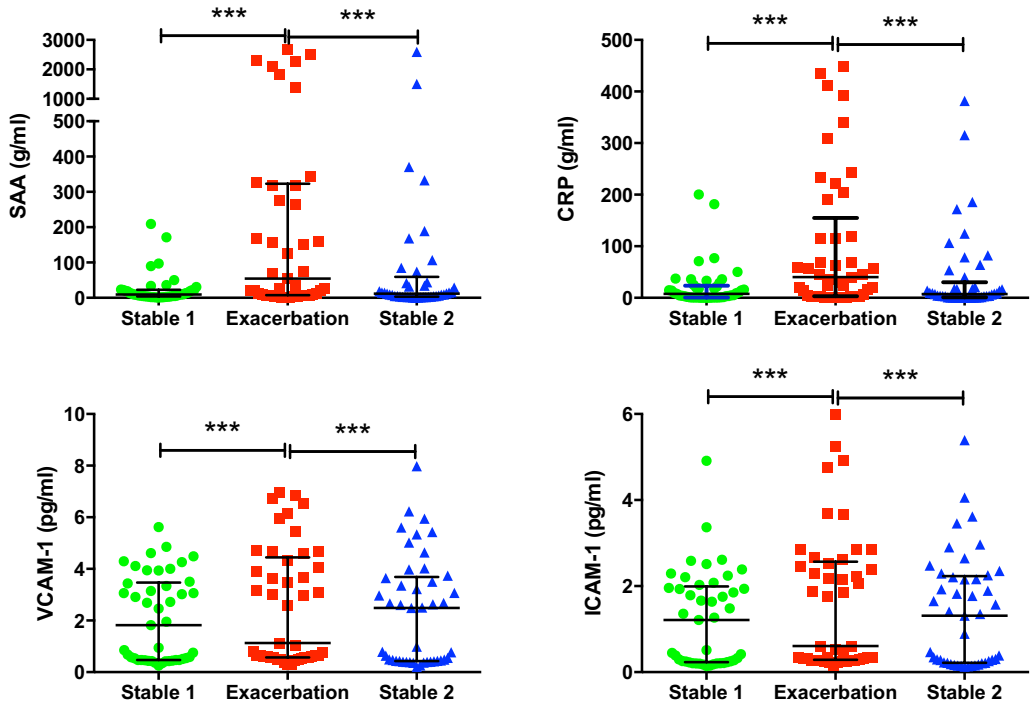


Figure 6.51. Serum vascular injury biomarkers with significantly different concentrations between the first stable, exacerbation presentation and second stable visits (n=45). ***p<0.0001. Error bars represent the median and interquartile range.

All biomarkers showed a significant difference between the exacerbation and both stable visits, but no significant difference between the two stable visits.

6.3.5 Correlations between airway and systemic biomarkers at the first stable visit and exacerbation presentation

Spearman's rank correlation matrices were performed to determine the relationship between airway and systemic biomarkers at both the first stable and exacerbation presentation visit. To enable a graphical display of the numerous correlation coefficients generated, as part of collaborative work with Pfizer, heatmaps were created, displaying the coefficients from -1.0 in blue to +1.0 in red.

At the first stable visit, the most highly correlated biomarkers were VEGF1 and VEGF both in serum. All the biomarkers with a correlation coefficient of >0.80 were from the same compartment i.e. sputum biomarkers correlated with other sputum biomarkers and serum biomarkers correlated with other serum biomarkers. In general, the expression patterns of particular biomarkers in serum and sputum have little correlation. [illegible] shows the heatmap for the first stable visit and the corresponding [illegible] shows the biomarkers with correlation coefficients >0.80.

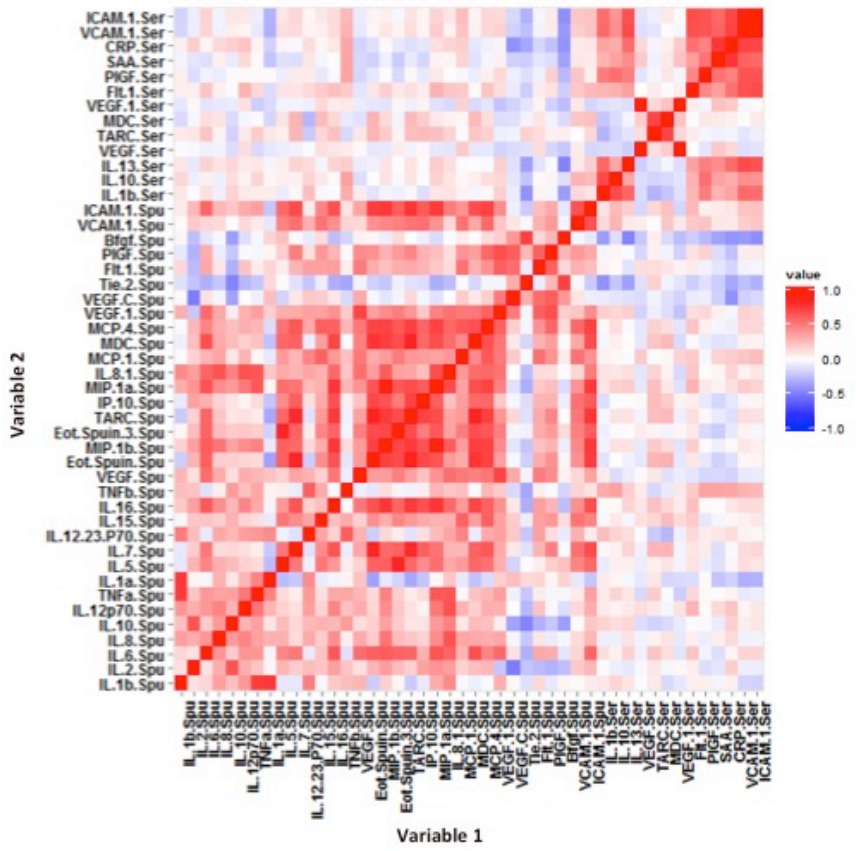


Figure 6.52. Heatmap showing correlations between serum and sputum biomarkers at the first stable visit. Red indicates a positive correlation (0<correlation coefficient□1.0) and blue indicates a negative correlation (-1.0<correlation coefficient<0.0). Graph generated by Pfizer.

□

Biomarker 1	Biomarker 1 compartment	Biomarker 2	Biomarker 2 compartment	Correlation coefficient
VEGF-1	serum	VEGF	serum	0.969
ICAM-1	serum	VCAM-1	serum	0.964
MIP-1□	sputum	MIP-1□	sputum	0.927
TARC	sputum	Eotaxin	sputum	0.878
MDC	sputum	TARC	sputum	0.849
Eotaxin-3	sputum	IL-5	sputum	0.845
Eotaxin	sputum	IL-7	sputum	0.837
TARC	sputum	IL-7	sputum	0.818

Table 6.41. The highest correlation coefficients >0.80 between biomarkers and their compartment (i.e. sample type) at the first stable visit.

□ □□□

At exacerbation presentation, more biomarker pairs were positively correlated, with 26 pairs all having correlation coefficients >0.80 (□□□□□□□□). This is reflected in the heatmap shown in □□□□□□□□□□, which demonstrated much larger areas of red reflecting the positive correlation coefficients. Similarly to the first stable visit, all highly correlated biomarker pairs were from the same compartment (either sputum or serum), and the majority of these pairs represented airway inflammation.

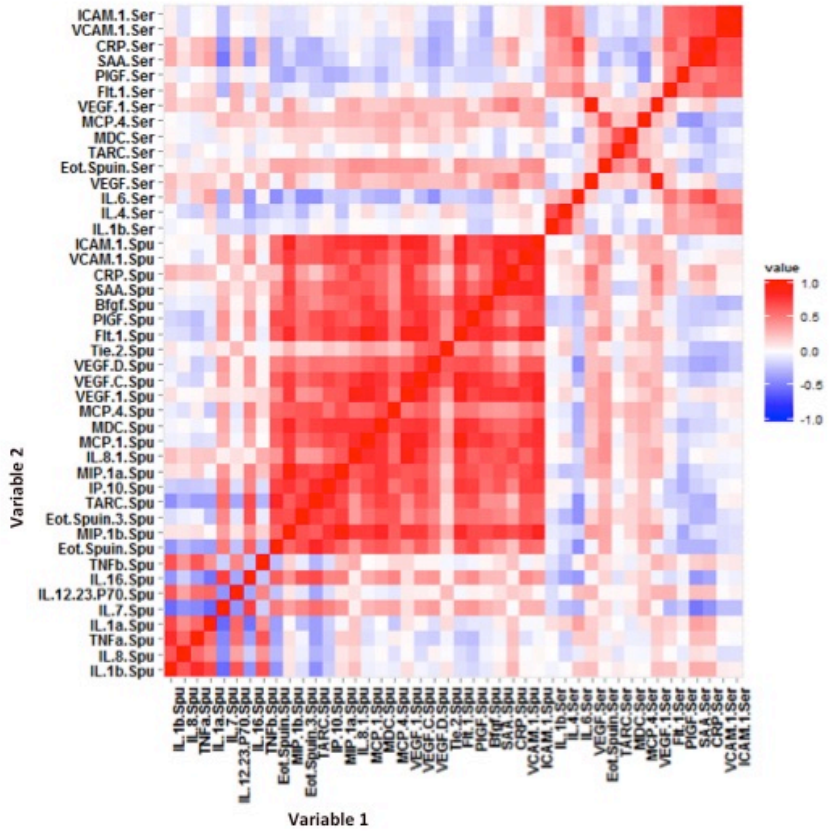


Figure 6.53. Heatmap showing correlations between serum and sputum biomarkers at the exacerbation presentation visit. Red indicates a positive correlation (0<correlation coefficient□1.0) and blue indicates a negative correlation (-1.0<correlation coefficient<0.0). Graph generated by Pfizer.

Biomarker 1	Biomarker 1 sample type	Biomarker 2	Biomarker 2 sample type	Correlation coefficient
VEGF-1	serum	VEGF	serum	0.973
ICAM-1	serum	VCAM-1	serum	0.968
MIP-1□	sputum	MIP-1□	sputum	0.944
Flt-1	sputum	MCP-1	sputum	0.934
VEGF-1	sputum	IL-8	sputum	0.899
Flt-1	sputum	VEGF-1	sputum	0.89
CRP	serum	SAA	serum	0.882
Flt-1	sputum	VEGF-C	sputum	0.879
ICAM-1	sputum	MIP-1□	sputum	0.873
VEGF-1	sputum	MCP-1	sputum	0.869
ICAM-1	sputum	Flt-1	sputum	0.864
ICAM-1	sputum	VCAM-1	sputum	0.861
VCAM-1	sputum	SAA	sputum	0.861
ICAM-1	sputum	VEGF-1	sputum	0.851
TARC	sputum	Eotaxin	sputum	0.841
VEGF-C	sputum	VEGF-1	sputum	0.838
VEGF-C	sputum	MCP-1	sputum	0.837
MDC	sputum	MIP-1□	sputum	0.836
VCAM-1	sputum	Flt-1	sputum	0.828
CRP	sputum	SAA	sputum	0.828
ICAM-1	sputum	VEGF-C	sputum	0.828
Flt-1	sputum	MIP-1□	sputum	0.825
IP-10	sputum	TARC	sputum	0.821
TNF□	sputum	IL-1□	sputum	0.82
ICAM-1	sputum	SAA	sputum	0.819
ICAM-1	sputum	CRP	sputum	0.818

Table 6.42. The highest correlation coefficients >0.80 between biomarkers and their compartment (i.e. sample type) at exacerbation presentation visit.

6.3.6 The effect of exacerbation on correlations between changes in airway and systemic biomarkers

To determine whether changes in biomarkers between stable and exacerbation states correlated between the airway and systemic compartment, Spearman's rank correlation matrices were performed using changes between the first stable visits and subsequent exacerbation, and from the exacerbation to subsequent second stable visit.

While there were numerous significant correlations between the biomarkers within each compartment, there were only three biomarkers where changes in their airway concentrations between first stable and exacerbation states were significantly associated with corresponding changes in the systemic compartments (IL-10: p=0.027; rho=0.33, IFN□: p=0.031; rho=0.32 and TNF□: p=0.004; rho=0.41, □□□□□□□□□□□).

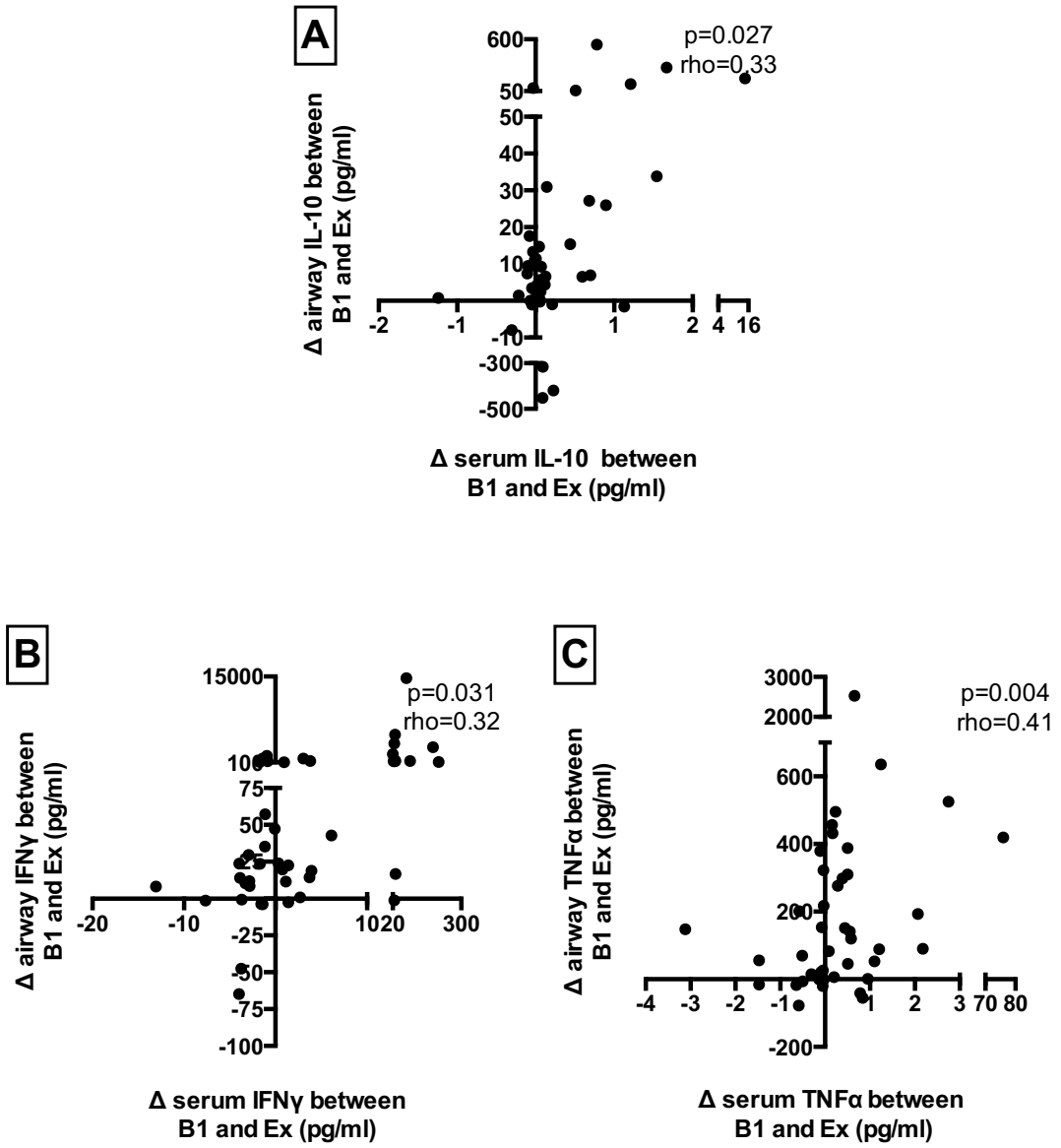


Figure 6.54. Relationship between changes in the same airway and systemic biomarkers between first stable visit (B1) and exacerbation (Ex). Graphs are shown for changes biomarkers significantly associated in both the airway and systemic compartments (A) IL-10, (B) IFN□and (C) TNF□. □: Change, n=45.

□

When changes between the exacerbation and subsequent stable state visit were considered, only two of the above three biomarkers demonstrated significant correlations between changes in their airway and systemic levels, although there was

□ □□□

a trend towards a significant association with TNF□(IL-10: p=0.002; rho=0.44, IFN□: p=0.030; rho=0.32 and TNF□: p=0.067; rho=0.27, □□□□□□□□□□).

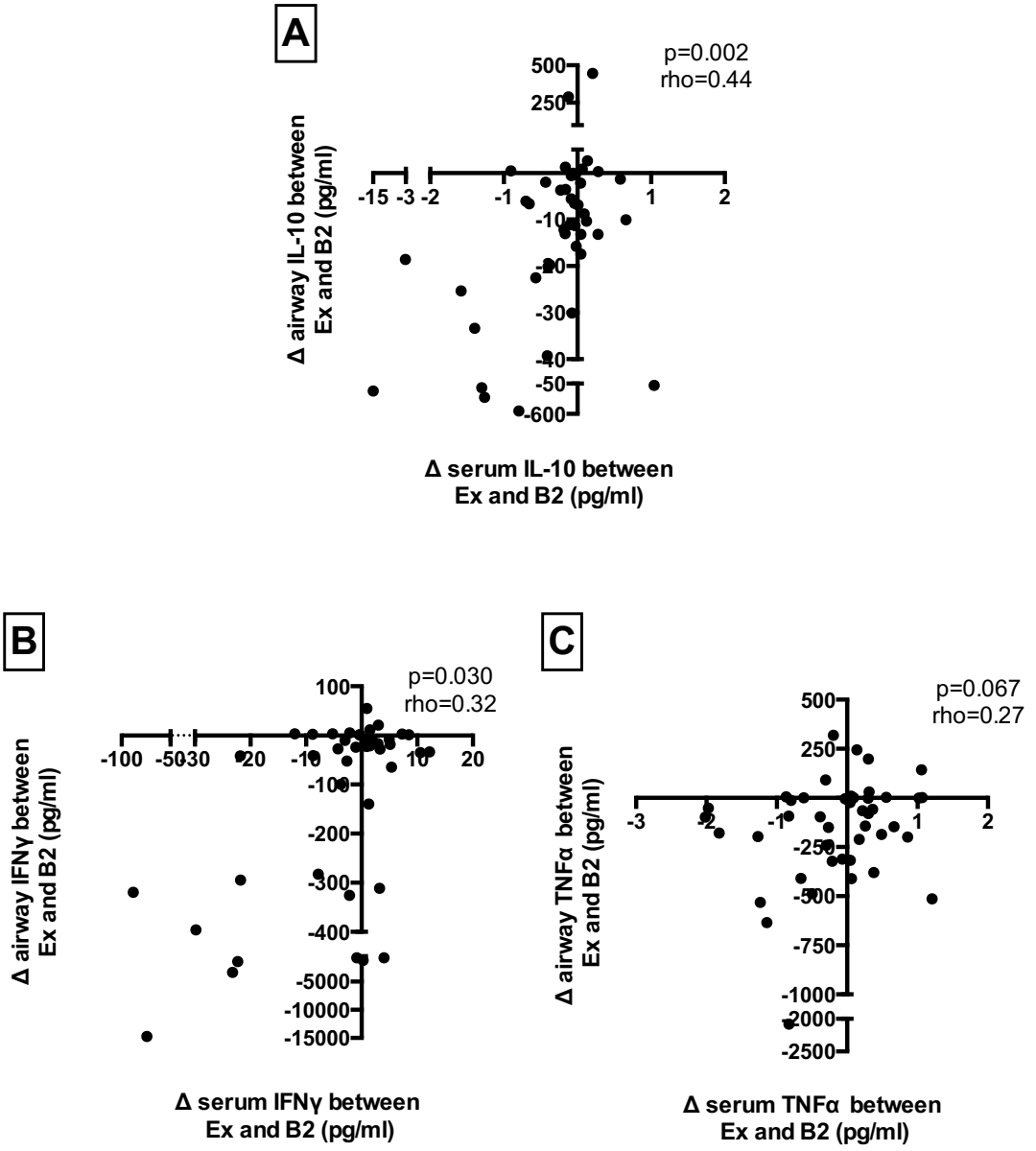


Figure 6.55. Relationship between changes in airway and systemic (A) IL-10, (B) IFNγ and (C) TNFα between exacerbation (Ex) and second stable (B2) visit. □: Change, n=45.

6.3.7 The effect of bacterial and/or viral pathogens on changes in inflammatory profile between stable and exacerbation states

To determine whether changes in airway and systemic inflammation between stable and exacerbation states were affected by the presence of bacterial and viral pathogens at exacerbation, differences in inflammatory profiles were considered between 4 groups; no pathogens, bacteria only, HRV only and co-infection with both HRV and bacteria. To take into account stable state total bacterial load, bacteria were only considered present at exacerbation when the total bacterial load increased from stable to exacerbation state (□□□□□□□□□B). If the total bacterial load decreased, or no bacteria were detected, the exacerbation sample was considered negative for bacteria. Therefore, in these analyses, 17/45 (38%) patients had no airway bacterial pathogens or HRV detected, 13/45 (29%) patients only bacterial pathogens isolated, 9/45 (20%) patients had only HRV isolated and 6/45 (13%) had co-infection with both HRV and bacterial pathogens detected.

6.3.7.1 Changes in airway biomarkers

In the chemokine panel, a significant difference between changes in concentrations of airway MCP-1 from stable to exacerbation state was seen between the four different exacerbation pathogen groups (-71 [-317-793] vs. -163 [-455-219] vs. 119 [-123-1578] vs. -1094 [-2245-75] pg/ml, for no pathogen, bacteria only, HRV only and co-infection respectively, p=0.025, □□□□□□□□□A). On multiple comparisons, the significant difference was only observed between HRV only and co-infection groups. There was a trend towards a difference in changes in TARC between the four different pathogen groups, but this did not reach statistical significance (p=0.079).

In the cytokine panel, a significance difference was observed between the four pathogen groups and changes in IL-5 between stable and exacerbation states (-0.05 [-2.3-1.3] vs. 1.0 [-0.2-15.3] vs. 1.3 [-6.1-6.4] vs. -1.8 [-16.4-0.6] pg/ml, for no pathogen, bacteria only, HRV only and co-infection respectively, p=0.019, [illegible] [illegible]B). On multiple comparisons, the significant difference was only observed between the bacteria only and co-infection group. There was a trend toward a difference in changes in IL-15 and IL-17A between the four groups, but this just missed statistical significance (p=0.052 and p=0.063, respectively).

In the pro-inflammatory panel, a significant difference was observed between the four pathogen groups and increases in IL-1□ between stable and exacerbation states (483 [117-1048] vs. 1018 [193-1787] vs. 85 [-255-434] vs. 1737 [315-1831], for no pathogen, bacteria only, HRV only and co-infection respectively, p=0.041, [illegible] [illegible]C). On multiple comparisons, the significant difference was observed between the HRV only and co-infection groups. There was a trend toward a difference in changes in TNF□ between the four groups, but this was not statistically significant (p=0.075).

In the angiogenesis panel, a significant difference was observed between the four pathogen groups and changes in Tie-2 between stable and exacerbation states (-73 [-184-62] vs. 1 [-88-170] vs. 2 [-375-18] vs. 169 [101-214], for no pathogen, bacteria only, HRV only and co-infection respectively, p=0.012, [illegible]D). On multiple comparisons, the significant difference was only observed between no pathogens and co-infection groups.

[illegible] [illegible]

[illegible] [illegible]

In the vascular injury panel, there were no significant differences between any of the biomarkers measured in the four-pathogen groups.

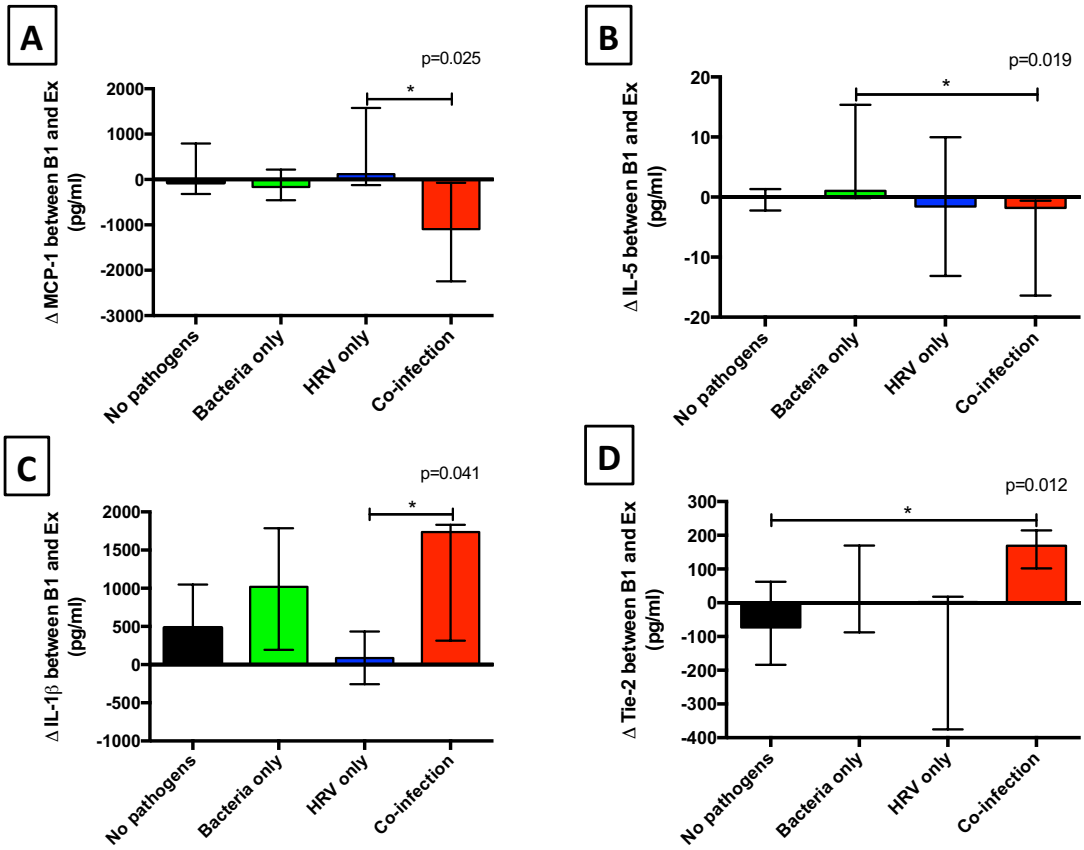


Figure 6.56. Changes in airway biomarkers between stable (B1) and exacerbation (Ex) states depending on the pathogens present at exacerbation. Graphs depict changes in (A) MCP-1, (B) IL-5, (C) IL-1□, and (D) Tie-2. 4 pathogen groups were defined; no pathogens, bacteria only, HRV only and co-infection with both HRV and bacteria. To take into account stable state total bacterial load, bacteria were only considered present at exacerbation when the total bacterial load increased from stable to exacerbation state (n=45). □: Change. HRV: human rhinovirus. *p<0.05 on multiple comparison.

6.3.7.2 Changes in systemic biomarkers

In the chemokine panel, a significant difference between changes in concentrations of systemic MCP-1 from stable to exacerbation state was seen between the four different exacerbation pathogen groups (-91 [-133-10] vs. 22 [-46-87] vs. -32 [-178-83] vs. -1023 [-140-33] pg/ml, for no pathogen, bacteria only, HRV only and co-infection respectively, p=0.030, [illegible]A). On multiple comparisons, the significant difference was only observed between the no pathogen and bacteria only groups. There was a trend toward a difference in changes in IP-10 between the four groups, but this just missed statistical significance (p=0.051).

In the cytokine panel, a significance difference was observed between the four pathogen groups and changes in IL-17A between stable and exacerbation states (0.10 [-0.26-0.53] vs. 0.52 [-0.19-1.45] vs. -0.21 [-1.00-0.10] vs. 1.00 [0.31-3.49] pg/ml, for no pathogen, bacteria only, HRV only and co-infection respectively, p=0.033, [illegible]B). On multiple comparisons, the significant difference was only observed between the HRV only and co-infection groups.

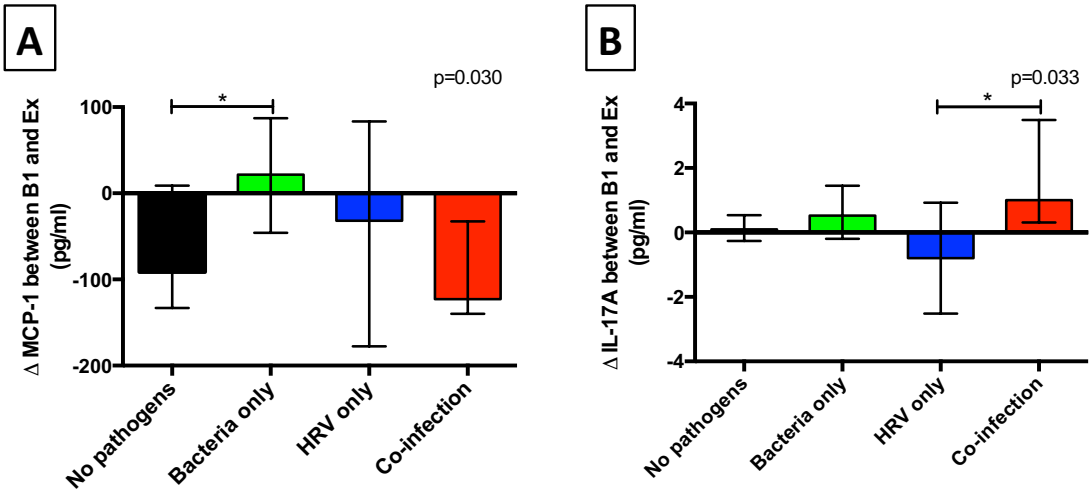


Figure 6.57. Changes in systemic biomarkers between stable (B1) and exacerbation (Ex) states depending on the pathogens present at exacerbation. Graphs depict changes in (A) MCP-1, and (B) IL-17A. 4 pathogen groups were defined; no pathogens, bacteria only, HRV only and co-infection with both HRV and bacteria. To take into account stable state total bacterial load, bacteria were only considered present at exacerbation when the total bacterial load increased from stable to exacerbation state (n=45). □: Change. HRV: human rhinovirus. *p<0.05 on multiple comparison.

There were no significant differences between the four pathogen groups and biomarkers in the serum pro-inflammatory, angiogenesis and vascular injury panels.

6.3.8 The independent effect of bacterial species and viral pathogens on biomarkers at exacerbation

Multiple linear regressions were used to assess the independent effect of the different bacterial species and HRV presence at exacerbation on airway and systemic inflammation. To take into account the presence of airway bacteria at the preceding first stable visit, the increase in bacterial load from first stable visit to exacerbation visit was determined for each bacterial pathogen.

□□□□□□□□ shows the prevalence of bacterial detection at the first stable visit, the number of patients with an increase in bacterial load and the mean load change. Due to low HRV prevalence during stable state, HRV was only measured at exacerbation presentation and considered positive if the HRV load was above the lower limit of detection.

Airway Pathogen	No. (%) positive* at stable 1 visit	No. (%) positive† at exacerbation visit	Mean (SD) □ bacterial load (log_{10}cfu/ml) or HRV (log_{10}pfu/ml) at exacerbation
H. influenzae	7 (16)	16 (36)	5.21 (1.08)
S. pneumoniae	5 (11)	8 (18)	6.84 (0.85)
M. catarrhalis	4 (9)	4 (9)	0.84 (0.09)
HRV	NA	15 (33)	4.15 (2.47)

Table 6.43. The detection of airway pathogens at first stable and exacerbation presentation visits. *At stable state, patients were defined as positive for airway bacteria if □l pathogens were detected in the stable sputum sample by qPCR. †At exacerbation state, patients were defined as positive for a bacterial pathogen if the load for that species increased at exacerbation presentation. HRV: human rhinovirus. □: Change between stable and exacerbation states.

At exacerbation *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* load increased from baseline in 16/45 (36%), 8/45 (18%) and 4/45 (9%) of patients' respectively. 15/45 (33%) of patients were also positive for HRV at exacerbation. Co-infection with two or more bacteria and/or HRV was observed in 11/45 (24%) of patients.

In patients positive for *H. influenzae,* there were higher levels at exacerbation relative to baseline of airway IL-1□ (p=0.004), but a significant decrease in Flt-1 (p=0.002)

and ICAM-1 (p=0.007), independent of the presence of *S. pneumoniae*, *M. catarrhalis* or HRV (□□□□□□□□□). No independent effects of *H. influenzae* were observed in changes in serum biomarkers at exacerbation.

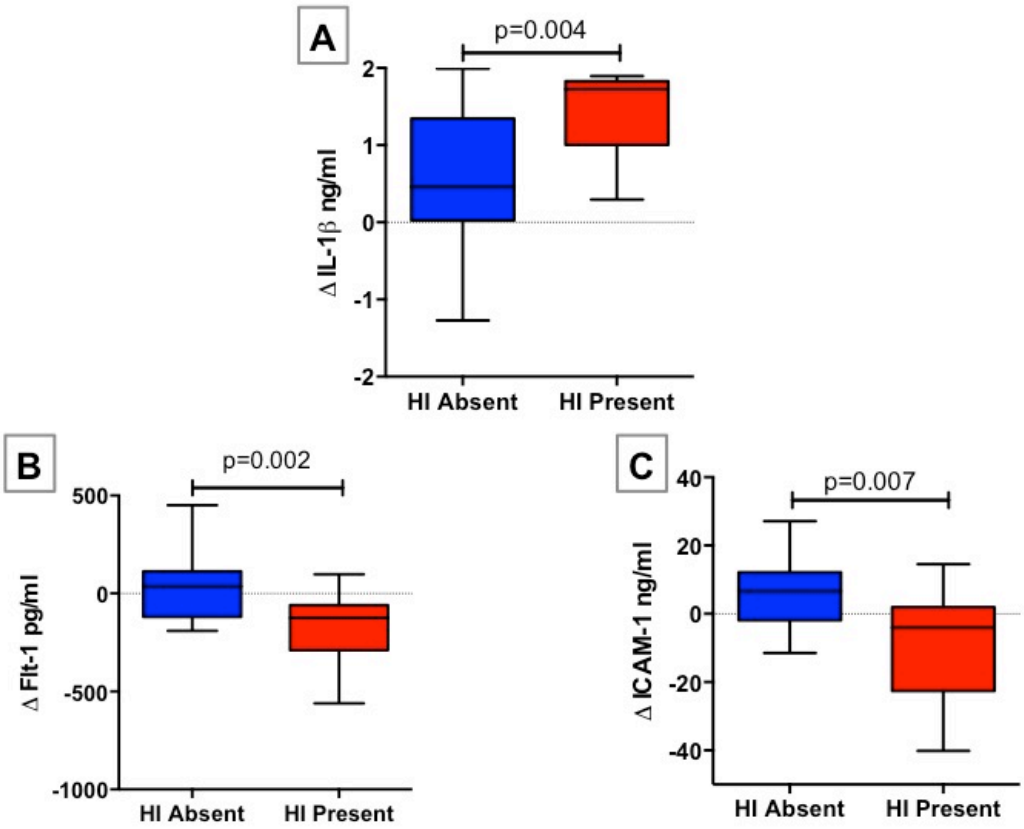


Figure 6.58. Change in airway (A) IL-1β (B) Flt-1 and (C) ICAM-1 at exacerbation relative to baseline in patients positive for *H. influenzae* (HI). Box-plots represent median and interquartile range, and whiskers represent ±(1.5 x IQR). p-value is independent of co-infection with either other bacteria or HRV using multiple regressions. □: Change.

□

In patients positive for *M. catarrhalis*, there were significantly higher levels of airway IL-12p70 at exacerbation relative to baseline (p=0.001), independent of co-infection with either other bacterial pathogens or HRV (□□□□□□□□□A). In addition, there was a

significantly lower concentration in serum VCAM-1 at exacerbation relative to baseline (□□□□□□□□□B).

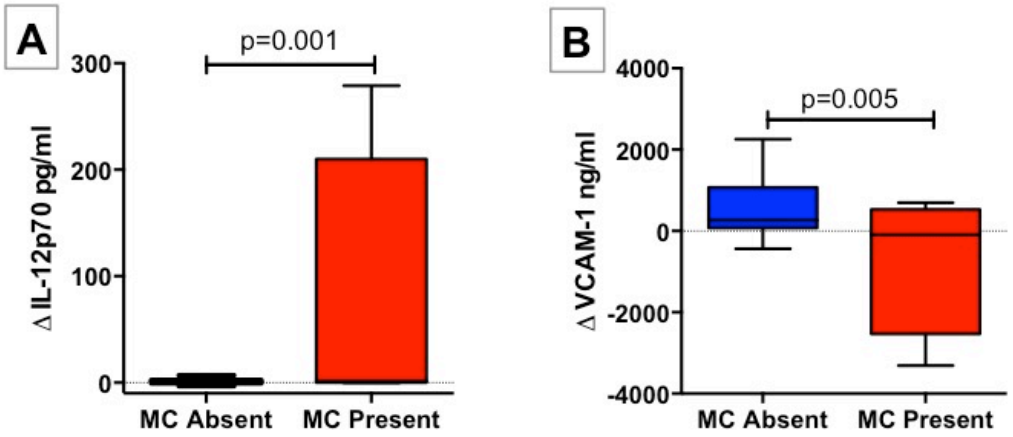


Figure 6.59. Change in (A) airway IL-12p70 and (B) serum VCAM-1 at exacerbation relative to baseline in patients positive for *M. catarrhalis* (MC). Box-plots represent median and interquartile range, and whiskers represent ±(1.5 x IQR). p-value is independent of co-infection with either other bacteria or HRV using multiple regressions. □: Change.

□

There were no significant changes in either airway or serum biomarkers between stable and exacerbation states independent of co-infection in patients positive for either *S. pneumoniae* or HRV at exacerbation.

6.4 Discussion

This study examined the detailed airway and systemic inflammatory profile at three consecutive time points.. There were numerous biomarkers that changed depending on clinical state in both the airway and systemic compartments, with the majority returning towards pre-exacerbation levels at subsequent stable visits. There was poor correlation between the same biomarker within both airway and systemic compartments at either stable state or exacerbation, although numerous biomarkers, generally within the same compartment correlated with each other. When changes in the inflammatory profile between stable and exacerbation states in both compartments were correlated, only changes in IL-10, IFN□ and TNF□ significantly correlated between the airway and systemic compartments. Changes in the inflammatory profile differed depending on the presence of bacteria and/or HRV at exacerbation, and only MCP-1 was significantly different in both airway and systemic compartments. In addition, a species-specific effect on inflammation at exacerbation was seen which was independent of the presence of other bacterial or viral pathogens, with both increases in *H. influenzae* and *M. catarrhalis* load associated with changes in airway inflammation but only increases in *M. catarrhalis* load associated with changes in systemic inflammation.

It is increasingly recognised that COPD is a heterogeneous condition, both in terms of clinical characteristics and stability but also the nature of the underlying chronic inflammation and pathogenesis. Respiratory symptoms and spirometry have been used as the mainstay of monitoring changes in clinical features, but these are inherently subjective measurements. Therefore, there has been increasing interest in the use of potential biomarkers to provide objective information that reflects disease

activity in COPD, represent biologically plausible pathways and alter according to disease state (Yoon and Sin, 2011). A wide variety of different potential biomarkers from blood, sputum and exhaled breath have been investigated (Agusti et al., 2015), and the study of cytokines to improve our understanding of the underlying inflammatory process and thus act as potential biomarkers has significantly increased.

In this study, the same 40 biomarkers were analysed in both sputum and serum samples to profile the airway and systemic inflammatory profile at three consecutive time points; stable, exacerbations and subsequent stable visit, with concurrent bacteriology and HRV detection at exacerbation. These exacerbations were defined using the London COPD cohort symptom-based definitions (section 3.3.1) and have been validated against quality of life (Seemungal et al., 1998) and FEV_1 decline (Donaldson et al., 2002). Airway pro-inflammatory and cytokine biomarkers predominantly increased at exacerbation from the first stable visit, with the majority returning to similar stable levels at the subsequent stable visit. Chemokine biomarkers tended to decrease at exacerbation, again returning to pre-exacerbation levels at the second stable visit, and there were only changes in two biomarkers in the vascular injury and angiogenesis panel measured in sputum. Systemic pro-inflammatory and cytokine panels showed similar increases, and chemokine panels decreases, in the overall biomarkers, although importantly the biomarkers that changed in these panels were not necessarily the same in both compartments. Furthermore, in serum, there were more biomarkers in both the vascular injury and angiogenesis panels that changed depending on clinical state.

Previous studies have demonstrated similar increases in both airway and systemic inflammation at exacerbation. However, considering the large number of biomarkers that have been investigated, especially with the use MSD or proteome arrays to test a broad panel of biomarkers, few are consistent amongst the studies, with airway IL-1□, IL-6, IL-10, CXCL8 (IL-8) and TNF□ (Aaron et al., 2001; Bafadhel et al., 2011; Bhowmik, 2000; Sethi et al., 2000) and serum IL-6, CRP, SAA, ICAM-1 (Bafadhel et al., 2011; Bozinovski et al., 2008; Hurst et al., 2006a) predominantly identified. This is partly due to the inherent variability in the measurement of biomarkers (Aaron et al., 2010; Sapey et al., 2008; Singh et al., 2010), especially true for airway biomarkers, where the type of sample itself (Bhowmik et al., 1998) and processing methods can alter absolute concentrations (Bafadhel et al., 2012a; Pedersen et al., 2015). This study not only adds further weight to the importance of the airway and systemic biomarkers consistently highlighted above, but demonstrates that post-exacerbation, inflammation returns to pre-baseline levels, and thus reflects on disease activity between stable and exacerbation state. Although previous studies have demonstrated that both airway and systemic inflammation increase over time leading to a faster decline in FEV_1 (Donaldson et al., 2005), this study was carried out over almost 3 years and therefore is unlikely that a similar finding would be observed in this dataset, where all three consecutive sampling time points were within the same year.

In this dataset, measurement of the same biomarkers within the airway and systemic compartments were performed, to enable a direct comparison between the two and to determine whether the systemic inflammatory profile was similar to that of the airway. This could not be tested using the dataset in chapter 4 due to serum levels

being below the level of detection for the ELISA assays. Systemic inflammation is assumed to result from a 'spill-over' of airway inflammatory mediators, (Barnes, 2010; Sinden and Stockley, 2010). There is increasing interest to find systemic markers that can be easily sampled by peripheral venepuncture and have limited processing needs, which reflects both the airway and systemic inflammatory process. In this study, the absolute levels of biomarkers in both the airway and systemic compartments were correlated against each other, at both stable and exacerbation state. The most highly associated biomarkers were all within the same compartment, but there were no significant correlations within the same biomarker in both compartments, similar to reports from previous studies (Sapey et al., 2009; Vernooy et al., 2002). Nevertheless, the lack of correlation does not disprove the 'spill-over' hypothesis, as other factors may influence the relationship between airway and systemic cytokines including the permeability of the epithelium and whether facilitated transport proteins are involved.

However, when changes in airway and systemic biomarkers between stable and exacerbation states were correlated against each other, there were three significant correlations, albeit weak ones, in changes in the same biomarkers between both compartments; IL-10, TNF□ and IFN□. This change in biomarker concentration during the onset of an exacerbation is likely to be rapid which may help to explain the correlation between both airway and systemic compartments, not seen with the absolute values at each state. IL-10, TNF□and IFN□are all between 19-26 kDa in size and molecules <40 kDa have been shown to rapidly appear in the blood following inhalation into the airways (Laube et al., 1993). Therefore, if rapid airway changes in airway concentrations occur at exacerbation, combined with increased permeability of

the epithelium, associated systemic levels may also increase. TNF□ is secreted by numerous inflammatory cells and is thought to play a central role as a neutrophil chemotactic protein including at exacerbation (Churg et al., 2004; Gan et al., 2004; Keatings et al., 1996). IFN□ is involved in numerous pathways and is a potent activator of macrophages priming them to increase their response to TLR ligands (Schroder et al., 2004), and hence its increase at exacerbation when increased pathogen recognition is important to host defences. IL-10 is a potent anti-inflammatory cytokine acting to inhibit numerous cytokines including TNF□, IFN□ and GM-CSF. Previous studies have demonstrated a reduction in IL-10 in stable COPD patients (Maneechotesuwan et al., 2013; Takanashi et al., 1999), although how it changes at exacerbation is less clear. The heightened response at exacerbation seen in this study may reflect the increases seen in TNF□ as an interacting pathway. However, it is likely that other complexities will influence the relationship between airway and systemic inflammation, and a 'spill-over' effect alone is too simplistic. It is clear that although numerous inflammatory biomarkers have been identified in COPD, their individual roles as well as their complex inter-relationships contributing to the pathophysiology remains unclear. Further studies using the developing field of network and pathway analysis (Agusti et al., 2012; Barnes, 2009) are needed to delineate these multifaceted cytokine networks and help identify inflammatory biomarkers and their relationship with disease states.

In this study, there were significant differences in the inflammatory profile of exacerbations based on the associated aetiology. Exacerbations were defined as either not associated with any pathogens, bacterial- or viral only associated, or co-infection with both bacteria and viruses. Bacterial-associated exacerbations were only

considered if total bacterial load as measured by qPCR increased from stable state, to account for the prevalence of airway bacteria detected at stable state. Significant differences were seen in changes in airway MCP-1, IL-5, IL-1□ and Tie-2 and systemic MCP-1 and IL-17A between the four different aetiologies, although no one airway or systemic biomarker was associated with the specific exacerbation aetiologies. Previous studies have identified specific biomarkers associated with exacerbations of different aetiologies. In a study of hospitalised exacerbations, raised C-reactive protein levels have been associated with bacterial-associated exacerbations (Clark et al., 2014). Furthermore, specific biomarkers within biologic clusters have been shown to be predictive of different exacerbation aetiologies (Bafadhel et al., 2011). In the observational study by Bafadhel and colleagues, 182 exacerbations were sampled from 86 patients, and a broad panel of serum and sputum biomarkers was measured using MSD analysis. Cluster analysis revealed four biologic clusters at exacerbation; bacteria-predominant, viral-predominant, eosinophilic predominant and pauci-inflammatory, each associated with specific airway and serum biomarkers. 55% of exacerbations were associated with bacteria, and airway IL-1□ and serum CRP were the most suitable biomarkers for determining the aetiology in this group, whereas 29% were exacerbations associated with viruses (either alone or co-infected with bacteria) and in this group, serum IP-10 was the best markers to determine a viral aetiology. Interestingly, the authors describe a constant biologic profile of exacerbations within the same patient.

The differences in the results between the Bafadhel study and the data presented in this chapter may be explained by several factors. Firstly, the study by Bafadhel and colleagues was considerably larger than the present study, with 182 exacerbations

sampled compared to 45. The increased exacerbation numbers reduces the inherent variability seen within the cohort when measuring biomarkers, which is evident in the large interquartile ranges seen in the data from this study, and furthermore, cluster analysis has limited value to explore relationships in a smaller dataset. In addition, the definition of bacteria-associated exacerbations was different between the two studies. The presence of bacteria at exacerbation, as used in the Bafadhel study, does not necessarily imply causality particularly as bacteria are present at stable state, and therefore changes in load between stable and exacerbation measured by qPCR is a more accurate reflection of bacterial-associated exacerbations.

In chapter 4, a species-specific effect of bacteria on airway inflammation was observed, and this effect was once again observed when analysing the large numbers of airway and systemic biomarkers in this study. Significant changes in airway IL-1□, Flt-1 and ICAM-1 were demonstrated with *H. influenzae*-associated exacerbations, and significant changes in airway IL-12p70 and serum VCAM-1 with *M. catarrhalis*-associated exacerbations. Importantly, these changes were independent of the inflammatory effect of both other bacterial pathogens and HRV, strengthening the finding of a species-specific inflammatory response. As discussed in chapter 4, species-specific effects have been demonstrated in stable disease and to both *H. influenzae* and *M. catarrhalis* (Hill et al., 2000; Marin et al., 2012; N'Guessan et al., 2014), but there have been few studies that have shown similar effects at exacerbation. In the study by Baker and colleagues, changes in only *M. catarrhalis* load between stable and exacerbation states was associated with changes in sputum TNF□ and IL-1□ (Barker et al., 2014), although these result were not independent of the presence of other bacterial and viral pathogens. Furthermore, a recent study has

demonstrated that IL-17A is elevated in exacerbations associated with *H. influenzae*, and IL-17A is functionally required to promote the neutrophilic response in an animal model of bacterial-exacerbations (Roos et al., 2015). The finding of a species-specific effect and biologic clustering based on exacerbation aetiology are important findings, as not only does this have the potential for targeted treatment of exacerbations, but also consideration of individualised treatment for exacerbation prevention (Miravitlles and Anzueto, 2015) with specific antibiotics and potentially in the future with novel cytokine inhibition (Barnes, 2009; Caramori et al., 2014). However, further work is needed both to validate the changes seen in these cytokines in other cohorts and also to fully explore the complex interrelationship of biomarkers and to identity those cytokines consistently related to viral- or bacterial- and specific bacterial- associated exacerbations before novel cytokine inhibition could be investigated.

Therefore, in conclusion, there are numerous airway and systemic biomarkers that change with clinical state, although there is not a clear correlation between the two compartments to enable a systemic biomarker to act as a surrogate for airway inflammation. Inflammatory profiles appear to be different depending on both the exacerbation aetiology and bacterial species, and further work is need to determine whether this species-specific effect may be used to provide a more targeted approach to the treatment of acute exacerbations.

Chapter 7:

The relationship between monocyte-derived macrophage phagocytosis and cytokine release with clinical phenotypes and lower airway bacterial colonisation in stable COPD

7.1 Introduction

Homeostasis of the lung microbiome is maintained through the complex interactions between different components of the innate immune system. This includes mucociliary clearance, secretion of antimicrobial peptides and inflammatory mediators, as well as cell mediated phagocytosis of inhaled particles and bacteria (Sethi and Murphy, 2008). The most prominent and highly adapted phagocyte within the lung is the alveolar macrophage (AM), which accounts for up to 95% of the cells identified in broncho-alveolar lavage (BAL) samples (Gordon and Read, 2002) and initiates the early immune-inflammatory responses following pathogen exposure.

There is evidence of disruption to the innate immune system in COPD, including airway epithelial structural and secretory abnormalities (Polosukhin et al., 2011; Verra et al., 1995), and despite a 10 to 20-fold increase in the number of AMs (Barnes, 2004a), lower airway bacterial colonisation (LABC) is seen in approximately 30-50% of patients (Banerjee et al., 2004; Patel et al., 2002). LABC is an important clinical outcome, associated with worsening underlying airway inflammation (Banerjee et al., 2004; Hill et al., 2000; Patel et al., 2002) and leading to a self-perpetuating cycle of airway infection and inflammation (Sethi and Murphy, 2008). This has been shown to alter the natural history of COPD, including increased exacerbation frequency (Patel et al., 2002) and faster FEV_1 decline (Wilkinson et al., 2003).

Previous studies investigating the phagocytic capacity of AMs from stable COPD patients have demonstrated an attenuated response to different pathogens, including fungal species and both Gram-negative and Gram-positive bacteria, and this supports the hypothesis that an intrinsic defect in macrophages is present. Early studies

focused on the phagocytosis of non-respiratory pathogens, including *Candida* spp. (Ferrara et al., 1996; Vecchiarelli et al., 1991) and *E. Coli* (Prieto et al., 2001; Taylor et al., 2010). Importantly, more recent studies have demonstrated attenuated macrophage phagocytosis in response to those respiratory pathogens commonly isolated from COPD patients, including *H. influenzae* and *S. pneumoniae* (Berenson et al., 2006a; Berenson et al., 2006b; Taylor et al., 2010).

However, obtaining AMs for such studies requires patients to undergo the invasive procedure of a flexible bronchoscopy and BAL. Although this procedure is safe in COPD patients after appropriate assessment (Hattotuwa et al., 2002), it does carry significant residual risk. In addition, BAL return and subsequent viable AM isolation may be poor. Many patients are therefore understandably reluctant to undergo such a procedure.

In order to bypass the need for invasive sampling, monocyte-derived macrophages (MDMs) are increasingly used to model AMs, which derive from circulating monocytes (Gordon and Taylor, 2005), and can be easily sampled by peripheral venepuncture. MDMs that have been differentiated from monocytes using GM-CSF have similar molecular and functional properties to AMs (Winkler et al., 2008), particularly a similar reduction in phagocytic capacity to pathogens (Taylor et al., 2010; Winkler et al., 2008). Although a study in COPD patients by Berenson and colleagues (Berenson et al., 2006a) found that this phagocytic defect was only observed in AMs and not MDMs, suggesting a compartmentalised defect, however their differentiation methodology used in this study did not appear to have been differentiated using GM-CSF.

Previous studies investigating defective macrophage phagocytosis, irrespective of whether AMs or MDMs were used, have suggested that this defect may contribute to LABC, and therefore influence the clinical consequences as detailed above. However, to date, no previous studies have examined airway bacterial presence and load in COPD patients and related this to an underlying defect of phagocytosis. Therefore, using MDMs to model AMs, and qPCR, which has a high diagnostic yield for the three most commonly isolated pathogens in COPD (*H. influenzae, S. pneumoniae* and *M. catarrhalis*) (Garcha et al., 2012), the hypothesis is that impaired phagocytosis would be related to both LABC status and the pathogen-load of the colonising bacteria, and underlying macrophage inflammatory responses to pathogens would contribute to airway inflammation in patients with stable COPD. Furthermore, the hypothesis that impaired phagocytosis would be related to the increased exacerbation susceptibility seen in patients with LABC, but would remain stable over time was investigated.

7.2 Methods

7.2.1 Patient recruitment

Ninety-two COPD patients enrolled in the London COPD cohort between November 2011 and February 2014 were included, as described in section 3.1.

7.2.2 MDM culture

Whole blood was collected from stable patients, as defined by daily symptom diary cards in section 3.3.1. Monocytes were isolated and cultured in the presence of GM-CSF for 12 days to generate MDMs (section 3.10.4 and section 3.10.5). Phagocytosis assays were performed using fluorescently labeled beads ($4.5x10^9$ microspheres/ml) and heat-killed *H. influenzae* or *S. pneumoniae* ($5x10^8$ cfu/ml) incubated with MDMs for 4 hours as described in section 3.11. In a sub-group of patients, repeat MDMs were cultured and phagocytosis measured at a subsequent stable visit.

7.2.3 Procedures

FEV_1 and FVC were measured in accordance with ATS/ERS guidelines using a Vitalograph Gold Standard spirometer (Vitalograph Ltd, Maids Moreton, UK). Patients completed daily diary cards for symptoms and were prospectively reviewed in clinic when stable during the study period. Stable state was defined as those patients without evidence of symptom-defined exacerbations in the preceding 4 weeks and the subsequent 2 weeks post-clinic visit. COPD exacerbation frequency was determined using diary card events or exacerbation recall as outlined in the methods (section 3.5).

Patients were asked to spontaneously expectorate sputum samples at the time of whole blood collection. Patients unable to expectorate sputum, underwent sputum induction as described in section 3.6.2. Only sputum of adequate quality on macroscopic appearance was included in the analysis. Collected sputum was subsequently processed using PBS-glass beads as described in section 3.6.4 and 3.7.3 for qPCR for typical bacteria.

7.2.4 Statistical Analysis

Data were analysed using GraphPad PRISM version 6.0 (GraphPad Software Inc., San Diego, CA, USA). Details of the statistical tests used are reported in 3.13.

7.2.5 Ethical considerations

Ethical approval for the study was granted from the Royal Free Hospital research ethics committee and all patients gave written informed consent.

7.3 Results

Ninety-two stable COPD patients between November 2011 and June 2014 were included in the analyses. Of these, 77/92 (84%) provided a concurrent sputum sample, enabling the relationship between MDM phagocytosis and airway bacteria to be investigated, and in 36/92 (39%) patients repeat MDMs were cultured at a subsequent stable visit for longitudinal analysis (□□□□□□□□□□).

The clinical demographics of the overall patients, and each sub-group, are shown in □□□□□□□□□. There were no clinically or statistically significant differences between the demographic data of each sub-group.

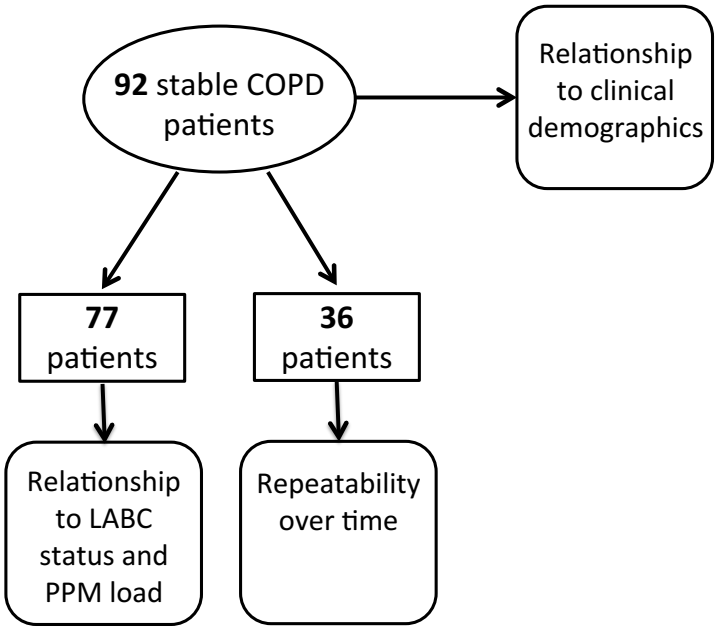


Figure 7.60. COPD patient groups analysed throughout the study. Patients may have been in more than one sub-analysis. LABC: lower airway bacterial colonisation. PPM: potentially pathogenic micro-organisms.

□
□
□
□

	All n=92	LABC analysis n=77	Repeatability analysis n=36	p-value†
Mean (SD) Age (years)	71.9 (8.7)	71.4 (8.7)	69.3 (8.7)	0.460
Mean (SD) FEV_1 (L)	1.34 (0.52)	1.38 (0.52)	1.47 (0.58)	0.641
Mean (SD) FEV_1 (% predicted)	54.6 (17.6)	56.3 (17.4)	58.1 (18.4)	0.775
Mean (SD) FVC (L)	2.86 (0.87)	2.86 (0.87)	2.90 (0.77)	0.982
Mean (SD) FEV_1/FVC (%)	47.6 (13.4)	48.7 (12.4)	50.7 (13.1)	0.675
Median [IQR] Exacerbation Frequency	2.0 [1.0-3.0]	2.0 [1.0-3.0]	2.0 [1.0-3.0]	0.505
Median [IQR] smoking pack years	50.0 [28.4-71.5]	50.0 [29.1-70.9]	45.3 [21.1-62.3]	0.944
Median [IQR] ICS dose (beclomethasone equivalent μg)	1000 [1000-2000]	1000 [1000-2000]	1500 [1000-2000]	0.982
N (%) Male sex	60 (65)	48 (62)	24 (67)	0.954
N (%) Current smokers	32 (35)	29 (38)	17 (47)	0.636
N (%) Chronic bronchitis	72 (78)	62 (81)	31 (86)	0.797

Table 7.44. Clinical demographics of the stable COPD patient subgroups analysed. Parametric data displayed as mean (SD), non-parametric data displayed as median (IQR) and categorical data displayed as N (%). †Groups compared using one-way ANOVA, Kruskal-Wallis or χ^2, as appropriate.

7.3.1. Relationship between MDM phagocytic capacity of *H. influenzae* and *S. pneumoniae*

Increasing phagocytic capacity of *H. influenzae* was significantly associated with increasing phagocytic capacity of *S. pneumoniae*, ($p<0.001$, $r=0.456$, [illegible]). No significant relationship was seen between the phagocytosis of the beads with either *H. influenzae* or *S. pneumoniae* ($p>0.05$ for both).

[illegible]

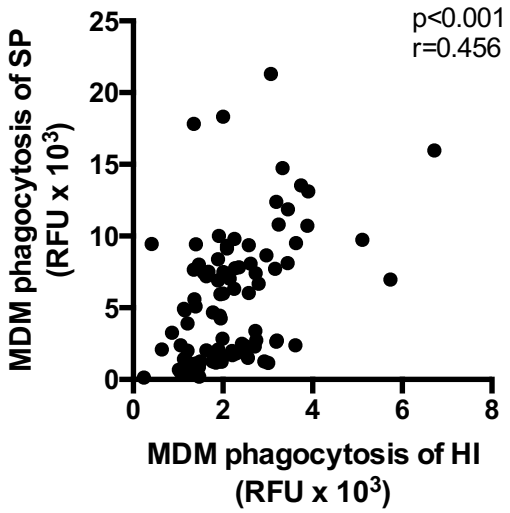


Figure 7.61. Relationship between the MDM phagocytic capacity of *H. influenzae* (HI) and *S. pneumoniae* (SP). Data analysed by Spearman rank correlation (n=92).

[illegible]

7.3.2 Relationship between MDM phagocytosis and clinical demographics

There was no significant relationship between the ability of MDMs to phagocytose any prey; beads or bacteria (*H. influenzae* or *S. pneumoniae*), with age (**Figure 7.62**), severity of lung disease as measured by FEV_1 % predicted (**Figure 7.63**), or FEV_1/FVC ratio (**Figure 7.64**) ($p>0.05$ for all correlations). MDM phagocytosis of bacterial prey only is shown in the subsequent figures.

[illegible] [illegible]

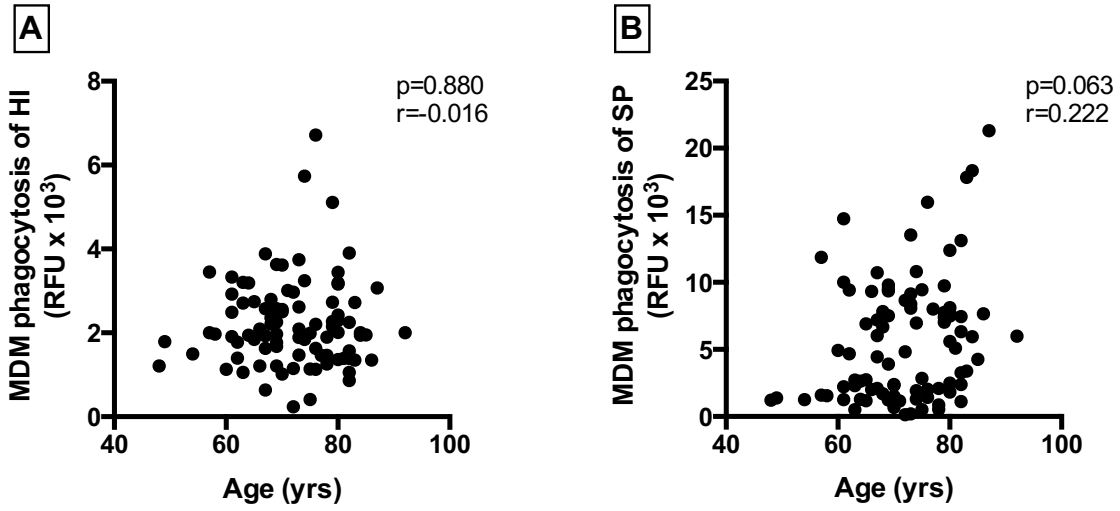


Figure 7.62. Relationship between age and MDM phagocytosis of (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP). Data analysed by Spearman rank correlation (n=92).

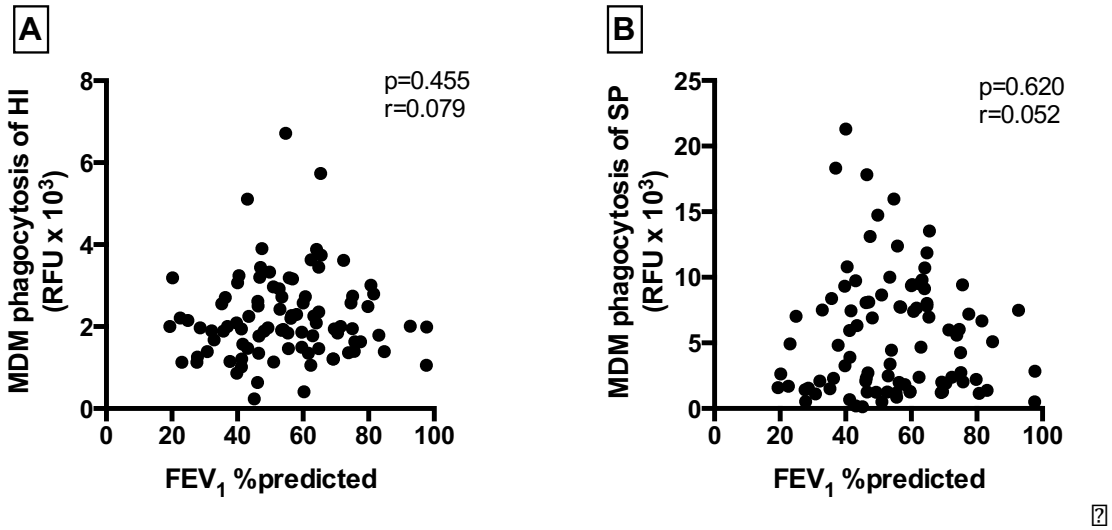


Figure 7.63. Relationship between FEV_1 % predicted and MDM phagocytosis of (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP). Data analysed by Spearman rank correlation (n=92).

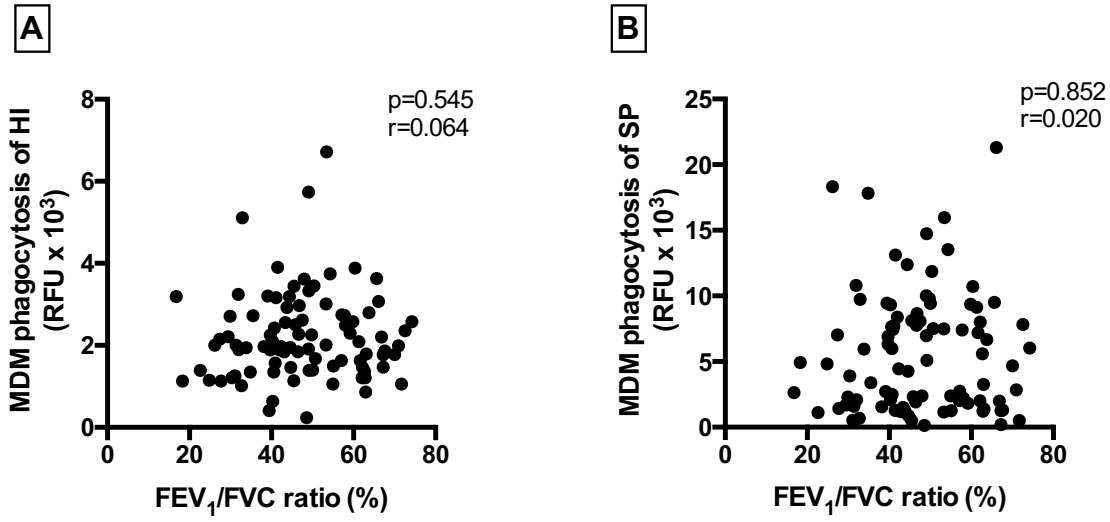


Figure 7.64. Relationship between FEV_1/FVC ratio (%) and MDM phagocytosis of (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP). Data analysed by Spearman rank correlation (n=92).

No significant association was seen between MDM phagocytosis of either beads (p=0.067), or bacteria with smoking history as measured by pack years (*H. influenzae*: p=0.827, [illegible]A; *S. pneumoniae*: p=0.763, [illegible]C), and there was no significant difference between the phagocytosis of beads (p=0.488) or bacteria in ex-smokers compared to current smokers (*H. influenzae*: p=0.595 [illegible]B; *S. pneumoniae*: p=0.139, [illegible]D).

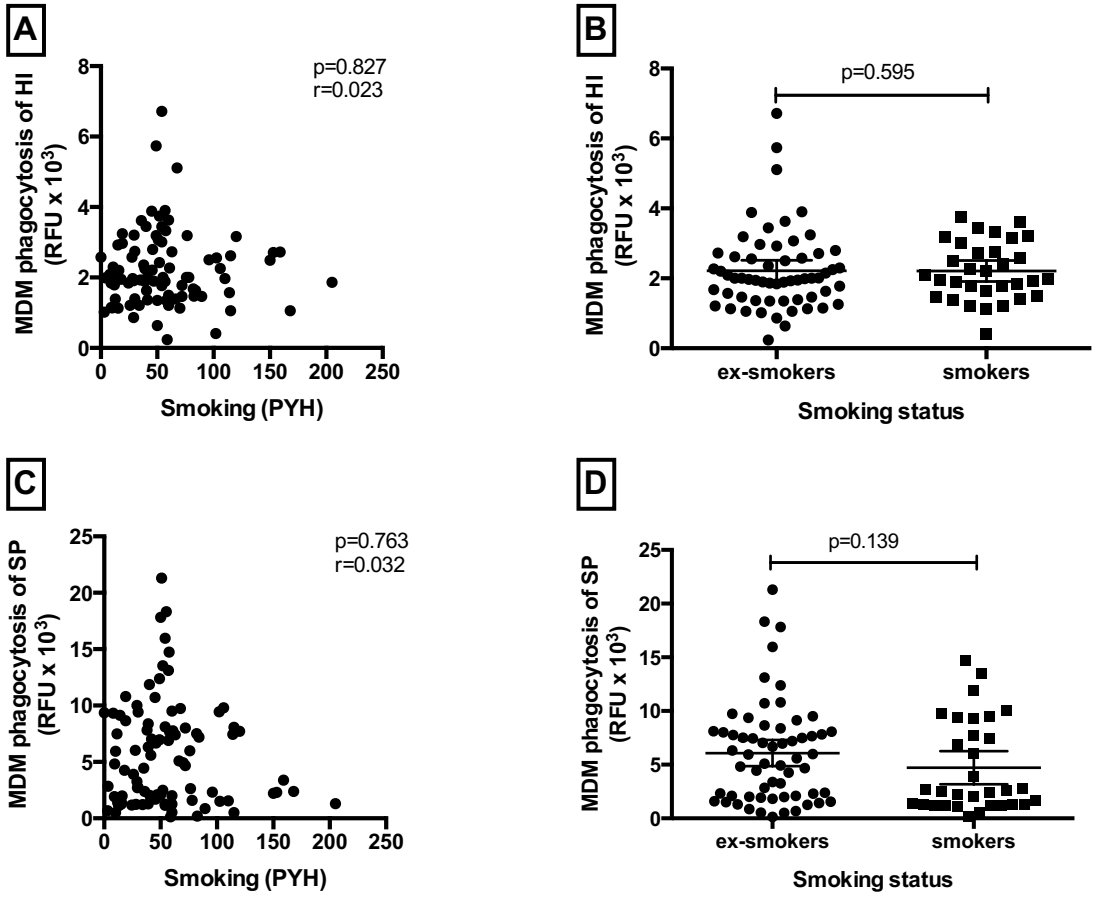


Figure 7.65. Relationship between smoking history and MDM phagocytosis to bacteria. Graphs A and B show the MDM phagocytosis of *H. influenzae* (HI) and graphs C and D show the MDM phagocytosis of *S. pneumoniae* (SP). Pack year history was analysed by Spearman rank correlation (graphs A and C, n=92). Current and ex-smokers were analysed by Mann-Whitney test (graphs B and D), with phagocytosis data presented as median and interquartile range.

7.3.3 The effect of concurrent inhaled medication on MDM phagocytosis

77/92 (84%) patients were using concurrent inhaled corticosteroids (ICS), with a median daily dose of 1000 [1000-2000] beclomethasone equivalent dose (μg). No significant relationship was observed between the MDM phagocytosis of beads or *H. influenzae* and *S. pneumoniae* with ICS dose ($p>0.05$ for all, **Figure 7.66**). In addition, there was no significant difference in the phagocytic capacity of either beads ($p=0.074$) or *H. influenzae* and *S. pneumoniae* between patients using different ICS formulations ($p=0.131$ and $p=0.729$, respectively,).

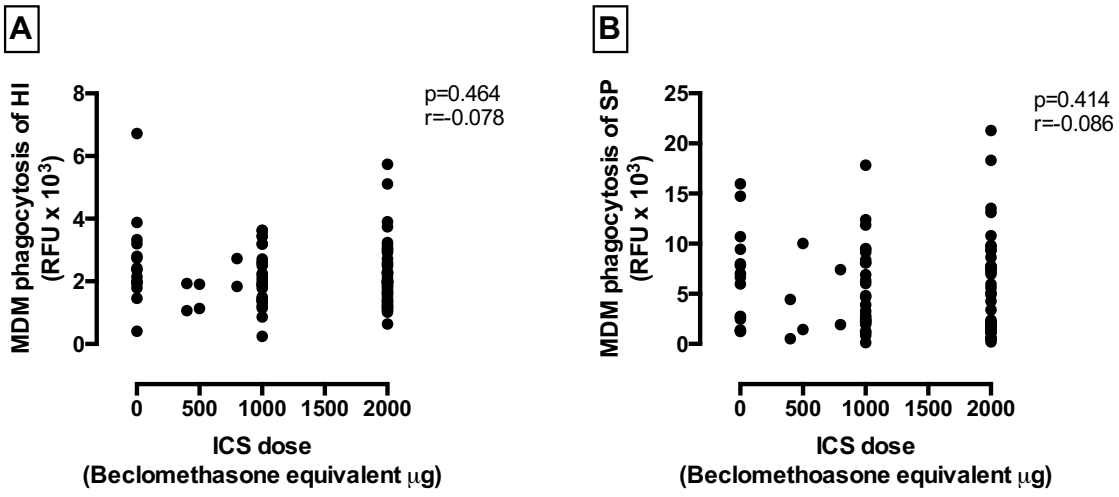


Figure 7.66. Relationship between MDM phagocytosis of (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP) with inhaled corticosteroid (ICS) dose. Data analysed by Spearman rank correlation (n=77).

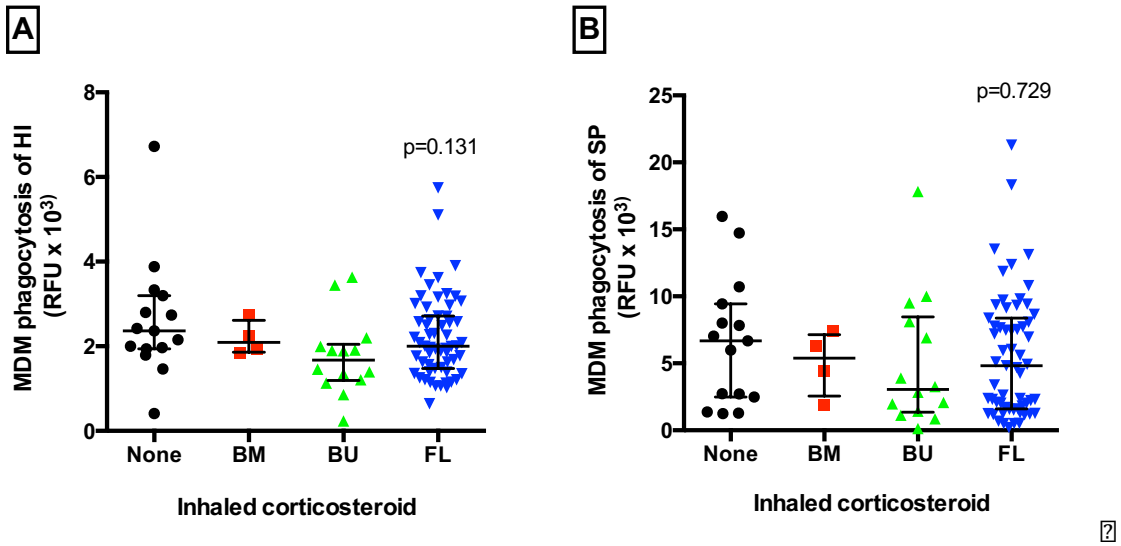


Figure 7.67. The effect of different inhaled corticosteroid formulations on MDM phagocytosis of (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP). Inhaled corticosteroids: None, n=15 (•); BM: beclomethasone, n=4 (■); BU: budesonide, n=14 (▲); FL: fluticasone propionate, n=59 (▼). Phagocytosis data presented as median and interquartile range.

80/92 (87%) of patients were using a long-acting muscarinic antagonist (LAMA), and in all cases, patients were using 18µg tiotropium bromide daily. There was no significant effect of concurrent LAMA use on the ability of MDMs to phagocytose either beads (p=0.412) or bacteria (*H. influenzae*: p=0.287 A; *S. pneumoniae*: p=0.095, B).

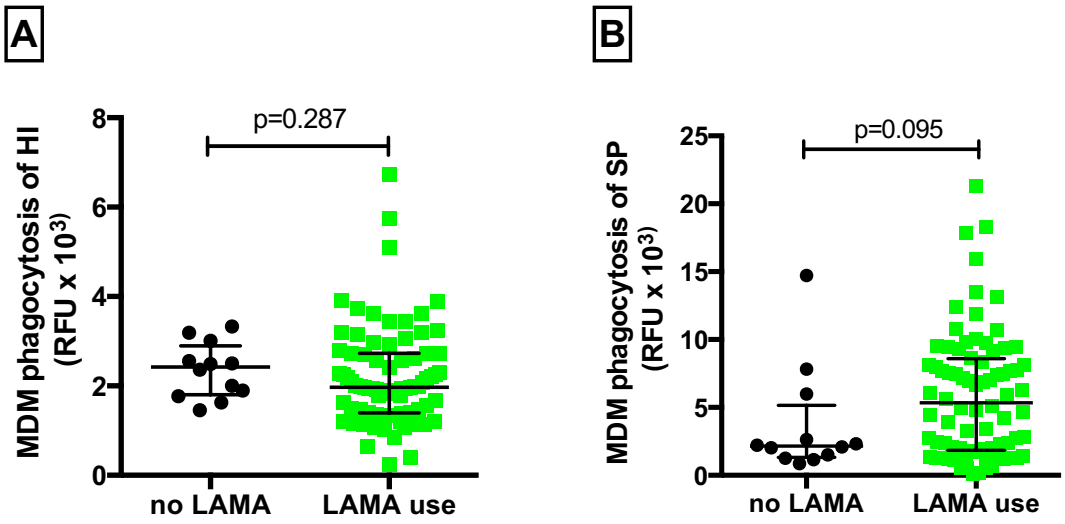


Figure 7.68. The effect of concurrent long-acting muscarinic antagonist (LAMA) use on MDM phagocytosis of (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP). All LAMA use was tiotropium bromide 18μg daily. Patients with LAMA (■, n=80); patients without LAMA use (•, n=12). Phagocytosis data presented as median and interquartile range.

72/92 (78%) of patients were using a long-acting β2 agonist (LABA), of which 15/72 (21%) were using formoterol fumerate 24 μg daily and 57/72 (79%) were using salmeterol 100 μg daily. Overall, there was no significant effect of concurrent LABA use on the ability of MDMs to phagocytose either beads (p=0.084) or bacteria (*H. influenzae*: p=0.219 [illegible]A; *S. pneumoniae*: p=0.796, [illegible]B).

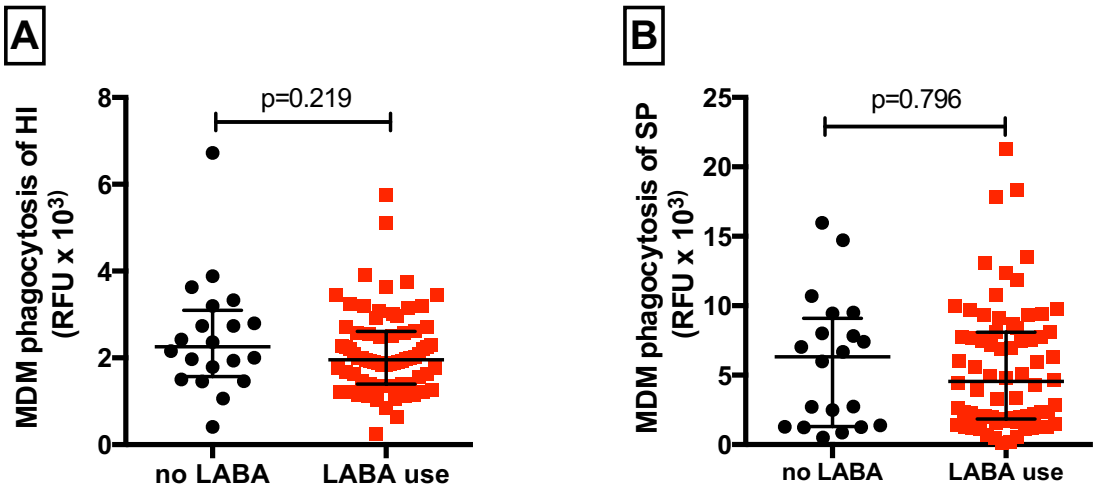


Figure 7.69. The effect of concurrent long-acting β2 agonist (LABA) use on MDM phagocytosis of (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP). Patients using LABA (■, n=72); patients not using LABA (•, n=20). Phagocytosis data presented as median and interquartile range.

When the different LABA formulations were compared, there was a significant difference in the ability of MDMs to phagocytose *H. influenzae* depending on whether a LABA was used, and the LABA formulation (p=0.034, [illegible]A). When each group was compared with each other, the phagocytosis of *H. influenzae* was significantly lower in the formoterol group compared to no LABA use ($p<0.05$), but there was no significant different between patients using either formoterol or salmeterol. There was no significant difference in the ability of MDMs to phagocytose *S. pneumoniae* between patients using a LABA and the two formulations (p=0.788, [illegible]).

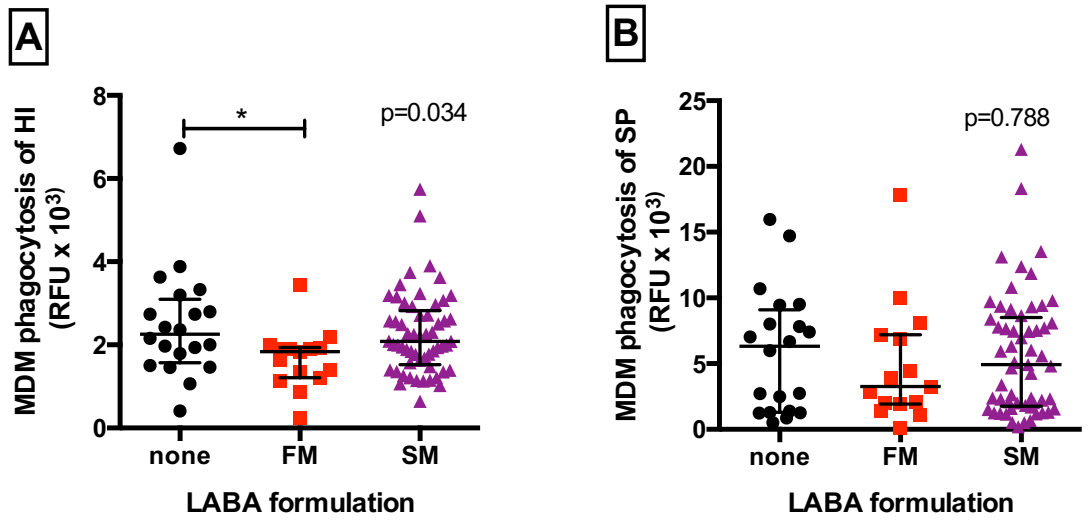


Figure 7.70. The effect of concurrent inhaled long-acting β2 agonist (LABA) formulations on MDM phagocytosis of (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP). LABA formulations: none, n=20 (•), FM: formoterol, n=15 (■), SM: salmeterol, n=57 (▲). Phagocytosis data presented as median and interquartile range. *p<0.05 on multiple comparison.

7.3.4 The effect of exacerbation frequency on MDM phagocytosis

Median exacerbation frequency per year was calculated for each patient based on diary card events collected between recruitment to the London COPD cohort and the recruitment to the current study. For recently recruited patients, with less than one year of diary card data, patient recall was used the determine the number of exacerbations in the previous year. When exacerbation frequency was used as a continuous variable, the phagocytosis of *H. influenzae* was significantly less with increasing exacerbation frequency (p<0.001; r=-0.440, **Figure 7.71**A), but there were no significant associations demonstrated between exacerbation frequency and phagocytosis of beads or *S. pneumoniae* (p>0.05, for all).

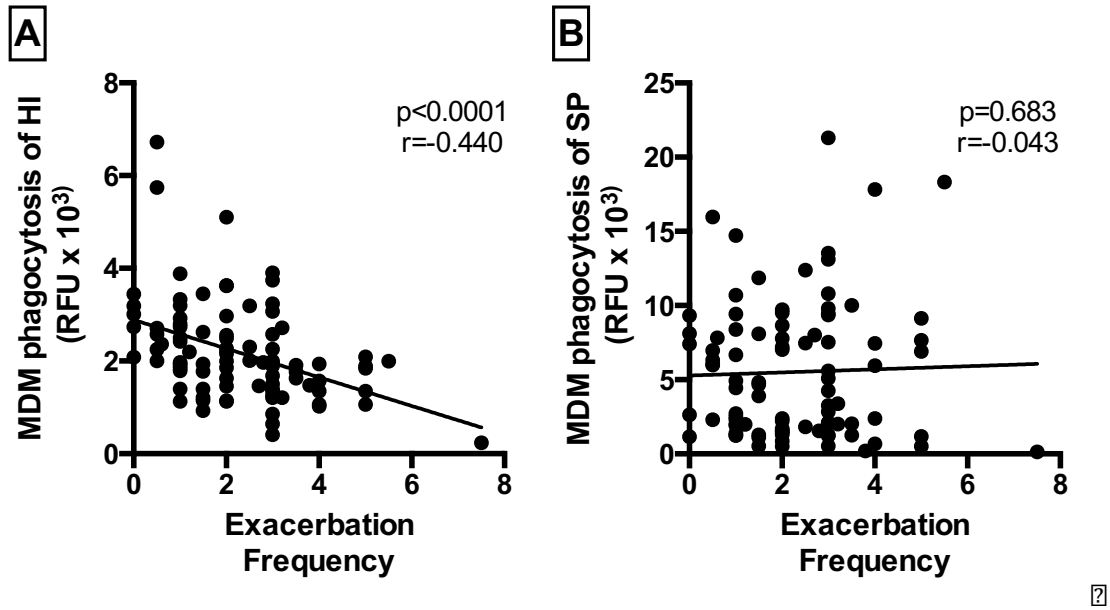


Figure 7.71. Relationship between MDM phagocytosis of (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP) with exacerbation frequency. Exacerbation frequency per year was used as a continuous variable (n=92).

When exacerbation frequency was used as a binary variable, patients were grouped into frequent and infrequent exacerbators, defined as □2 exacerbations/year and <2 exacerbations/year respectively. As above, MDM phagocytosis of *H. influenzae* was significantly lower in the frequent exacerbators patients than the infrequent exacerbators (p=0.002, **Figure 7.72**A). Exacerbation frequency did not affect the phagocytosis of *S. pneumoniae* (p=0.697, **Figure 7.72**B).

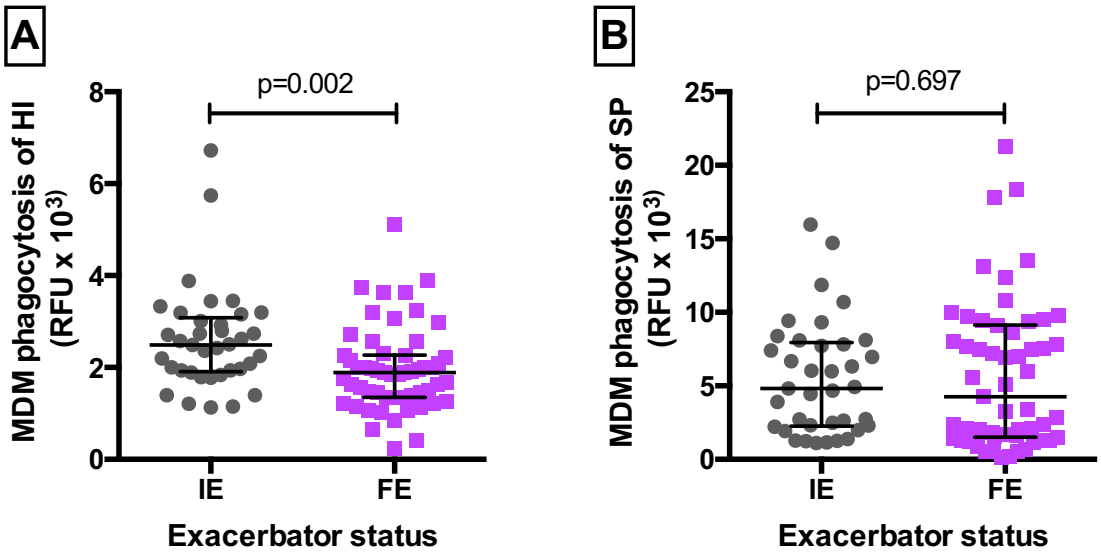


Figure 7.72. Effect of exacerbator status on the ability of MDMs to phagocytose (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP). Exacerbator status was defined either as frequent exacerbator (FE) with □2 exacerbations/year (■, n=55), or infrequent exacerbators (IE) with <2 exacerbations/year (•, n=37). Phagocytosis data presented as median and interquartile range.

□

6.3.5 Relationship between MDM phagocytosis, LABC status and bacterial load

Paired sputum samples, in addition to the culture of MDMs, were available in 77 patients. One or more PPMs were identified by qPCR in 37/77 (48%) patients, and these patients were defined as having evidence of LABC.

Mono-microbial isolation with *S. pneumoniae* was identified in 16/37 (43%) of patients, with mono-microbial isolation of *H. influenzae* identified in 15/37 (41%) of patients. Only 1/37 (3%) patient had evidence of mono-microbial *M. catarrhalis* isolation. 5/37 (14%) patients had evidence of polymicrobial detection; three patients had both *H. influenzae* and *S. pneumoniae* detected, one patient had *H. influenzae* and

M. catarrhalis detected and one patient had all three PPMs identified by qPCR detected (□□□□□□□□).

□

PPM	Single Bacterial isolated (n)	Mixed isolates (n)	Bacterial load Log_{10} (SD) (cfu/ml)
H. influenzae	15	5	6.04 (1.39)
S. pneumoniae	16	4	6.85 (1.09)
M. catarrhalis	1	2	8.21 (1.79)

□

Table 7.45. Number and load of potentially pathogenic micro-organisms (PPMs) isolated from patients with lower airway bacterial colonisation. qPCR was used to detect the three common PPMs; *H. influenzae, S. pneumoniae* and *M. catarrhalis* (n=37). Five patients had evidence of polymicrobial colonisation (>1 PPM).

□
□

There was no significant difference in the MDM phagocytosis of *H. influenzae* between patients who had evidence of LABC with any bacteria, compared to those patients without evidence of LABC (p=0.913, □□□□□□□□A). Although there was a trend towards a reduction in MDM phagocytosis of *S. pneumoniae* in patients with LABC compared to those without, this did not reach statistical significance (p=0.115, □□□□□□□□B).

In order to determine whether the ability of MDMs to phagocytose either *H. influenzae* or *S. pneumoniae* was related to colonisation by the same PPM, patients without evidence of LABC were compared to those with either mono-microbial *H. influenzae* (n=15) or *S. pneumoniae* (n=16) presence.

□

In the 15 patients with mono-microbial *H. influenzae* colonisation, no significant difference was seen between the phagocytic capacity of either *H. influenzae* or *S. pneumoniae* and those patients without any evidence of LABC (p=0.402 and p=0.371, respectively, □□□□□□□□□□B and C).

In the 16 patients with mono-microbial *S. pneumoniae* colonisation, no significant difference was seen between their ability to phagocytose *H. influenzae* and those patients without evidence of LABC (p=0.709, □□□□□□□□□□E).

However, there was a trend towards decreased MDM phagocytosis of *S. pneumoniae* in those patients colonised only with *S. pneumoniae* compared to those patients without evidence of LABC, although this did not reach statistical significance (p=0.208, □□□□□□□□□□F).

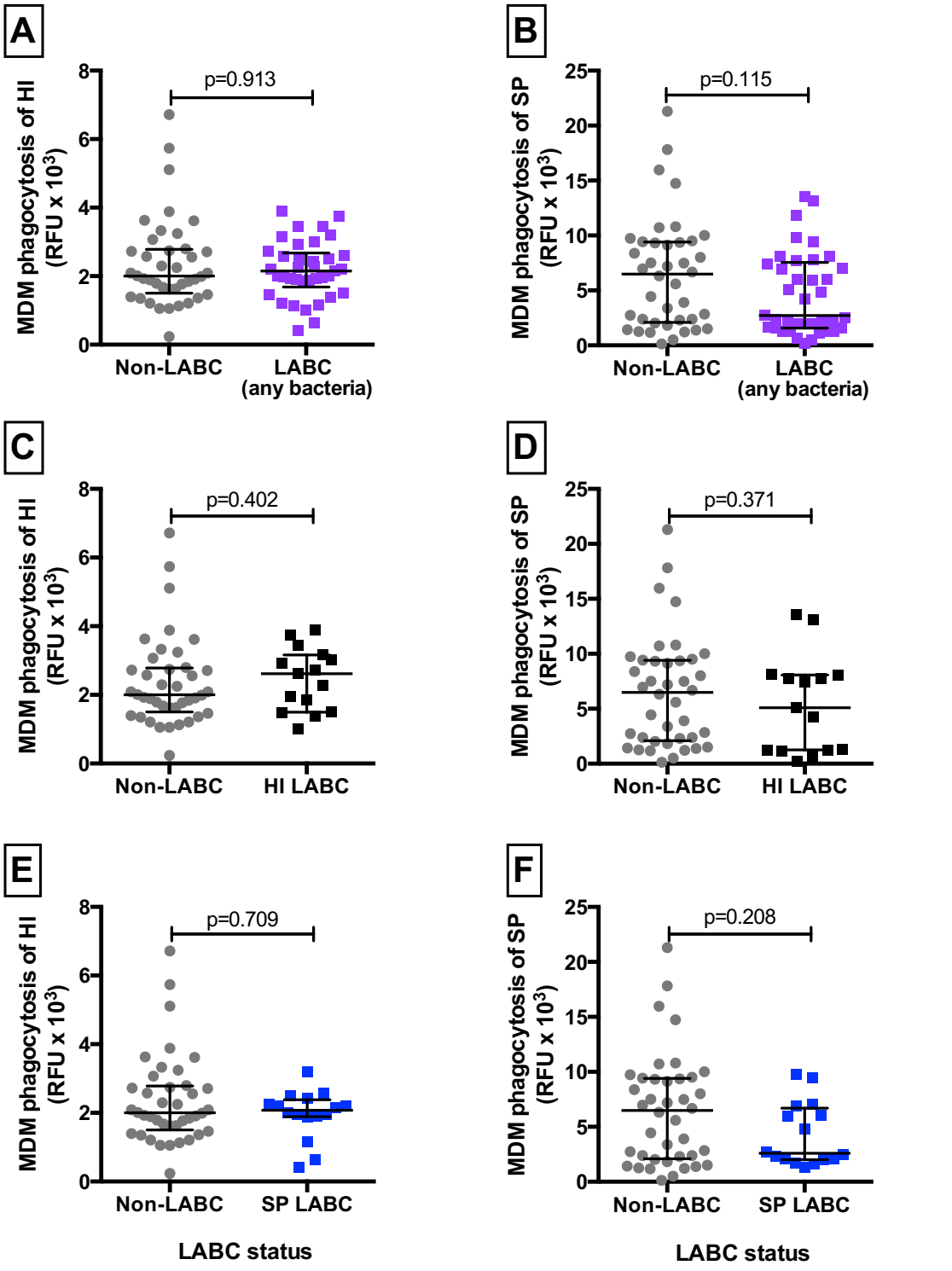


Figure 7.73. Difference between MDM phagocytosis in patients with and without evidence of lower airway bacterial colonisation (LABC). MDM phagocytosis of *H. influenzae* (HI) is shown in graphs A, C and E. MDM phagocytosis of *S. pneumoniae* (SP) is shown in graphs B, D and F. LABC status was defined as non-LABC (•, n=40), LABC with any bacteria (■, n=37), LABC with *H. influenzae* (HI) only (■, n=15), and LABC with *S. pneu*moniae (SP) only (■, n=16). Comparison of MDM

phagocytosis of bacteria in patients without evidence of LABC and those with LABC with any bacteria are shown in graphs A and B. Comparison of MDM phagocytosis between patients without evidence of LABC and those with HI-LABC only are shown in graphs C and D. Comparison of MDM phagocytosis between patients without evidence of LABC and those with SP-LABC only are shown in graphs E and F. Phagocytosis data presented as median and interquartile range.

No significant relationship was seen between the phagocytic capacity of *H. influenzae* or *S. pneumoniae* and total bacterial load (p=0.743, [illegible]A and p=0.955, [illegible]B, n=37). In addition, there was no significant relationship between the bacterial load and phagocytic capacity of the same colonising PPM (*H. influenzae*: p=0.524, [illegible]C, n=15; *S. pneumoniae*: p=0.686, [illegible]D, n=16).

[illegible]

[illegible] [illegible]

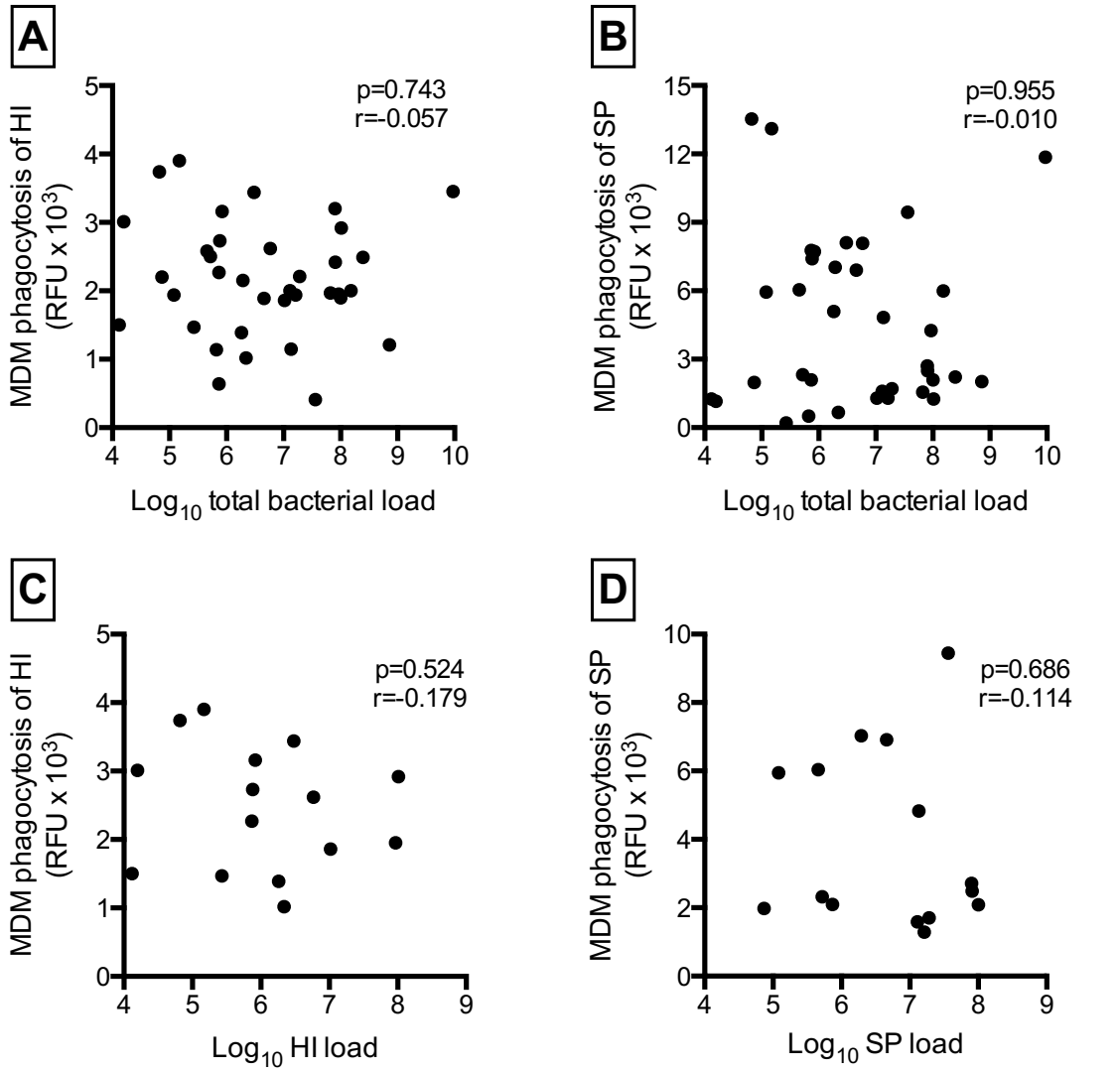


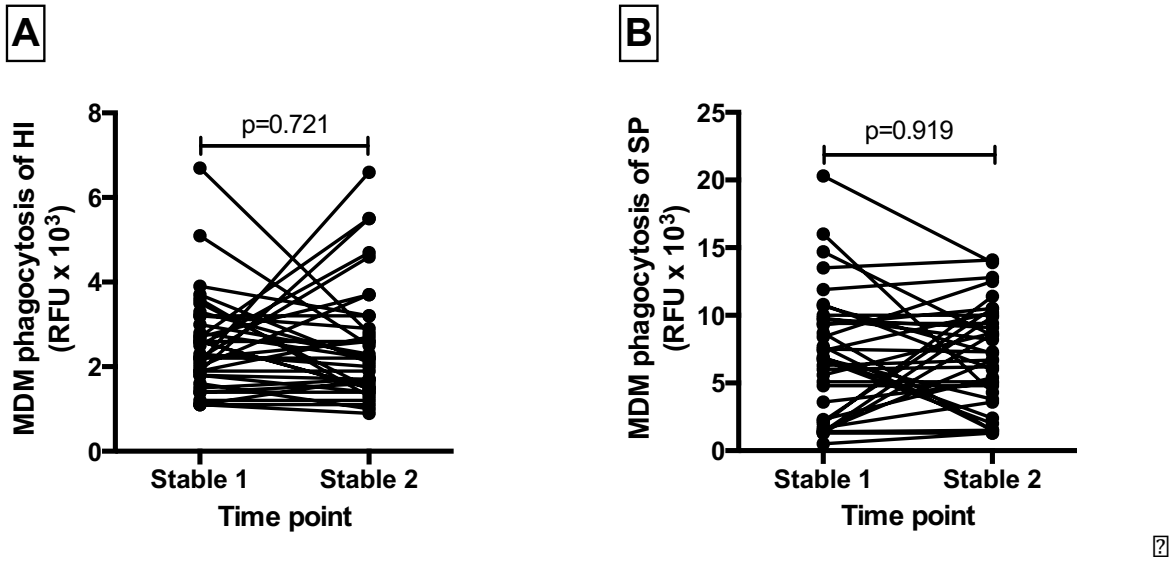
Figure 7.74. Relationship between MDM phagocytosis and airway bacterial load. MDM phagocytosis of *H. influenzae* (HI) is shown in graphs A and C. MDM phagocytosis of *S. pneumoniae* (SP) is shown in graphs B and D. The relationship between MDM phagocytosis and total bacterial load is shown in graphs A and B (n=37). The relationship between MDM phagocytosis and load of the same bacteria is shown in graphs C and D (HI, n=15 and SP, n=16).

7.3.6 Stability of MDM phagocytosis

In 36 patients, repeat stable MDMs were cultured and phagocytosis assays performed after a median time of 9.5 (6.0-14.0) months. There were no significant differences in clinical demographics in this sub-group of patients compared to the overall stable COPD patients (□□□□□□□□□).

In this group of patients, there was no significant change in phagocytic capacity to either beads (p=0.179) or bacteria over time (p>0.05 for both, □□□□□□□□□).

□



□

Figure 7.75. Stability of MDM phagocytosis to (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP) over time. Repeat stable samples were collected over a median time of 9.5 [6.0-14.0] months.

□

As LABC is a dynamic process, changes in phagocytosis in 34/36 (94%) patients with paired sputum samples at both stable time points were investigated. Patients were defined as positive for bacteria if one or more of the three PPMs identified by qPCR were detected in the sputum sample. Patients were described as never colonised, if both samples were negative for bacterial pathogen detection; persistently colonised, if both samples were positive; gaining colonisation, if the first sample was negative and

the second positive; and losing colonisation if the first sample was positive and the second negative. Due to small numbers in these four different groups, changes between the bacteria species could not be analysed statistically.

□□□□□□□□ shows the dynamics of colonisation status in this group of patients and the time between the two stable samples. There was a significant difference between the proportions of patients in the four different groups representing the changes in colonisation status, with a larger proportion of patients being never colonised (p=0.026). There was no significant effect of time between samples and the changing colonisation status (p=0.999).

	Dynamics of colonisation				
	Never	Gain	Lose	Persistent	p-value†
No. (%)	15 (44)	7 (21)	5 (15)	7 (21)	0.026
Median [IQR] Months between samples	9.0 [6.0-14.0]	13.0 [3.0-15.0]	11.0 [6.0-13.5]	7.0 [6.0-19.0]	0.999

Table 7.46. Dynamics of airway colonisation status in paired stable samples. Positive samples were defined as □ 1 PPM detected in sputum by qPCR. Four groups were defined to reflect the dynamics of colonisation; never, if both samples were negative; gain, if the first sample was negative and the second positive; lose, if the first sample was positive and the second sample negative and persistent, if both samples were positive. † Groups were analysed using $\square^2$ and Kruskal-Wallis, as appropriate.

□

There was no significant change in the stable macrophage phagocytosis of either *H. influenzae* or *S. pneumoniae* in patients who were never colonised (p=0.292 and p=0.463, □□□□□□□□□A and B respectively) or in those who gained colonisation status (p=0.750 and p=0.063, □□□□□□□□□C and D respectively).

□ □□□

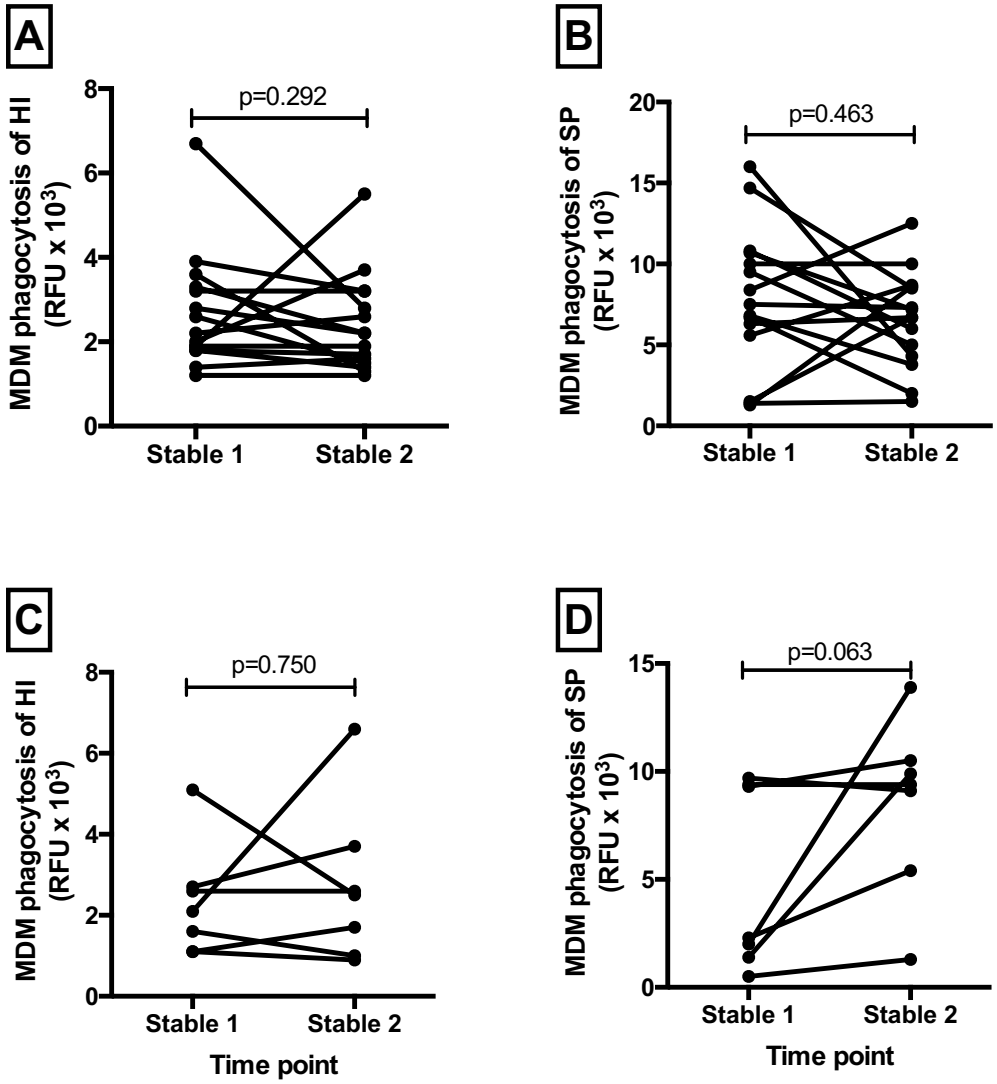


Figure 7.76. The effect of being never colonised or gaining colonisation status on MDM phagocytosis of bacteria. Graphs A and C show the phagocytosis of *H. influenzae* (HI) and graphs B and D show the phagocytosis of *S. pneumoniae* (SP). Patients who were never colonised, defined as no bacteria detected by qPCR in both sputum samples taken at the two time points, are shown in graphs A and B. Patients who gained colonisation, defined as no bacteria detected in the first sputum sample, but □l bacteria detected at the second time point are shown in graphs C and D.

There was no significant change in the stable macrophage phagocytosis of either *H. influenzae* or *S. pneumoniae* in patients who lose colonisation (p=0.625 and p=0.375,

Figure 7.77A and B respectively) or in those who were persistently colonised (p=1.00 and p=0.625, Figure 7.77C and D respectively).

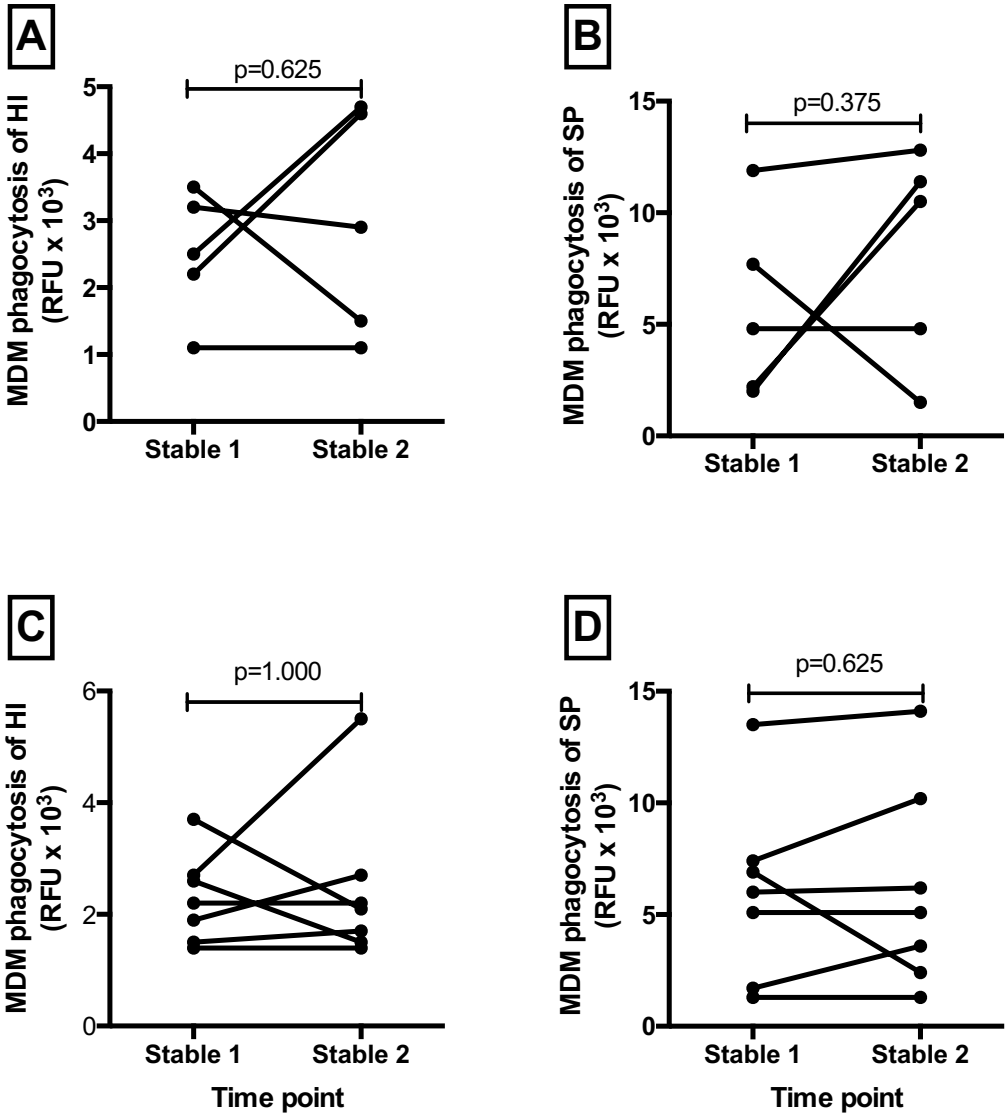


Figure 7.77. The effect of losing colonisation or being persistently colonised on MDM phagocytosis of bacteria. Graphs A and C show the phagocytosis of *H. influenzae* (HI) and graphs B and D show the phagocytosis of *S. pneumoniae* (SP). Patients losing colonisation status were defined as no bacteria detected by qPCR in the second sputum samples after having had bacteria detected in the first sample, and are shown in graphs A and B. Patients who were persistently colonised, defined as □1 bacteria detected in both sputum samples, are shown in graphs C and D.

7.4 Discussion

In this study, MDMs were used to model AMs to investigate phagocytosis from COPD patients during periods of stability. Phagocytosis to any prey, either inert beads or bacteria, was not significantly related to any clinically relevant demographic data including age, severity of lung disease, smoking or the use and type of ICS or LAMA maintenance therapy, although MDMs from patients using formoterol were less able to phagocytosis *H. influenzae* than patients without concurrent LABA use. Importantly, in repeat stable samples, the ability of MDMs to phagocytose either bacteria or beads did not alter over time. However, a significant relationship between decreasing MDM phagocytosis of *H. influenzae* and worsening exacerbation frequency was observed, although no significant relationship was observed with *S. pneumoniae* or inert beads phagocytosis. This suggests that frequent exacerbators exhibit a worsening phagocytic defect in their innate response to Gram-negative bacteria.

Although there was no significant difference in the ability of MDMs to phagocytose bacteria between non-colonised patients and those colonised with any bacteria, or those patients colonised with either *H. influenzae* or *S. pneumoniae* only, there was a trend towards decreased phagocytic ability of *S. pneumoniae* in patients with colonisation by any bacteria and also those only colonised with *S. pneumoniae*. Changes in the presence of airway bacteria in paired stable samples over time could not be explained by associated changes in phagocytosis, implying that while macrophage phagocytosis plays an important role in the homeostasis of the airway microbiome, defective phagocytosis it is not the sole causative factor resulting in the airway pathogen persistence or contributing to the dynamics of LABC.

As demonstrated in this data, there was no significant relationship between macrophage phagocytosis of either *H. influenzae* or *S. pneumoniae* with age, lung function or smoking history in patients with established COPD. This supports the view that defective phagocytosis is a result of an intrinsic mechanism within the macrophage and/or its monocyte pre-cursor, and is not a result of ageing, severity of underlying lung disease or differences in smoking exposure. These results are in keeping with previous work from our group using a different patient dataset (unpublished - CMR Thomas PhD thesis). However, one study carried out by Berenson and colleagues reported a significant relationship between macrophage phagocytosis and disease severity (Berenson et al., 2013). In this study, AM phagocytosis of 3 different bacterial prey, *H. influenzae, M. catarrhalis* and *S. pneumoniae* in healthy, non-COPD patients with ex- and current-COPD smoking patients was compared. Unlike previous studies, they demonstrated that defective phagocytosis was only evident to the Gram-negative bacteria, and more so to *H. influenzae* than *M. catarrhalis.* They also noted that worsening phagocytic capacity to *H. influenzae* and *M. catarrhalis* was associated with worsening COPD disease severity. However, in their analysis, all study participants were included, including the healthy patients with an FEV_1 % predicted of greater than 100, which appears to skew the data set. In addition, no association was reported between worsening phagocytosis and FEV_1 % predicted in only the COPD patients. Furthermore, the pathogen-specificity of their results may be inaccurate, as the methodologies used to assess phagocytosis differed between the pathogens investigated. Thus, although the results from the Berenson study are contradictory to the findings presented herein, their results should be interpreted with caution.

In healthy smokers, smoking status and the degree of exposure has been shown to have an effect on both macrophage recognition of bacteria (Chen et al., 2007; Hodge et al., 2007) and subsequent phagocytosis compared to non-smokers (Berenson et al., 2006a; Hodge et al., 2007; King et al., 1988; Taylor et al., 2010). In the current study, only MDMs from COPD patients were used, and all patients had considerable exposure to tobacco smoke with a median pack years of 50. No difference in responsiveness was observed in MDM between current smokers and ex-smokers, and no significant association was observed between defective phagocytosis and pack year history. This is keeping with previous studies that suggest smoking exposure, possibly via the down-regulation of microRNAs and thus altered gene expression, may have an early but long-lasting effect on macrophage function, promoting the more pro-inflammatory phenotype which contributes to disease progression (Graff et al., 2012; Gross et al., 2014).

Medication commonly used to treat COPD may inadvertently affect macrophage phagocytosis. Early *in vitro* studies have suggested that corticosteroids may further suppress the defective phagocytosis observed in COPD patients (Zetterlund et al., 1998). This could be an important mechanism contributing to LABC and the paradoxical risk of pneumonia despite a reduction of exacerbation frequency observed in patients with COPD taking ICS therapy (Calverley et al., 2007; Calverley et al., 2011; Crim et al., 2009; Drummond et al., 2008; Singh et al., 2009). However, conversely, more recent *in vitro* studies have shown that pre-treatment of MDMs from both COPD patients and healthy smokers with budesonide improved phagocytic responses to both *H. influenzae* and *S. pneumoniae* (Taylor et al., 2010), although a similar effect with fluticasone propionate was not observed. This may possibly help to

explain the differential risk of pneumonia with different formulations of ICS. In this dataset, all patients were investigated when stable as defined using daily symptom diary cards, and would not have been treated with systemic treatment including oral steroids and antibiotics for a minimum of 4 weeks before sampling. Therefore, any possible systemic effects of corticosteroids would result from prescribed inhaled therapy, which would have considerably lower systemic concentrations than those used in the *in vitro* studies. However, no relationship between MDM phagocytosis was observed between ICS dose, as measured by beclomethasone equivalent dose, or between the different ICS formulations. There was a trend towards concurrent budesonide use causing a greater impairment in phagocytosis than the other formulations, although this was not statistically significant. In keeping with these findings, a study in severe asthmatics similarly demonstrated that dexamethasone use did not significantly change the MDM phagocytic response to bacteria, although the majority of patients in this study were using concurrent oral corticosteroids (Liang et al., 2014).

LAMA and LABA are key maintenance therapies in COPD. While they are used primarily for their bronchodilatory effects, both medications have been shown to reduce the underlying chronic inflammation characteristic of COPD (Antoniu, 2010; Buhling et al., 2007; Karakiulakis and Roth, 2012; Santus et al., 2012). In addition, formoterol but not salmeterol has been shown to reduce inflammatory cytokine release from MDM from healthy patients (Donnelly et al., 2010). However, the effects of bronchodilators on macrophage phagocytic function have been less well studied. In this study, over three-quarters of patients were using either one or both types of bronchodilators. No significant effect of either concurrent LAMA or LABA use was

observed in the MDM phagocytosis of either beads or bacteria, although when the different LABA formulations were compared, in patients using formoterol, there was a significant difference in the ability of MDMs to phagocytose *H. influenzae* but not *S. pneumoniae*. Formoterol is a full β_2-receptor agonist in comparison to salmeterol, which is a partial agonist, and therefore formoterol-induced activation leads to increased cyclic AMP (cAMP) generation, which has been shown to suppress phagocytosis (Cox and Karnovsky, 1973). In addition, an early *in vitro* study demonstrated significant impairments in both alveolar macrophage superoxide anion release, used as a marker of phagocytosis, and bacterial killing following exposure to formoterol (Capelli et al., 1993). However, more recently, formoterol have not been shown to have any significant effect on the *ex vivo* MDM phagocytic response in either severe asthmatics (Liang et al., 2014) or in patients with COPD (Taylor et al., 2010). Thus, whether LABAs, and particularly formoterol, have any deleterious effects on macrophage function, as suggested from these data, warrants further studies.

The role of long-term antibiotics, particularly the macrolide azithromycin, which has been shown to modulate the innate immune system, decreasing chronic inflammation in addition to its anti-bacterial effects (Martinez et al., 2008), has been of increasing interest in the management of stable COPD (Albert et al., 2011; Han et al., 2014). Short-term use of azithromycin for 12-weeks has been shown to improve macrophage phagocytic function in COPD, albeit in a small number of patients (Hodge et al., 2008; Hodge and Reynolds, 2012). Although this effect has not been studied in longer-term studies, in view of this potential effect, patients using long-term

antibiotics, including azithromycin, were excluded from this dataset to avoid any influence on the data presented herein.

Importantly, this study demonstrated a significant relationship between decreasing phagocytosis of *H. influenzae* and increasing exacerbation frequency. *H. influenzae* is the most common bacterial pathogen isolated in both stable and exacerbated COPD (Beasley et al., 2012; Hill et al., 2000; Marin et al., 2012; Rosell et al., 2005; Sethi and Murphy, 2008). Unlike *S. pneumoniae*, a key pathogenic mechanism of *H. influenzae* is its ability to persist in the lower airways, adhering to the epithelium or residing between the epithelial and subepithelial tissues (King, 2012; Read et al., 1991), which together with impaired phagocytosis, contributes to its persistence in the airways. LABC has been shown to increase airway inflammation and exacerbation frequency (Patel et al., 2002), and there is evidence of a species-specific inflammatory response, with greater airway inflammatory response to *H. influenzae* than other commonly isolated pathogens, including *S. pneumoniae* (Hill et al., 2000; Marin et al., 2012) (Chapter 4). Therefore, if defective macrophage phagocytosis potentially increases the risk of *H. influenzae* colonisation, the subsequent higher chronic airway inflammation may contribute to the increase in exacerbation susceptibility observed.

Some patients, irrespective of disease severity, are particularly susceptible to exacerbations, and these patients have been termed ‘frequent exacerbators’ (Hurst et al., 2010; Wedzicha et al., 2013a). In the large Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints (ECLIPSE) exacerbation study, this phenotype was shown to remain stable over time in approximately 70% of patients (Hurst et al., 2010), and investigators used an arbitrary value of two exacerbations per

year to define frequent and infrequent exacerbators. However, this cut-off does not reflect different exacerbation frequencies within each group; for example, a patient with an exacerbation frequency of 5 may have different disease characteristics than a patient with an exacerbation frequency of 2, despite both being defined as a 'frequent exacerbator'. Therefore in the present analysis, exacerbation frequency was examined both as continuous variable, to represent the variation in exacerbation frequency, in addition to the binary variable of frequent and infrequent exacerbators. Irrespective of the means of describing the exacerbation frequency variable, MDM phagocytosis of *H. influenzae* only was significantly lower in patients with a greater exacerbation frequency. As acute exacerbations of COPD are major determinants of the mortality and morbidity associated with COPD, they are a key clinical outcome in pharmacological studies evaluating new therapies. Thus, potentially correcting the defective macrophage phagocytosis, which appears to be associated with increased exacerbation frequency in patients with COPD, may result in their reduction and improved clinical outcomes.

Defective macrophage phagocytosis is thought to one of the mechanisms contributing to LABC commonly observed in patients with COPD (Banerjee et al., 2004; Patel et al., 2002). However, as no previous studies have related the presence of airway pathogens in stable COPD to the underlying defect in macrophage phagocytosis, paired stable sputum samples were analysed using qPCR, to detect the three common pathogens isolated in COPD (Patel et al., 2002; Rosell et al., 2005; Sethi and Murphy, 2008). qPCR was used over culture diagnostic methodology, in view of its high diagnostic yield (Garcha et al., 2012) (Chapter 4), which is especially important in stable COPD. However, there were no significant differences in MDM phagocytosis

of either beads or bacteria between patients with and without evidence of LABC, including colonisation with any bacteria, or colonisation with either *H. influenzae* or *S. pneumoniae* alone. There was a trend towards decreased MDM phagocytosis to *S. pneumoniae* in patients with both colonisation by any bacteria and also those colonised with only *S. pneumoniae*, but this did not reach statistical significance. In addition, in colonised patients there was no significant relationship observed between MDM phagocytic capacity to the different bacterial prey and the load of the same pathogens. Furthermore, as the presence of airway bacteria in stable COPD appears to be a dynamic process, with patients changing their colonisation status, species and strain of bacteria, the change in MDM phagocytosis over time was addressed using repeated stable visit sampling. There was no significant change in the ability of MDMs to phagocytose bacteria over time. In these paired samples with concurrent microbiological data, the majority of samples remained never colonised between the two samples. However, in the samples that changed colonisation status between the two stable visits, there was no change in the phagocytic capacity, suggesting stability in the mechanisms causing defective macrophage phagocytosis.

LABC is likely to be a cumulative consequence resulting from the impairment of different mechanisms and pathways of the innate immune response seen in patients with COPD. Although macrophage phagocytosis is the most important cellular component to clear particulate matter, including bacteria, it is not the only mechanism involved. As previously mentioned, *H. influenzae* is able to adhere to the respiratory mucosa and also reside between the epithelial tissues (King, 2012; Read et al., 1991), and could potentially evade macrophage recognition and subsequent phagocytosis, contributing to its colonisation of the airways but not be directly related to impaired

phagocytosis. In addition, following phagocytosis, to fully clear airway bacteria, any internalised bacteria must be killed. Studies have shown that failure of intracellular killing of bacteria increases the risk of bacterial persistence within macrophages (Cano et al., 2015; Jubrail et al., 2015), and hence when macrophages undergo cell lysis, any viable bacteria may be re-released into the airway. Further studies are needed to fully investigate the different macrophage functions and how these together contribute to the failure of airway microbiome homeostasis in COPD.

In this dataset, LABC, defined by the presence of one or more PPM on qPCR, was evident in 48% of patients. Defective macrophage phagocytosis appears to be non-pathogen specific, (Berenson et al., 2006a; Berenson et al., 2006b; Ferrara et al., 1996; Prieto et al., 2001; Taylor et al., 2010; Vecchiarelli et al., 1991), and in this data, the phagocytosis of both Gram-negative *H. influenzae* and Gram-positive *S. pneumoniae* were positively correlated. It is possible that the attenuated phagocytosis of a specific pathogen would be more likely to be associated with the presence and greater load of the same pathogen. However, in this data, there were no significant differences in phagocytosis of the same pathogen that was present in isolation in the sputum or between the pathogen load and phagocytosis of the same bacteria.

Just over 40% of the colonised patients had evidence of mono-microbial colonisation and hence comparing this sub-group of mono-microbial colonised patients against the non-colonised patients significantly reduces the size of the colonised group. As a result, the sample size may be underpowered to detect any differences. There was evidence of a trend in a reduction of MDM phagocytosis to *S. pneumoniae* in patients colonised with *S. pneumoniae* alone, and also those patients colonised with any

pathogen, compared to non-colonised patients. Therefore, increasing the sample size of colonised patients, may allow sufficient data comparison to demonstrate a significant difference across phagocytosis of both Gram-negative and Gram-positive bacteria compared to patients without colonisation and relate the MDM phagocytic capacity of the pathogens to their load in sputum. Furthermore, when dynamic changes in airway bacteria were considered, due to the small number of patients, analyses were only performed using a binary definition of whether a bacteria was present, as the potential number of variations between the presence or absence of bacteria, the species change and the load, were too great to enable a meaningful comparison in paired samples.

In conclusion, defective macrophage phagocytosis appears to be non-pathogen specific and likely a result of an intrinsic defect within the macrophage. This defect appears to confer susceptibility to exacerbations, but cannot be fully explained by the relationship to airway pathogen presence, species or load due to the limited sample size. Therefore, further work, is needed to explore and explain the potential link between defective phagocytosis and LABC in patients with COPD, as this may provide a novel target for novel pharmacological therapies to help improve the considerable morbidity and mortality associated with the disease.

As the majority of exacerbations are associated with respiratory infections, the change in macrophage function at the time of acute exacerbation will be investigated to determine whether further functional impairments may contribute to the heterogeneity in exacerbation characteristics.

Chapter 8:

The effect of acute exacerbations of COPD on monocyte-derived macrophage phagocytosis and cytokine release

8.1 Introduction

Respiratory infections with bacterial pathogens, respiratory virus or co-infection with both have been shown to play a role in the onset of COPD exacerbations, whereby an infectious pathogen may be isolated at exacerbation in up to 78% of exacerbations (Papi et al., 2006). Exacerbations are complex and heterogeneous events in the natural history of COPD, with considerable inter- and intra-patient variability in the individual exacerbation events' severity, duration and aetiological triggers. The relative contributions of respiratory pathogens, together with environmental factors, to the characteristics of exacerbation episodes have not been well established (Wedzicha and Seemungal, 2007). However, there are some patients that are more susceptible to exacerbations than others, irrespective of disease severity, and these patients are termed 'frequent exacerbators' (Hurst et al., 2010).

There has been increasing interest in the effect of bacterial and viral co-infection at exacerbation. Considerable interplay between bacterial and viral pathogens at exacerbation has been demonstrated in both experimental and naturally occurring exacerbations (George et al., 2014; Mallia et al., 2012). Therefore, viruses may modulate the innate immune system and specifically macrophage function, further reducing the already impaired phagocytic defect enabling secondary bacterial infection to occur. Indeed, exposure of tissue macrophages to human rhinovirus (HRV) impairs bacterial phagocytosis (Oliver et al., 2008), and bacterial load at exacerbation significantly increases in the presence of HRV (Wilkinson et al., 2006a).

In the previous Chapter, a significant relationship between decreasing MDM phagocytosis of *H. influenzae* and increasing exacerbation frequency was seen.

Although this data did not show a significant relationship between impaired macrophage phagocytosis and bacterial presence or load during stable disease, it is likely that this macrophage defect is one of several mechanisms contributing to susceptibility of bacterial infection in COPD. Therefore, it may be postulated that if impaired macrophage phagocytosis is related to exacerbation frequency, it may modulate the characteristics of the exacerbation itself, including the associated pathogens at exacerbation onset, the severity and duration.

In addition, chronic inflammation is a hallmark of COPD, and this inflammatory process is heightened at times of acute exacerbation (Bhowmik, 2000; Hurst et al., 2006a; Hurst et al., 2006b; Papi et al., 2006; Wedzicha et al., 2000b). Activated macrophages play a pivotal role in this process by further promoting the vicious cycle of inflammation (Barnes, 2004a). Macrophages may be activated by several different stimuli including cigarette smoke and bacterial proteins, and the chemokine CXCL8 and the inflammatory cytokine TNF□, are among the best-studied measures of activation. However, alveolar macrophage inflammatory responses to *H. influenzae* and *M. catarrhalis* have been shown to be blunted (Berenson et al., 2014; Berenson et al., 2005b), and this may be a factor contributing to airway bacterial persistence. Furthermore, this impaired activation and subsequent decrease in the inflammatory response may be associated with an increased risk of exacerbation (Berenson et al., 2014).

Therefore, in this Chapter, the hypothesis that MDM function may further worsen at times of acute exacerbation, and this would relate to airway bacterial infection and influence the characteristics of the exacerbation was examined. In addition, the

hypothesis is that viral associated exacerbations would cause a further impairment in phagocytosis and provide a plausible biological mechanism for secondary bacterial infections seen following HRV infection. Furthermore, the stable state activation of MDMs was investigated and the hypothesis that cytokine induction between stable and exacerbation states would differ, and this would reflect the stimulating respiratory pathogen was investigated.

8.2 Methods

8.2.1 Patient recruitment

Forty-seven exacerbations were sampled from 47 COPD patients enrolled in the London COPD cohort between March 2012 and June 2014, as described in section 3.1. Exacerbations were defined according to daily symptom diary card criteria (section 3.3.2) and only exacerbations reported to the study team were sampled prior to the addition of any systemic therapy. In 39 patients, paired stable-exacerbation data was available, with the stable sample taken a minimum of two weeks prior to the exacerbation onset (section 3.3.1). In 20 patients, paired exacerbation follow-up data at two weeks post exacerbation treatment were available (section 3.4.4).

8.2.2 MDM culture and phagocytosis assay

Monocytes were isolated from whole blood collected from COPD patients, at either exacerbation or stable states, and were cultured in the presence of GM-CSF for 12 days to generate MDMs (section 3.10.4 and section 3.10.5). Phagocytosis assays were performed using fluorescently labelled beads ($4.5x10^9$ microspheres/ml) and heat-killed *H. influenzae* or *S. pneumoniae* ($5x10^8$ cfu/ml) incubated with MDMs for 4 hours as described in section 3.11.

8.2.3 Exacerbation characteristics

Exacerbation duration and severity, as defined by symptom count at exacerbation onset, was determined using daily symptom diary cards (section 3.3.2). Exacerbation severity at presentation was also captured using the CAT questionnaire (section 3.2.2). The presence of a viral prodrome before exacerbation onset was determined

using cold-like symptoms or sore throat documented on the daily symptom diary card for □7 days prior to exacerbation onset.

8.2.4 Aetiology of the exacerbations

Sputum collected at exacerbation presentation was homogenised using PBS-glass beads, as described in section 3.6.4, and where sufficient volume was obtained, a sample was analysed by qualitative bacterial culture (section 3.7.3). Homogenised sputum was batch analysed by qPCR for the detection and measurement of load for both bacteria (*H. influenzae*, *S. pneumoniae* and *M. catarrhalis* only) and human rhinovirus (section 3.8). Changes in MDM phagocytosis between paired stable and exacerbation samples were analysed by the pathogen isolated at exacerbation presentation and also by change in bacterial load between stable and exacerbation presentation.

8.2.6 Cytokine release from MDM

In a subset of the paired stable and exacerbation MDM samples, MDM cell supernatants were collected after 4 hours incubation with either beads or bacteria, and subsequently analysed by ELISA to measure CXCL8 and TNF□, as described in section 3.12.

8.2.6 Statistical Analysis

Data were analysed using GraphPad PRISM version 6.0 (GraphPad Software Inc., San Diego, CA, USA). Details of the statistical tests used are reported in section 3.13.

8.2.6 Ethical considerations

Ethical approval for the study was granted from the Royal Free Hospital research ethics committee and all patients gave written informed consent.

8.3 Results

Forty-seven COPD patients were included in the analyses. The stable clinical demographics of these patients and the 39 with paired stable visits are shown in [illegible]. There were no clinically or statistically significant differences between the two patient groups.

	All patients n=47	Paired patients n=39	p-value†
Mean (SD) Age (years)	71.4 (8.0)	71.5 (8.0)	0.952
Mean (SD) FEV_1 (L)	1.30 (0.59)	1.27 (0.55)	0.810
Mean (SD) FEV_1 (% predicted)	52.7 (19.0)	52.7 (18.8)	0.989
Mean (SD) FVC (L)	2.79 (0.97)	2.69 (0.93)	0.624
Mean (SD) FEV_1/FVC (%)	47.7 (15.8)	48.2 (15.4)	0.875
Median [IQR] Exacerbation Frequency	2.5 [1.5-3.5]	2.5 [1.5-3.5]	0.915
Median [IQR] smoking pack years	46.0 [29.0-70.0]	45.6 [24.5-76.5]	0.954
Median [IQR] ICS dose (beclomethasone equivalent μg)	1000 [500-2000]	2000 [1000-2000]	0.598
N (%) Male sex	29 (62)	23 (59)	0.828
N (%) Current smokers	13 (28)	11 (28)	1.000
N (%) Chronic bronchitis	36 (77)	35 (90)	0.155

Table 8.47 Stable clinical demographics of COPD patients with monocyte-derived macrophages sampled at exacerbation. Parametric data displayed as mean (SD), non-parametric data displayed as median [IQR] and categorical data displayed as N (%). †Groups compared using unpaired t-test, Mann-Whitney U test or χ^2, as appropriate. ICS: inhaled corticosteroids.

8.3.1 The effect of MDM phagocytic capacity on exacerbation characteristics

The 47 patients included were sampled at exacerbation presentation, prior to starting additional systemic therapy with antibiotics and/or oral glucocorticoids. The median time between exacerbation onset and presentation was 3 [2-5] days.

Exacerbation duration was available in 46 patients (98%). One patient recorded symptoms continuously for >99 days and was excluded from this analysis. There was no significant association between the duration of the exacerbation and the ability of MDMs to phagocytose either beads (p=0.342) or bacteria (*H. influenzae*: rho=0.107; p=0.205, [illegible]A; *S. pneumoniae*: rho=0.115; p=0.206, [illegible]B).

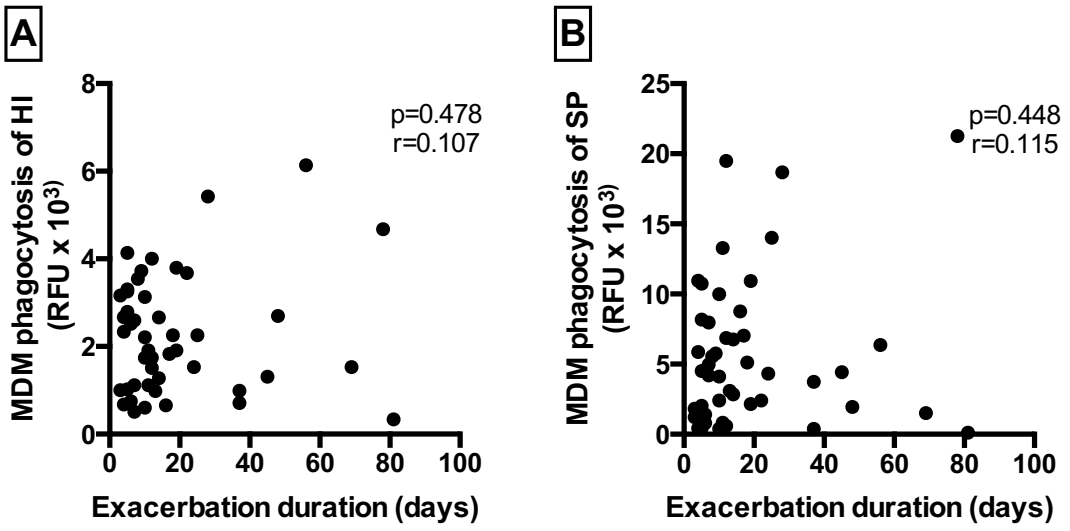


Figure 8.78. Correlation between exacerbation duration and MDM phagocytosis of (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP). Monocytes from COPD patients at exacerbation presentation were cultured in complete media containing GM-CSF (2ng/ml) for 12 days to generate MDM. Phagocytosis assays were performed and the phagocytosis analysed by fluorimetry. Exacerbation duration was determined from daily symptom card data (n=46).

[illegible]

[illegible] [illegible]

CAT scores at exacerbation presentation were available in 42 (89%) patients. There was no significant association between the MDM phagocytosis of either beads or bacteria and exacerbation severity, as measured by both the exacerbation symptom count and the CAT score (p>0.05 for all, [illegible]).

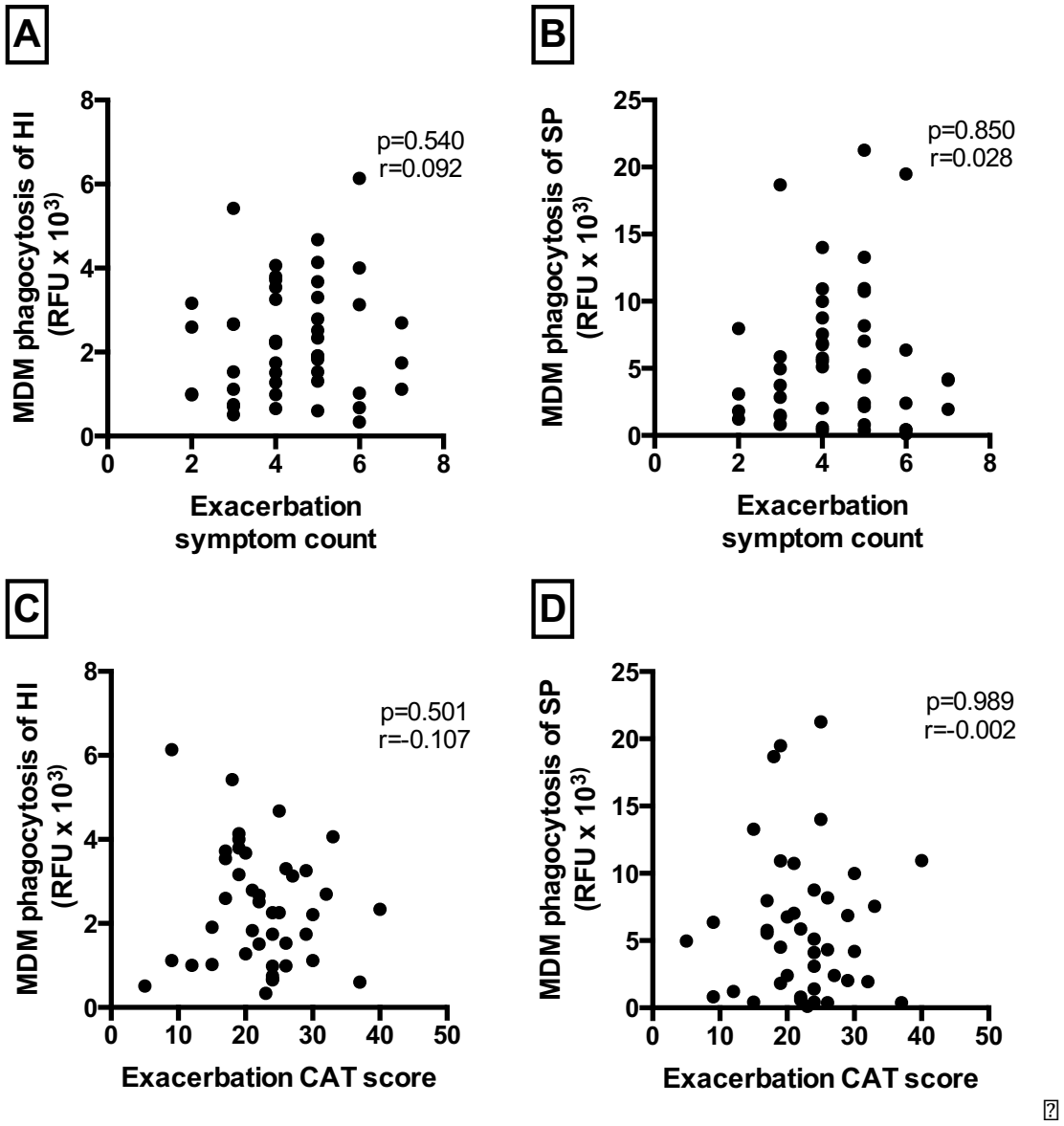


Figure 8.79. Relationship between exacerbation MDM phagocytosis and exacerbation severity, as measured by exacerbation symptom count (A and B) and CAT score (C and D). Exacerbation symptom count was determined from daily symptom diary cards (n=47). CAT scores were available in 42 patients. Phagocytosis is shown for bacteria only: *H. influenzae* (HI) (figures A and C), and *S. pneumoniae* (SP) (figures B and D).

Purulent sputum was present at exacerbation presentation in 34/47 (79%) patients and the presence of cold symptoms and/or sore throat was evident in 25/47 (53%) patients. There was no significant difference between the MDM phagocytosis of beads or bacteria in patients with or without the presence of these symptoms ($p>0.05$ for all, [illegible]).

Prey	MDM phagocytosis (RFU x 10^3)		p-value
	Purulent sputum at exacerbation (n=34)	No purulent sputum at exacerbation (n=13)	
Beads	19.9 [13.5-24.9]	18.4 [15.3-23.0]	0.740
H. influenzae	2.27 [1.24-3.37]	1.83 [0.88-2.88]	0.436
S. pneumoniae	4.37 [2.13-8.32]	4.97 [1.31-7.75]	0.812
	Cold symptoms or sore throat at exacerbation (n=25)	No cold symptoms or sore throat at exacerbation (n=22)	p-value
Beads	17.9 [11.9-21.9]	21.5 [16.0-27.5]	0.061
H. influenzae	1.91 [1.01-3.21]	2.30 [1.42-3.58]	0.530
S. pneumoniae	4.12 [1.88-7.15]	5.82 [1.49-9.26]	0.213

Table 8.48. The effect of exacerbation symptoms on MDM phagocytosis at exacerbation. Presence of purulent sputum and cold symptoms or presence of a sore throat were taken from daily symptom diary cards at exacerbation presentation. Data is presented as median [IQR], n=47.

7.3.2 MDM phagocytosis and pathogen presence at exacerbation

Paired sputum samples were obtained in 39/47 (83%) patients at exacerbation presentation. One or more bacterial pathogens were detected in 29/39 (74%) of patients.

Mono-microbial isolation with *H. influenzae* was identified in 12/29 (41%) of patients, with mono-microbial isolation of *S. pneumoniae* identified in 6/29 (21%) of patients. 4/29 (14%) patients had evidence of mono-microbial *M. catarrhalis* isolation. 7/29 (24%) patients had evidence of mixed detection; all patients with mixed bacterial pathogen detection had *H. influenzae* isolated together with *M. catarrhalis* in 4 patients and *S. pneumoniae* in 3 patients (□□□□□□□□□).

PPM	Single Bacterial isolated (n)	Mixed isolates (n)	Mean bacterial load (SD) (Log_{10} cfu/ml)
H. influenzae	12	7	5.78 (1.77)
S. pneumoniae	6	3	8.08 (1.55)
M. catarrhalis	4	4	5.77 (1.38)

Table 8.49. Number and load of potentially pathogenic microorganisms (PPMs) isolated by qPCR from sputum obtained at exacerbation presentation (n=39). Numbers are given for both mono-microbial and mixed-microbial (>1 bacterial pathogen) detection.

Similarly to stable state phagocytosis as discussed in chapter 7, there was no significant difference in the exacerbation state phagocytosis of either beads or bacteria between patients with and without any bacterial pathogens detected at exacerbation (beads: p=0.811; *H. influenzae*: p=0.985, □□□□□□□□□□A; *S. pneumoniae*: 0.845, □□□□□□□□□□B). In addition, there was no significant difference in the phagocytosis of either beads or bacteria in patients without any bacterial pathogens detected and those with either *H. influenzae* isolation alone (□□□□□□□□□□C and D) or those with *S. pneumoniae* isolation alone (□□□□□□□□□□E and F).

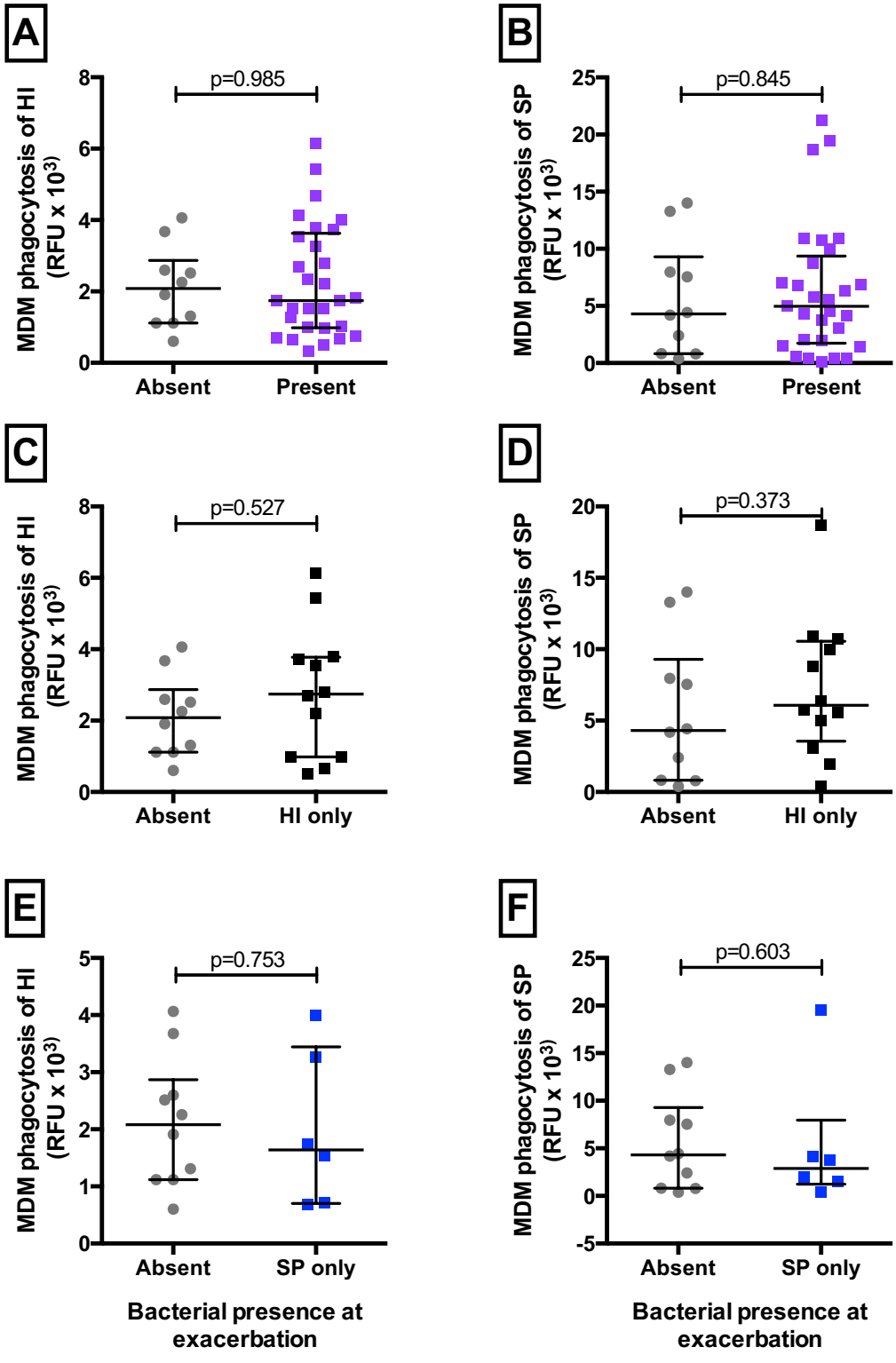


Figure 8.80. MDM phagocytosis of *H. influenzae* (HI) and *S. pneumoniae* (SP) in patients with and without potentially pathogenic microorganisms (PPMs) detected at exacerbation. PPMs presence was defined as absent (•, n=10), any bacteria present (A and B, ■, n=29), HI only (C and D, ■, n=12) and SP only (E and F, ■, n=6). Comparison of MDM phagocytosis of bacteria at exacerbation in patients

with and without evidence of airway bacteria are shown in graphs A and B. Comparison of MDM phagocytosis at exacerbation between patients without evidence of airway bacteria and those with HI-only are shown in graphs C and D. Comparison of MDM phagocytosis at exacerbation between patients without evidence of airway bacteria and those with SP-only are shown in graphs E and F. Phagocytosis data presented as median and interquartile range.

When HRV presence at exacerbation was included, co-infection was detected in 8/29 (28%) of samples with bacterial pathogens isolated. Only 1/10 (10%) of samples had HRV isolated alone, without any bacterial pathogens isolated. To determine whether co-infection altered the MDM phagocytic capacity at exacerbation, the co-infected patients (n=8) were compared with the patients with absent pathogens at exacerbation (neither HRV or PPMs detected, n=9) and those with only bacterial pathogens detected (n=21). Due to the small number, the single patient with HRV alone was not included in this analysis. Co-infection did not alter the exacerbation MDM phagocytic capacity to either beads or bacteria, with no significant difference observed between the three groups (beads: p=0.878; *H. influenzae*: p=0.419, [illegible]A; *S. pneumoniae*: 0.866, [illegible]B)

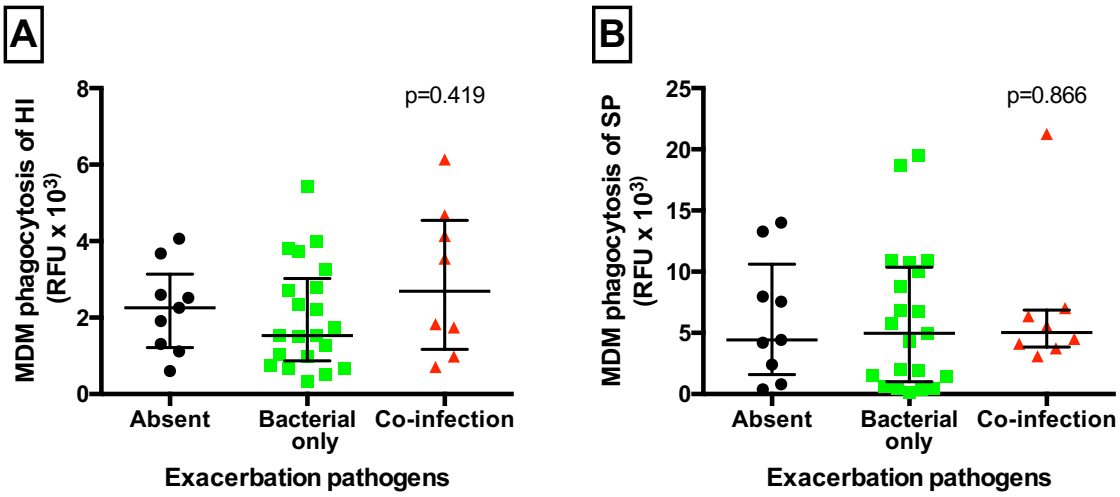


Figure 8.81. MDM phagocytosis of (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP) by the pathogens isolated at exacerbation. Bacterial and human rhinovirus (HRV) pathogens were absent in 9 patients (•); bacterial pathogens alone were isolated in 21 patients (■) and co-infection with bacteria and HRV was detected in 8 patients (▲). One patient had HRV detected alone, but was not included in this comparison. Phagocytosis data presented as median and interquartile range.

As with stable state phagocytosis, no significant relationship was seen between the total airway bacterial load at exacerbation and the MDM phagocytic capacity of *H. influenzae* (n=29, rho=-0.118; p=0.541, A) or *S. pneumoniae* (n=29, rho=-0.236; p=0.218, B). In addition, there was no significant relationship between the bacterial load and phagocytic capacity of the same bacteria at exacerbation (*H. influenzae*: n=12, rho=-0.118; p=0.541, C; *S. pneumoniae*: n=6, rho=0.714; p=0.375, D).

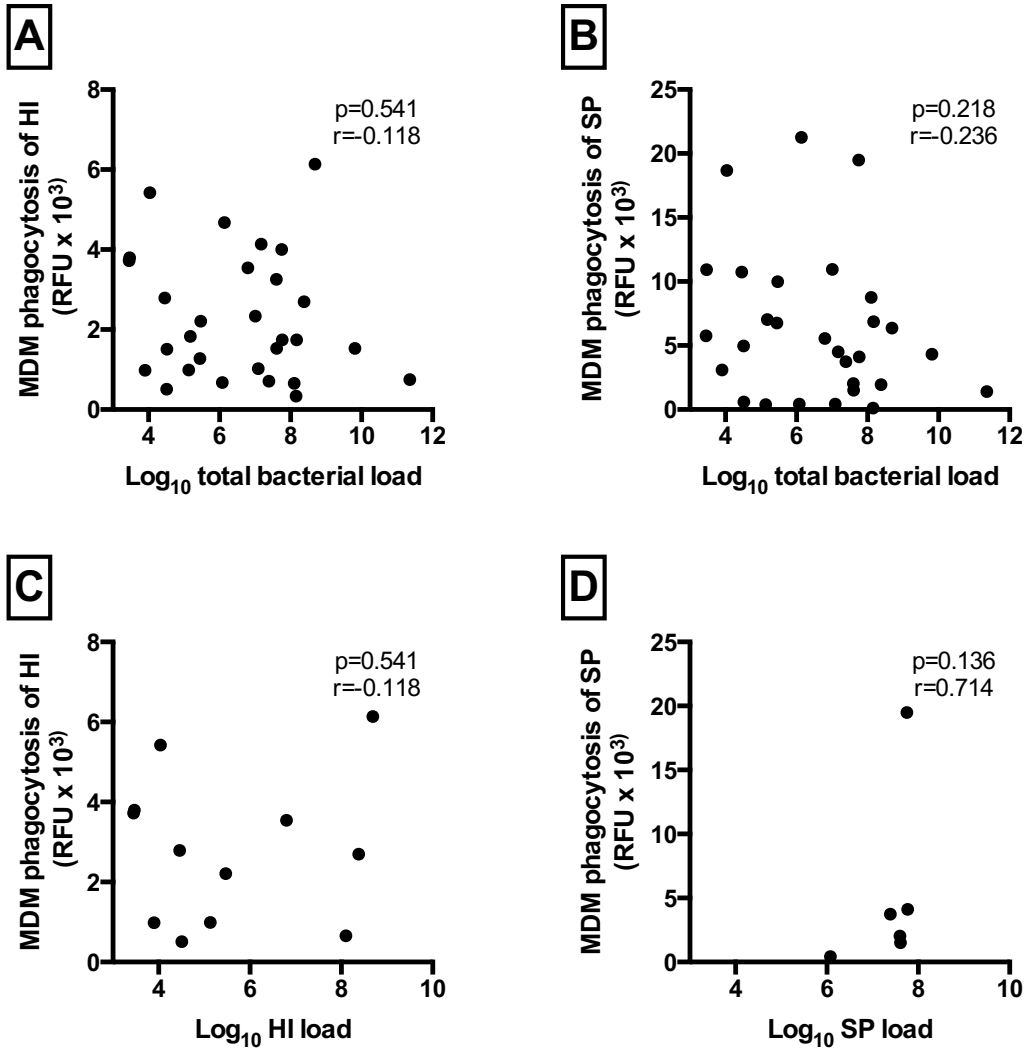


Figure 8.82. Relationship between MDM phagocytosis and bacterial load at exacerbation. Correlation are shown between the phagocytosis of *H. influenzae* (HI) with (A) total bacterial load (n=29) and (C) HI load in patients with mono-microbial isolation (n=12), and between the phagocytosis of *S. pneumoniae* (SP) with (B) total bacterial load (n=29) and (D) SP load in patients with mono-microbial isolation (n=6).

8.3.3 Change in MDM phagocytosis between stable and exacerbation states – unpaired analysis

To determine whether MDM phagocytic capacity changed between stable and exacerbation states, the 47 exacerbation samples were compared with the 92 stable samples described in chapter 7. There was no significant difference between the ability of stable and exacerbation MDMs to phagocytose beads or bacteria (beads: p=0.282; *H. influenzae*: p=0.886; *S. pneumoniae*: 0.727,).

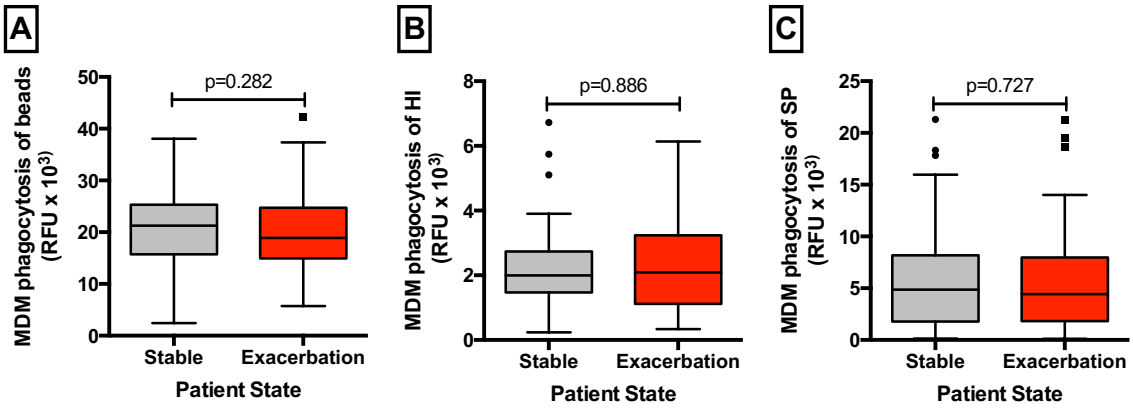


Figure 8.83. Change in MDM phagocytosis of (A) beads, (B) *H. influenzae* (HI), and (C) *S. pneumoniae* (SP) between stable and exacerbation states. Unpaired analysis comparing stable cohort described in chapter 7 (n=92) with exacerbation cohort (n=47). Box plots represent the median and interquartile range and the whiskers represent ±(1.5 x interquartile range), with the outliers plotted separately. (■) stable state and (■) exacerbation state.

8.3.4 Change in MDM phagocytosis between stable and exacerbation states – paired analysis

To further investigate MDM phagocytosis at stable and exacerbation states and whether changes in phagocytosis contribute to the aetiology of the exacerbations, paired analysis was undertaken in 39 patients. The median time between stable and

exacerbation samples was 5 [3-7] months. As with the unpaired analysis, overall there was no significant difference between the ability of stable and exacerbation MDMs to phagocytose beads or bacteria (beads: p=0.189; *H. influenzae*: p=0.735; *S. pneumoniae*: 0.809,).

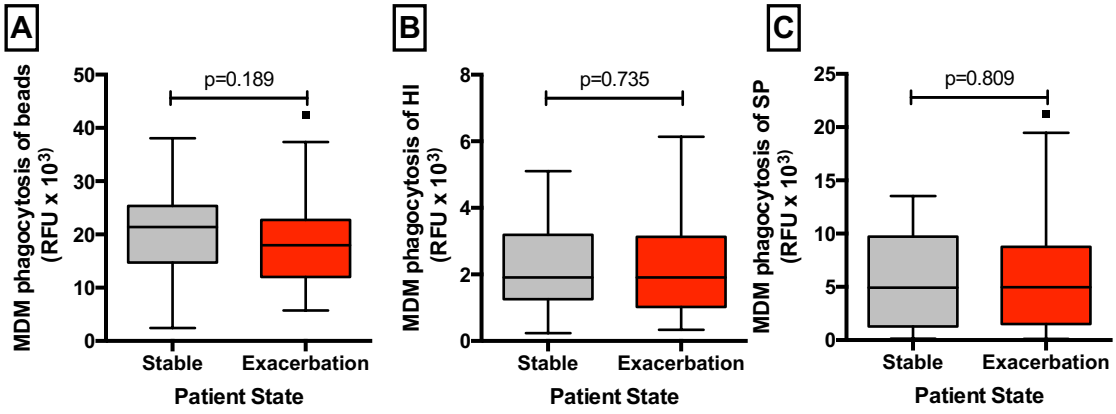


Figure 8.84. Change in MDM phagocytosis of (A) beads, (B) *H. influenzae* (HI), and (C) *S. pneumoniae* (SP) in COPD patients with paired stable and exacerbation samples (n=39). Box plots represent the median and interquartile range and the whiskers represent ±(1.5 x interquartile range), with the outliers plotted separately. (■) stable state and (■) exacerbation state.

The changes in paired stable and exacerbation phagocytosis were then examined to take into account the different pathogens isolated at exacerbation, to determine whether changes in phagocytosis could contribute to the aetiology of exacerbation. Concurrent exacerbation sputum samples were available in 30/39 (77%) patients and were examined by both qualitative culture and qPCR for bacterial pathogens and by qPCR for HRV. 12/30 (40%) exacerbation sputum samples were positive for bacterial pathogens by qualitative culture compared to 23/30 (77%) by qPCR (p=0.008), and 8/30 (27%) exacerbation samples were positive for HRV, of which 7/8 (89%) were

co-infected with a bacterial pathogen. The number of mono- and mixed-microbial and HRV samples, and their loads are shown in [illegible].

PPM	Single pathogen isolated (n)	Mixed isolates (n)	Mean pathogen load (SD) PPMs: (Log_{10} cfu/ml) HRV: (Log_{10} pfu/ml)
H. influenzae	10	6	5.78 (1.72)
S. pneumoniae	4	3	8.19 (0.53)
M. catarrhalis	3	3	6.17 (1.39)
Human rhinovirus	1	7	4.30 (1.86)

Table 8.50. Number and load of potentially pathogenic microorganisms (PPMs) and human rhinovirus (HRV) isolated using qPCR from patients at exacerbation with paired stable samples (n=30).

In the 12 patients with bacterial pathogens detected at exacerbation using qualitative culture, there was no significant difference between the MDM phagocytosis at stable and exacerbation states to beads (23.4 [15.0-26.7] vs. 20.4 [12.4-24.1] RFU x 10^3, p=0.681), *H. influenzae* (2.31 [1.18-3.19] vs. 1.78 [1.07-2.59] RFU x 10^3, p=0.519) or *S. pneumoniae* (4.87 [2.69-10.46] vs. 6.31 [4.36-8.33] RFU x 10^3, p=0.678). Similarly, there was no significant difference between MDM phagocytosis of either beads or bacteria at stable and exacerbation states in the 18 patients without any pathogens detected by qualitative culture (p>0.05 for all).

Using qPCR, 23 patients had bacterial pathogens detected at exacerbation. In these 23 patients, there was no significant difference between the phagocytosis of either beads

or bacteria between paired stable and exacerbation states (p>0.05 for all, [illegible]).

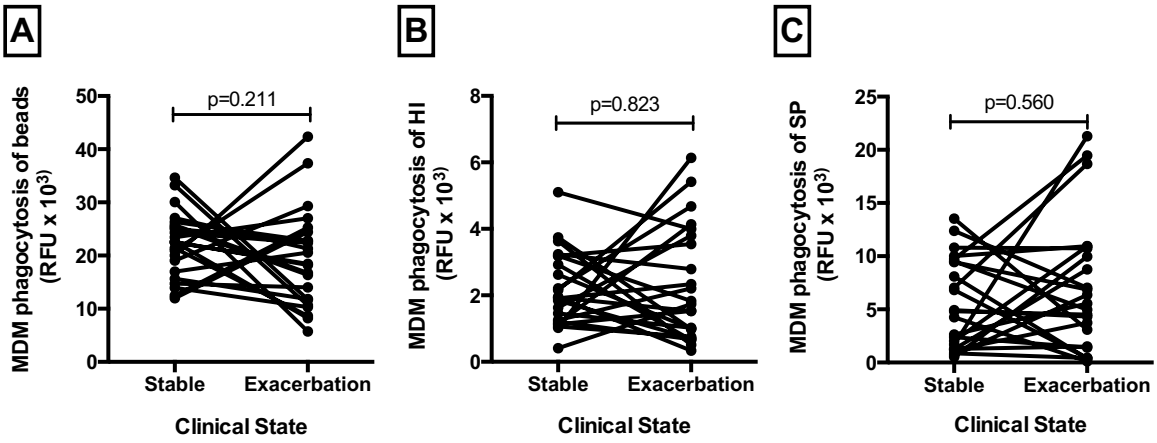


Figure 8.85. Change in MDM phagocytosis of (A) beads, (B) *H. influenzae* (HI) and (C) *S. pneumoniae* (SP) between paired stable and exacerbation samples in patients with any bacterial pathogen detected by qPCR at exacerbation (n=23).

Similarly, in 7 patients without a bacterial pathogen detected by qPCR at exacerbation, there was no significant difference between the MDM phagocytosis at stable and exacerbation states to beads (18.9 [6.40-25.1] vs. 18.4 [14.9-22.4] RFU x 10^3, p=0.513), *H. influenzae* (1.68 [0.87-2.36] vs. 1.91 [1.12-2.52] RFU x 10^3, p=0.469) or *S. pneumoniae* (3.27 [0.20-7.83] vs. 4.43 [0.80-13.29] RFU x 10^3, p=0.469).

To determine whether the presence of airway *H. influenzae* or *S. pneumoniae* at exacerbation presentation was associated with a change in the ability of the MDM to phagocytose the same pathogen between stable and exacerbation states, MDM phagocytosis was compared in all patients with detection of *H. influenzae* or *S. pneumoniae*. Due to small numbers, patients with either mono-microbial or poly-microbial detection were included in the analysis.

In patients with *H. influenzae* detection at exacerbation, no significant difference was seen in the phagocytosis of *H. influenzae* between stable and exacerbation states (n=16, p=0.940, [illegible]A). Similarly, in patients with *S. pneumoniae* detection at exacerbation, there was no significant difference in the phagocytosis of *S. pneumoniae* between stable and exacerbation states (n=7, p=0.813, [illegible]B).

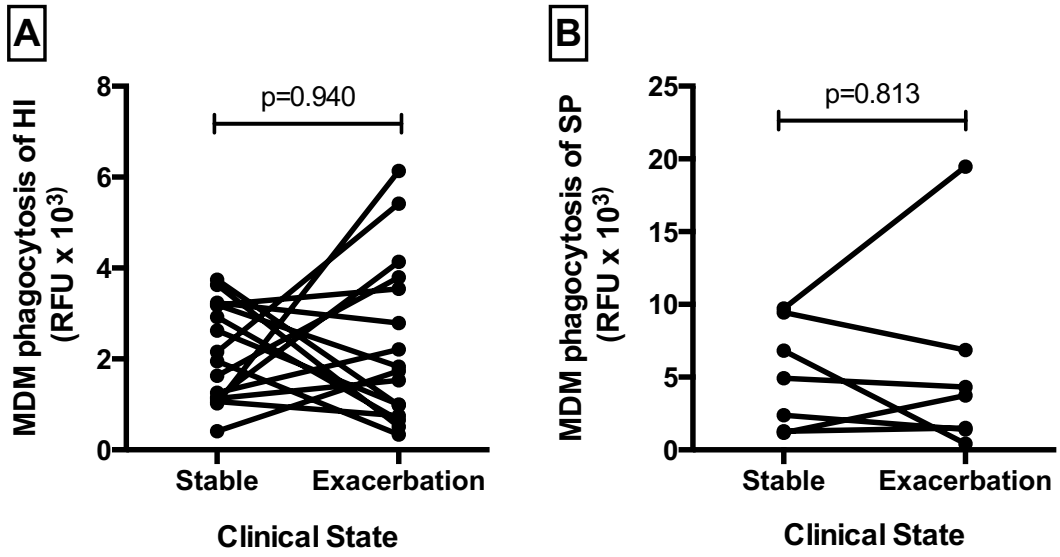


Figure 8.86. Change in MDM phagocytosis to bacteria between stable and exacerbation states by the detection of the same bacteria at exacerbation. (A) Phagocytosis capacity to *H. influenzae* (HI) in patients with *H. influenzae* detected at exacerbation (n=16). (A) Phagocytosis capacity to *S. pneumoniae* (SP) in patients with *S. pneumoniae* detected at exacerbation (n=7).

The effect of human rhinovirus presence at exacerbation on change in MDM phagocytosis between stable and exacerbation states was then investigated. As 7/8 patients with HRV detected had evidence of co-infection with a bacterial pathogen, the patients were considered as together irrespective of co-infection status. Neither HRV presence (n=8, [illegible]) nor the presence of a viral prodrome [illegible]7 days before

exacerbation onset (n=19, □□□□□ □□□) had any significant effect on MDM phagocytosis of either beads or bacteria between stable or exacerbation states.

	MDM phagocytosis (RFU x 10^3)		
Prey	Stable	Exacerbation	p-value
Beads	23.2 [14.1-31.4]	16.6 [12.5-28.3]	0.844
HI	2.03 [0.23-3.19]	2.69 [1.02-4.53]	0.641
SP	2.17 [0.80-10.49]	5.03 [3.26-6.87]	0.547

Table 8.51. The effect of human rhinovirus presence at exacerbation on the change in MDM phagocytosis of beads, *H. influenzae* (HI) and *S. pneumoniae* (SP) between stable and exacerbation states. Data is presented as median [IQR], (n=8).

□

	MDM phagocytosis (RFU x 10^3)		
Prey	Stable	Exacerbation	p-value
Beads	18.1 [14.7-22.6]	17.8 [11.8-24.4]	1.000
HI	1.74 [1.13-2.93]	1.74 [1.03-4.00]	0.824
SP	4.26 [0.86-9.45]	4.32 [0.82-8.77]	0.646

Table 8.52. The effect of a viral prodrome within 7 days of exacerbation onset on the change in MDM phagocytosis of beads, *H. influenzae* (HI) and *S. pneumoniae* (SP) between stable and exacerbation states. Viral prodrome was defined as the presence of cold-like symptoms or sore throat documented on daily symptom diary cards. Data is presented as median [IQR], (n=19).

8.3.5 Change in MDM phagocytosis with paired sputum samples between stable and exacerbation states

As the presence of a bacterial pathogen at exacerbation does not necessarily indicate the aetiology of the exacerbation, especially as lower bacterial colonisation is often present at stable state, the change in MDM phagocytosis between stable and exacerbation states was further investigated in a sub-set of 21 patients with both paired MDM and sputum samples at baseline and exacerbation.

10/21 (47%) patients had evidence of one or more bacterial pathogen detected by qPCR at stable visit, compared to 17/21 (81%) patients at exacerbation presentation (p=0.052). In 13/21 (62%) patients, the total bacterial load increased at exacerbation onset and in 8/21 (38%) patients the total bacterial load decreased or did not change (i.e. no bacterial pathogen was detected at both stable and exacerbation states). [illegible] shows the pathogens isolated at stable and exacerbation states in the 21 patients with sputum samples at both visits.

Pathogen present	Stable		Exacerbation	
	N (%)	Mean (SD) Log_{10} load*	N (%)	Mean (SD) Log_{10} load*
Overall	10 (48)	6.78 (1.06)	17 (81)	6.60 (2.13)
H. influenzae	5 (24)	6.51 (1.45)	12 (57)	5.76 (1.97)
S. pneumoniae	5 (24)	7.04 (0.51)	7 (33)	7.93 (1.78)
M. catarrhalis	0 (0)	0	3 (14)	6.42 (0.85)
Human rhinovirus	NA	NA	6 (29)	4.64 (1.78)

Table 8.53. Number and load of potentially pathogenic microorganisms (PPMs) and human rhinovirus (HRV) isolated using qPCR at stable and exacerbation states with paired stable samples (n=21). *Mean load PPM: cfu/ml. HRV: pfu/ml.

Change in the total bacterial load between stable and exacerbation clinical states was not significantly associated with a change in MDM phagocytosis to either beads (p=0.103) or either bacteria (*H. influenzae*: rho=-0.046; p=0.845, [illegible]A; *S. pneumoniae*: rho=-0.151; p=0.514, [illegible]B).

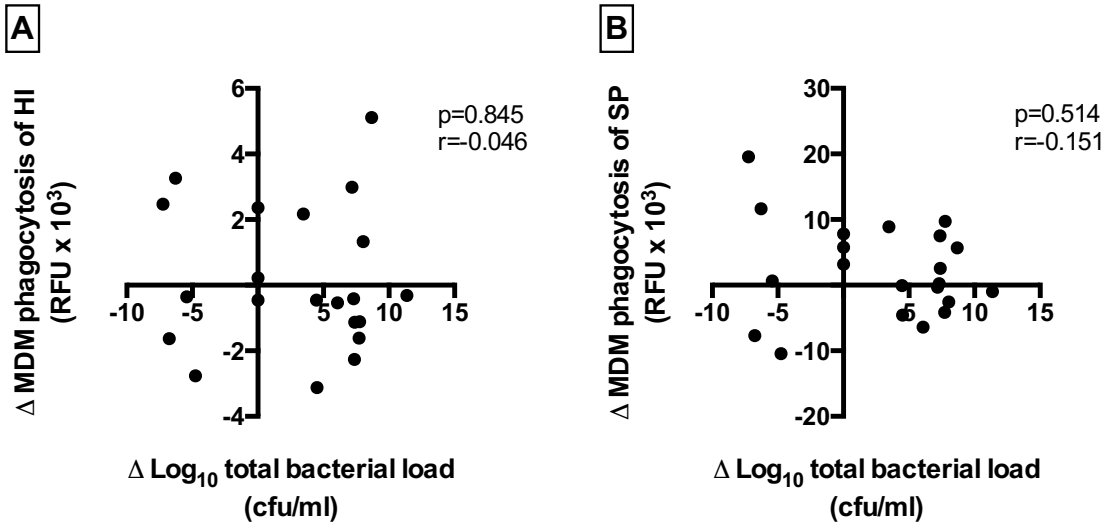


Figure 8.87. The relationship between change in MDM phagocytosis and change in total bacterial load between stable and exacerbation states. Phagocytosis to bacteria only is shown; (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP). Airway bacterial load was measured in paired sputum samples by qPCR (n=21).

When only the 13 patients with an increase in total bacterial load were considered, there was no significant difference in phagocytosis at stable and exacerbation states to either beads (22.6 [16.3-28.2] vs. 18.2 [10.5-24.6] RFU x 10^3, p=0.053) or bacteria (*H. influenzae*: 1.84 [1.11-3.08] vs. 1.53 [0.67-3.90] RFU x 10^3, p=0.735; *S. pneumoniae*: 4.26 [1.28-9.48] vs. 4.97 [1.46-9.76] RFU x 10^3, p=0.685).

When the 8 patients with a decrease or no change in total bacterial load were considered alone, no significant difference was observed between stable and exacerbation state phagocytosis to either beads (18.7 [11.4-20.7] vs. 22.4 [16.5-35.4] RFU x 10^3, p=0.109) or bacteria (*H. influenzae*: 1.96 [1.52-2.52] vs. 1.61 [1.03-4.16] RFU x 10^3, p=0.844; *S. pneumoniae*: 4.37 [0.46-7.94] vs. 6.19 [1.39-17.33] RFU x 10^3, p=0.313).

In addition, change in *H. influenzae* load between stable and exacerbation states was not significantly associated with a change in MDM phagocytosis to *H. influenzae* (rho=0.226; p=0.325, [illegible]A). Similarly, change in *S. pneumoniae* load was not significantly related to a change in the phagocytic capacity to *S. pneumoniae* between stable and exacerbation states (rho=-0.151; p=0.394, [illegible]B).

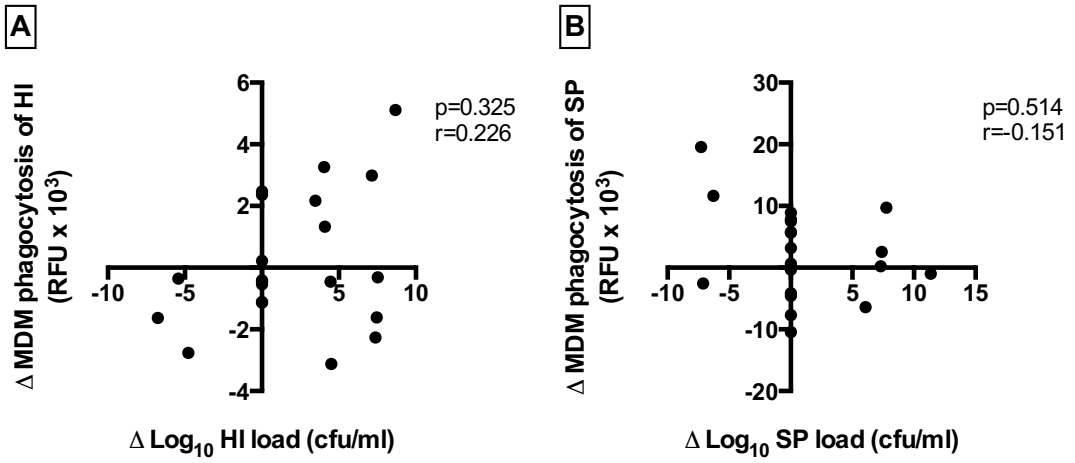


Figure 8.88. The relationship between change in MDM phagocytosis and change in bacterial load of the same pathogen between stable and exacerbation states. Phagocytosis to bacteria only is shown; (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP). Airway bacterial load was measured in paired sputum samples by qPCR (n=21).

8.3.6 The effect of exacerbation recovery on MDM phagocytosis

In a subset of the 39 patients with paired stable and exacerbation MDM samples, exacerbation follow-up samples were taken from 20/39 (51%) patients 2 weeks after exacerbation presentation. All patients had been given systemic treatment with a 7-day course of both antibiotics and oral corticosteroids at exacerbation presentation. At the 2-week follow-up visit, only 9 (45%) patients had fully recovered from their exacerbation.

In the 20 patients with paired stable-exacerbation-2-week samples, no significant difference was observed between the phagocytic capacity of either beads (p=0.259) or bacteria (*H. influenzae*: p=0.325 and *S. pneumoniae*: p=0.819,).

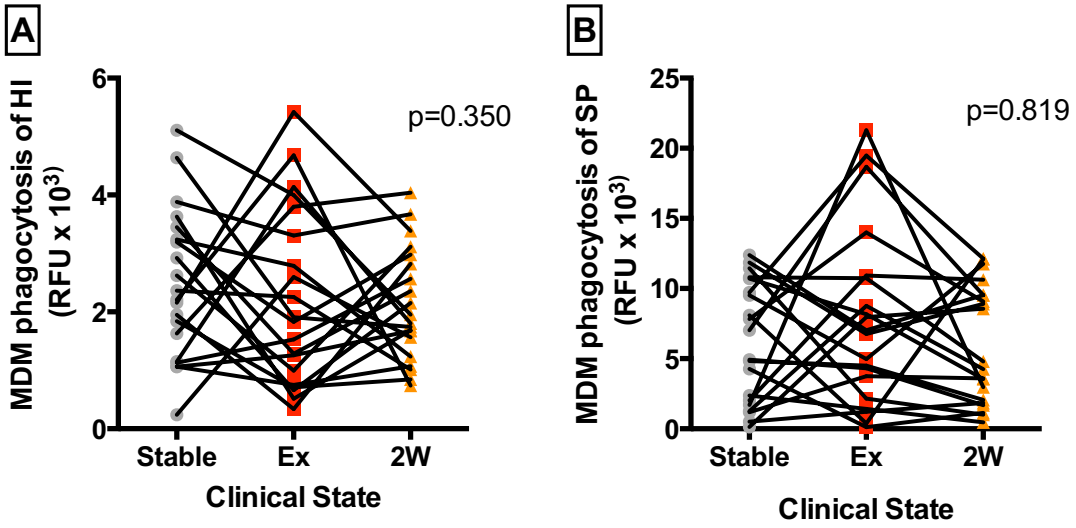


Figure 8.89. Time course of phagocytic capacity to (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP). Paired samples were taken at stable visit (•), exacerbation presentation (Ex, ■) and exacerbation follow-up at 2 weeks (2W, ▲). Analysis was performed by Friedman test (n=20).

The change in phagocytosis between exacerbation presentation and 2-week exacerbation follow-up was analysed by bacterial and viral pathogens detected at exacerbation. Exacerbation sputum samples for pathogen analysis were available in 16/20 (80%) of patients.

In patients with at least one bacterial pathogen detected at exacerbation presentation by qPCR (14/16 (89%)), there was no significant difference in the MDM phagocytosis of either beads or bacteria between exacerbation presentation and 2-

week follow-up (beads: 18.3 [11.5-23.4] vs. 20.4 [11.6-23.8] RFU x 10^3, p=0.358; *H. influenzae*: 1.68 [0.70-4.03] vs. 2.05 [1.19-3.01], RFU x 10^3 p=1.000; *S. pneumoniae*: 6.00 [3.16-12.86] vs. 4.52 [1.97-10.91] RFU x 10^3, p=0.241).

As only 2 patients did not have pathogens detected at exacerbation and only 4 had HRV detected, both patient groups were too small to analyse statistically. However, in 3 of the 4 patients with HRV detected, the paired exacerbation-2-week samples showed a trend decrease in phagocytosis at exacerbation follow-up.

8.3.7 Cytokine release from MDM in response to different phagocytic prey

The cytokine release from activated MDMs was used as another assessment of their function in different disease states and was available from 37/39 (95%) patients with paired stable and exacerbation samples.

The effect of basal cytokine release and the effect of the three different prey incubated with MDMs in the stable state was first studied. There was a significant difference in both CXCL8 and TNF release between the basal levels (MDMs incubated with the control (RPMI alone)) and from the MDMs incubated with either beads or bacteria ($p<0.0001$ for both cytokines,). Inert beads did not activate an inflammatory response, with no significant difference in cytokine release observed between the control and inert bead incubated cells. However, MDMs incubated with bacteria demonstrated significantly higher levels of both cytokines compared to control. Although there was a trend towards a species-specific effect on cytokine release, particularly with TNF release, this was not statistically significant on multiple comparisons.

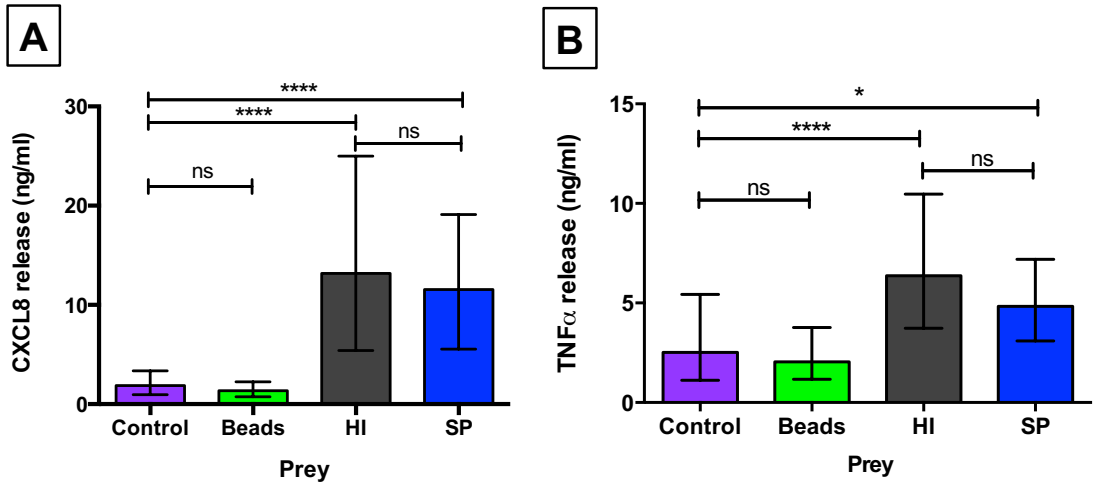


Figure 8.90. Cytokine release from stable MDMs after 4 hours incubation with phagocytic prey. Supernatants were taken after stable MDM incubation with both RPMI alone (control, ■) and prey; inert beads (■), *H. influenzae* (HI, ■) and *S. pneumoniae* (SP, ■) and ELISAs for CXCL8 (A) and TNF□(B) performed, n=37. * represents p<0.05, ****p<0.0001. ns = not statistically significant. Data presented as median values with the error bars representing the interquartile range.

□

Similarly in MDMs taken at exacerbation presentation, there was a significant difference in cytokine release between the control and different prey (p<0.0001 for both cytokines). As in the stable state, inert beads did not cause a heightened inflammatory response, but MDMs incubated with bacteria demonstrated significantly higher levels of CXCL8 and TNF□compared to control (p<0.001 for all) although, no species-specific response was observed (□□□□□□□□□□).

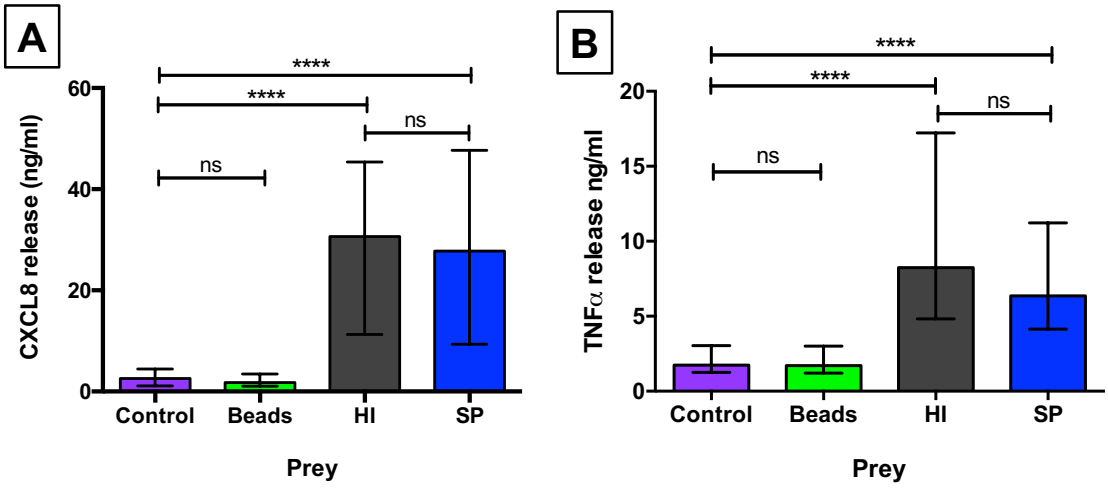


Figure 8.91. Cytokine release from exacerbation MDMs after 4 hours incubation with phagocytic prey. Supernatants were taken after exacerbation MDM incubation with both RPMI alone (control, ■) and prey; inert beads (■), *H. influenzae* (HI, ■) and *S. pneumoniae* (SP, ■) and ELISAs for CXCL8 (A) and TNF□ (B) performed, n=37. **** represents p<0.0001. ns = not statistically significant. Data presented as median values with the error bars representing the interquartile range.

□

8.3.7 The effect of stable state characteristics on stable MDM cytokine release

To determine whether stable state characteristics affect the cytokine release from MDMs taken at stable state, CXCL8 and TNF□ was compared between patients with and without concurrent inhaled corticosteroid use, and between frequent and infrequent exacerbators, defined as □2 exacerbations/year and <2 exacerbations/year respectively.

32/37 (86%) patients were using a concurrent inhaled corticosteroid and the median dose was 2000 [1000-2000] beclomethasone equivalent dose (μg). There was no significant difference in CXCL8 release, following incubation with either *H. influenzae* or *S. pneumoniae*, between those patients with and without concurrent inhaled corticosteroid use (p>0.05 for all, □□□□□□□□□).

□ □□□

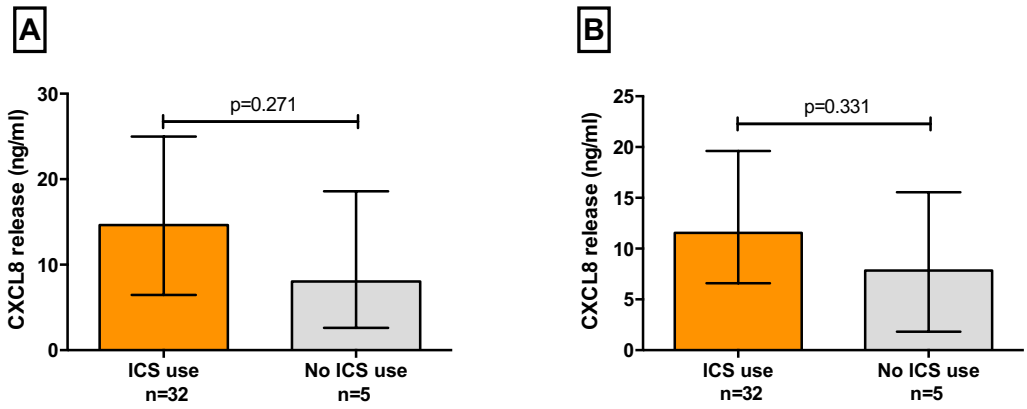


Figure 8.92. The effect of inhaled corticosteroid use on stable state CXCL8 release from MDMs following incubation with (A) *H. influenzae* and (B) *S. pneumoniae.* Data presented as median values with the error bars representing the interquartile range (n=37). ICS: inhaled corticosteroids. (■) ICS use and (■) no ICS use.

Similarly, there was no significant difference in TNF□ release following incubation with bacteria between those patients with and without concurrent inhaled corticosteroids use (*H. influenzae*: 6.37 [3.66-11.78] vs. 5.35 [3.52-7.70], p=0.428, and *S. pneumoniae*: 4.87 [2.91-7.98] vs. 4.51 [3.40-5.62], p=0.719).

30/37 (81%) patients were defined as frequent exacerbators, with a median exacerbation frequency of 3.0 [2.3-3.6] events/year. Exacerbation frequency did not significantly affect the induction of either CXCL8 or TNF□ in response to either *H. influenzae* or *S. pneumoniae* (□□□□□□□□□ and □□□□□□□□□, respectively).

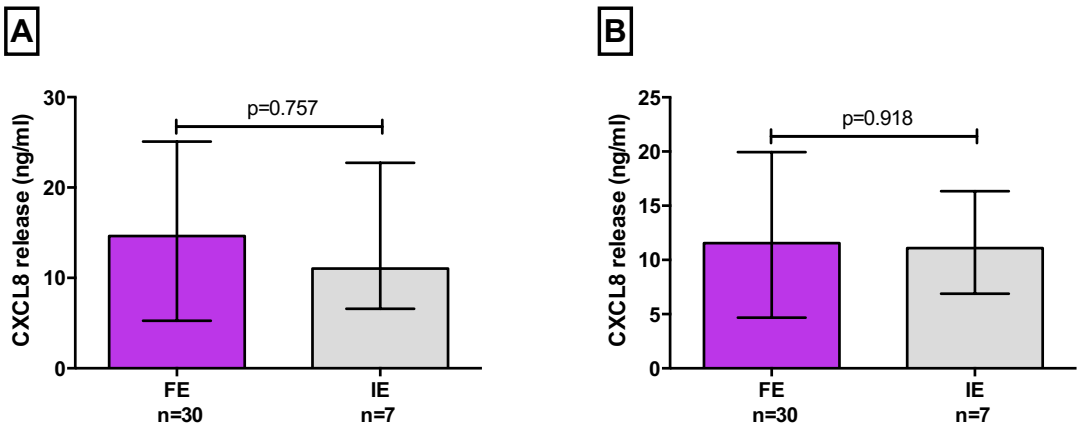


Figure 8.93. The effect of exacerbation frequency on stable state CXCL8 release from MDMs following incubation with (A) *H. influenzae* and (B) *S. pneumoniae*. Data presented as median values, with the error bars representing the interquartile range (n=37). FE: frequent exacerbators, ≥2 exacerbations/year (■); IE: infrequent exacerbators, <2 exacerbations/year (■).

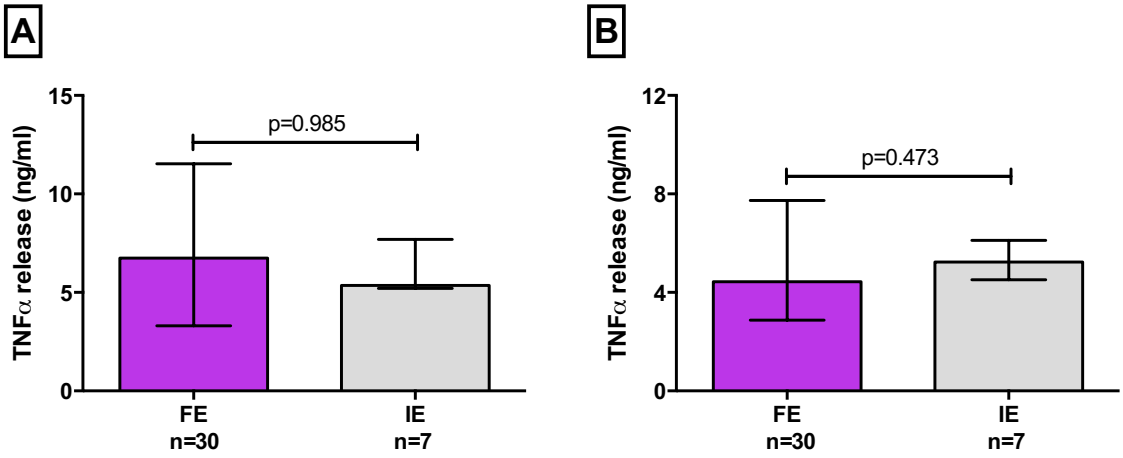


Figure 8.94. The effect of exacerbation frequency on stable state TNFα release from MDMs following incubation with (A) *H. influenzae* and (B) *S. pneumoniae*. Data presented as median values, with the error bars representing the interquartile range (n=37). FE: frequent exacerbators, ≥2 exacerbations/year (■); IE: infrequent exacerbators, <2 exacerbations/year (■).

8.3.8 The relationship between MDM activation and phagocytosis in stable and exacerbated states

To determine whether the two functional outputs measured from the MDMs were related, the cytokine release following incubation with either *H. influenzae* or *S. pneumoniae* was correlated with the phagocytic capacity of the MDMs to the same bacteria at both stable and exacerbation states.

There was no significant relationship between CXCL8 release and the phagocytic capacity of MDM to either bacteria at stable state (*H. influenzae*: rho=0.168; p=0.328, *S. pneumoniae*: rho=-0.083; p=0.630,).

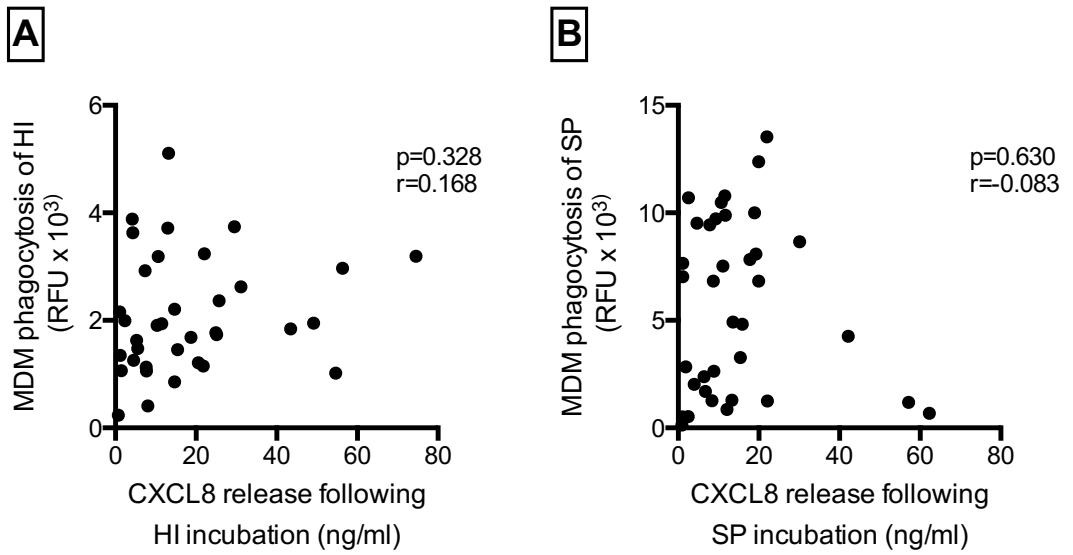


Figure 8.95. Relationship between CXCL8 release and phagocytosis of MDMs to (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP) at stable state (n=37).

Similarly, at exacerbation, there was no significant relationship between CXCL8 release and phagocytic capacity of MDMs to *H. influenzae* (rho=-0.075; p=0.654) or *S. pneumoniae* (rho=-0.049; p=0.770).

At stable state, there was no significant relationship between TNF release and phagocytic capacity of MDMs to *H. influenzae* (rho=0.187; p=0.208) or *S. pneumoniae* (rho=0.154; p=0.308).

However, at exacerbation, increasing TNF release in response to incubation with *H. influenzae* was associated with higher phagocytic capacity to *H. influenzae* (rho=0.388; p=0.029, A). No such relationship was seen with TNF release in response to incubation with *S. pneumoniae* and the ability of MDMs to phagocytose *S. pneumoniae* (rho=0.100; p=0.580, B).

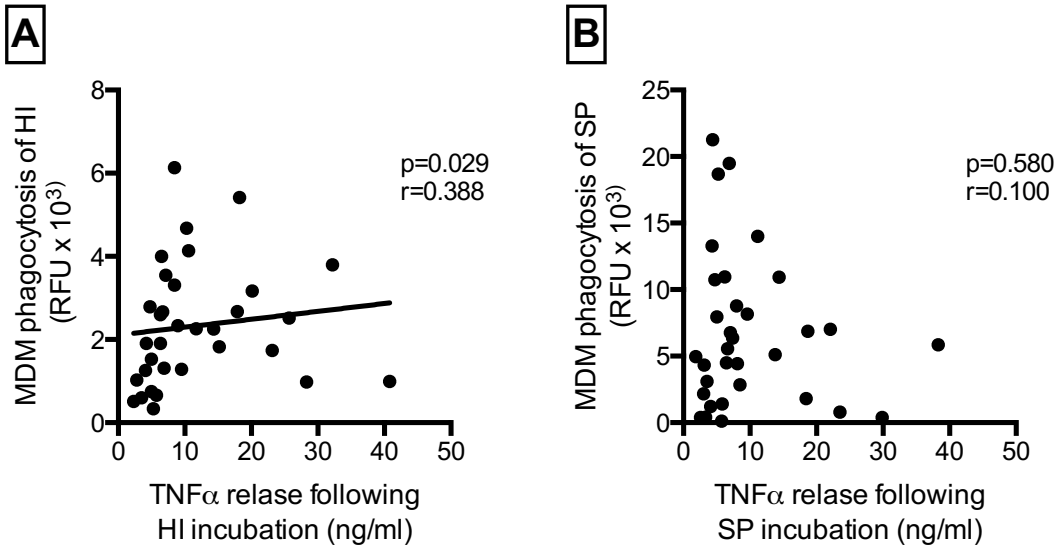


Figure 8.96. Relationship between TNFα release and phagocytosis of MDMs to (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP) at exacerbation state (n=37).

8.3.9 Cytokine release at stable and exacerbated states

To determine whether there was any change in cytokine release between disease states, CXCL8 and TNF levels were compared between the paired stable and exacerbation states in 37 patients.

There was no significant difference in basal or inert bead induction of CXCL8 between stable and exacerbation states (1.86 [0.94-3.35] vs. 2.73 [1.35-4.46], p=0.788 and 1.36 [0.74-2.25] vs. 1.92 [1.18-3.54], p=0.295 respectively). However, following incubation with either *H. influenzae* or *S. pneumoniae*, the induction of CXCL8 significantly increased at exacerbation (13.18 [5.40-25.00] vs. 30.64 [11.53-46.78], p=0.004 and 11.55 [5.54-19.11] vs. 29.96 [9.36-49.14], p<0.001, respectively, [illegible]).

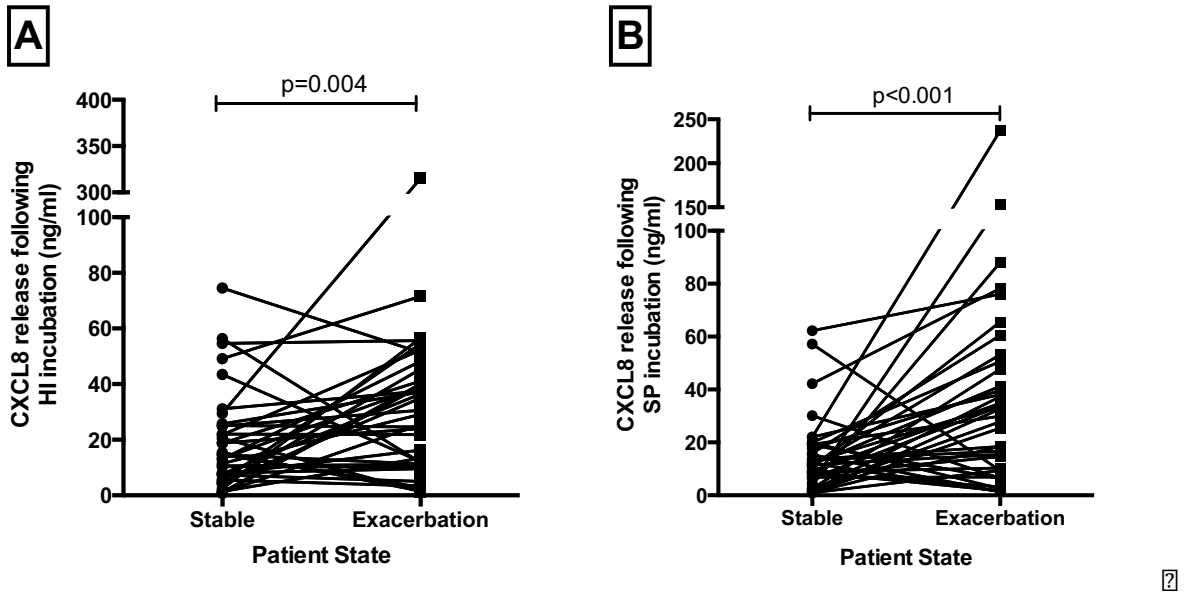


Figure 8.97. Change in CXCL8 release between stable and exacerbation states following incubation with (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP). Paired samples were taken from 37 patients.

Similarly, there was no significant difference in basal or inert bead induction of TNF between stable and exacerbation states (2.52 [1.21-5.43] vs. 2.23 [1.40-4.06], p=0.184 and 2.05 [1.16-3.76] vs. 2.04 [1.42-3.25], p=0.535 respectively).

Following incubation with *S. pneumoniae*, the induction of TNF□ significantly increased at exacerbation (4.83 [3.09-7.19] vs. 6.63 [4.32-14.07], p=0.002, □□□□□□□□□B), but a similar increase in TNF□ release was not seen in response to *H. influenzae* (6.37 [3.73-10.46] vs. 8.05 [4.96-18.07], p=0.233, □□□□□□□□□□A).

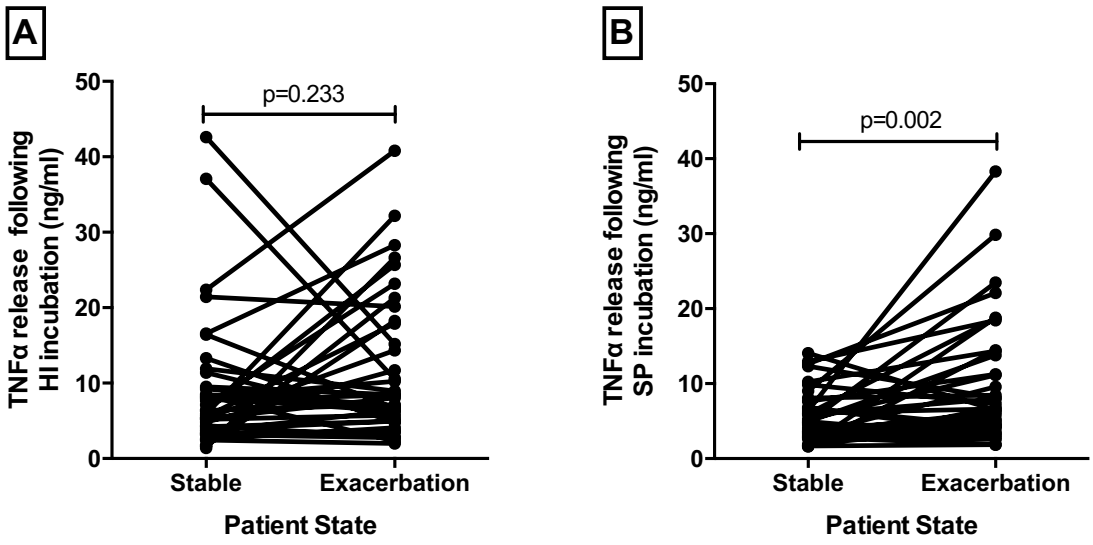


Figure 8.98. The change in TNFα release between stable and exacerbation states following incubation with (A) *H. influenzae* (HI) and (B) *S. pneumoniae* (SP). Paired samples were taken from 37 patients.

8.4 Discussion

For the first time, this study examined the detailed relationships between macrophage function and the characteristics, pathogen-related aetiology and recovery of acute exacerbations in a well-characterised cohort of patients with COPD. Impaired MDM phagocytosis at exacerbation was not significantly associated with exacerbation severity, duration, symptoms or airway bacterial pathogen presence. Furthermore, there were no significant changes in MDM phagocytosis between stable and exacerbation states and in paired analyses, changes in phagocytosis could not explain changes in airway bacterial load between these two states nor provide a biological mechanism for the interaction between bacterial and viral pathogens often observed following co-infection. Thus, these detailed results suggest that the impaired phagocytic response seen in patients with COPD may be due to an intrinsic monocyte defect, conferring susceptibility to exacerbation frequency in stable disease, as discussed in Chapter 7, but is not modulated at exacerbation and does not influence exacerbation characteristics.

In addition, MDMs from patients with COPD are activated following exposure to bacteria, releasing CXCL8 and TNF□, but no species-specific effect was seen. Inhaled corticosteroids or exacerbation frequency did not influence MDM cytokine release, and the basal cytokine release was not significantly different between stable and exacerbation states. However, following incubation with either *H. influenzae* or *S. pneumoniae*, CXCL8 significantly increased between stable and exacerbation states, whereas TNF□ only increased in response to *S. pneumoniae,* suggesting either differential activation or priming of these cells at exacerbation, or as the MDMs are

cultured from monocytes for 12 days, this effect may represent a different monocyte precursor at exacerbation.

Exacerbations are key events for both patients and healthcare providers, contributing both to the morbidity and mortality of the disease (Decramer et al., 2012) as well as being a significant financial burden due to direct healthcare costs including increased medication use and prolonged hospital stays (2010; Niewoehner, 2006). Hence, the prevention and timely treatment of acute exacerbations are key targets for healthcare professionals. However, exacerbations are particularly heterogeneous and this is likely to be a reflection of the complex interactions that occur between host pathophysiological and environmental factors (Wedzicha and Seemungal, 2007). The identification of patient and exacerbation phenotype may identify a novel mechanism for future therapeutics and this is an important area of research.

The data in this thesis presented previously, showed a significant relationship between MDM phagocytosis of *H. influenzae* and exacerbation frequency in stable disease, albeit, this could not be explained by its association with lower airway bacterial presence in stable disease. As macrophages are the most prominent phagocyte within the lung and play a vital role in innate immunity, including bacterial clearance, it is feasible that changes in macrophage phagocytosis could be one plausible biological determinant of exacerbation heterogeneity. However, to date, the study of macrophage function during COPD exacerbations has been neglected, and the focus has predominantly been on the subsequent exacerbation risk due to the dysfunction seen in stable disease (Berenson et al., 2014; Eltboli et al., 2014). The use of alveolar macrophages requires invasive sampling, which would be contraindicated at times of

acute exacerbation. However, using MDMs as a model of alveolar macrophages, allows their function at exacerbation to be examined in depth together with detailed exacerbation characteristics in this study.

If the defective macrophage phagocytosis observed in stable COPD was altered at exacerbation, a further deterioration in susceptible patients may enable airway bacterial load to increase, resulting in an associated increase in airway inflammation which may contribute to exacerbation symptomatology (Wedzicha and Seemungal, 2007). However, the role of airway bacterial pathogens in the aetiology of exacerbations remains controversial. As previously discussed in Chapter 5, airway bacterial detection in exacerbation studies is conflicting, with some studies demonstrating an increase in prevalence (Hurst et al., 2006b; Rosell et al., 2005; Wilkinson et al., 2006b) and also bacterial load (Garcha et al., 2012; Hurst et al., 2006b; Wilkinson et al., 2006b), while others do not show such a change (Bafadhel et al., 2011; Papi et al., 2006; Sethi et al., 2007). In this study, several methods were used to assess the presence of a bacterial pathogen at exacerbation. Bacterial presence was detected by qualitative culture, which is often used in hospital clinical microbiology laboratories, by qPCR for the three common bacterial pathogens, which has greater diagnostic yield than culture methods (Garcha et al., 2012) (Chapter 4), and also by the presence of sputum purulence, which has been shown to be a surrogate marker of bacterial load (Miravitlles et al., 2010) and is often the only available method in primary care and community settings. Neither of these methods was associated with either a significant relationship with exacerbation state MDM phagocytosis or a significant change in phagocytosis between stable and exacerbation states.

One of the criticisms of using the presence of bacteria alone to determine their role at exacerbation is that this does not take into account the presence of lower airway bacteria in stable disease, which is seen in 30-50% of patients (Banerjee et al., 2004; Patel et al., 2002; Rosell et al., 2005; Sethi et al., 2006). Therefore, to better characterise this relationship, the change in bacterial load between stable and exacerbation states and its relationship to change in phagocytosis was examined. In this analyses, the patient numbers significantly decreased from the overall cohort of 39 to 21 patients, as this required sputum samples being obtained at both time points. While no significant relationship was seen, this patient sample size may be underpowered to detect any significant relationship and so further research in a larger dataset should be performed. However, as in stable disease, macrophage phagocytosis is only one of numerous mechanisms of the innate immunity to maintain the homeostasis of the airway microbiome, and it is likely that these other mechanisms also play a contributory role, rather than the defective phagocytosis being the sole factor.

Co-infection with airway bacteria and viruses has been of considerable interest in the study of COPD exacerbations, and bacterial infection following influenza viral infection has been well documented (McCullers, 2014). While the role of bacteria in acute exacerbations remains debated, it is clear that the majority of exacerbations have an infective component (Papi et al., 2006; Wedzicha and Seemungal, 2007; Wilkinson et al., 2006a). Respiratory viruses have been detected in up to 60% of exacerbations (Seemungal et al., 2001) and HRV, which is one of the main causes of the common cold, is thought to be a major viral pathogen contributing to COPD exacerbations (Kurai et al., 2013). Several studies have demonstrated interplay

between HRV and bacterial pathogens, with increasing airway bacterial load (Molyneaux et al., 2013; Wilkinson et al., 2006a) or secondary bacterial infection occurring after an initial viral insult.

In the study by Mallia and colleagues, healthy smokers, non-smokers and patients with moderate COPD were infected using their HRV model of acute exacerbations (Mallia et al., 2012). Following HRV infection, 60% of COPD patients had evidence of secondary bacterial infection, compared to 10% of the non-COPD study participants, with a peak in bacterial load at day-15 post inoculation. Importantly, this work was replicated in naturally occurring HRV exacerbations (George et al., 2014), where patients had worse disease severity compared to the patients included in the experimental model, FEV_1 % predicted 44.9 vs. 68.1. In addition, over half of the patients with a secondary rise in bacterial load in this study reported worsening of their respiratory symptoms at day 14, which coincided with the peak bacterial load, suggesting that this is a clinically important occurrence.

The biological mechanism for this secondary bacterial infection following HRV infection is likely to be due to several insults on the host's innate immune system. Indeed in the study by Mallia and colleagues (Mallia et al., 2012), the authors also demonstrated a significant fall in the sputum antimicrobial peptides SLPI and elafin in the COPD patients with secondary bacterial infection. In addition, rhinovirus infected macrophages have been shown to have an impaired pro-inflammatory cytokine TNF and CXCL8 production in response to the Gram positive and Gram negative bacterial products, lipopolysaccharide and lipoteichoic acid respectively, as well as demonstrating impaired phagocytosis to bacterial particles (Oliver et al., 2008).

Therefore, the effects of HRV on macrophage function may be a key mechanism resulting in this phenomenon

In the present study, the presence of HRV was studied using both upper airway symptoms of colds or presence of a sore throat at exacerbation onset but also as a prodrome prior to onset, and directly using qPCR of sputum samples. Both methods were used as cold and sore throat symptoms have been shown to be associated with high HRV loads (George et al., 2014) and therefore may indicate its presence when molecular testing is unavailable or may imply the presence of another respiratory viral pathogen. In this dataset, there was no significant difference in MDM phagocytosis in those patients presenting with and without cold symptoms at exacerbation onset.

Viral replication within the macrophage has been shown to take up to 3 days and viral RNA expression lasts up to 10 days (Oliver et al., 2006). Therefore, the presence of a viral prodrome documented on daily diary cards within 7 days of the exacerbation presentation, was taken a surrogate marker of viral infection, to enable sufficient time for viral replication within macrophages to occur and thus exert the effect on phagocytosis. However, in patients with a viral prodrome, there was no significant change between stable and exacerbation MDM phagocytosis of bacteria. Only 8 of the 39 patients with paired stable and exacerbation samples had definitive evidence of human rhinovirus infection, as detected by qPCR at exacerbation presentation, so again the limited numbers may suggest that this study is underpowered. Similarly in this group, there was no significant change in MDM phagocytosis between the stable and exacerbation states. It could be argued that at exacerbation presentation, the effect of HRV may be too early to be observed, as secondary bacterial infection occurs after

14 days post viral insult. Therefore, the exacerbation recovery samples taken at 2-weeks post exacerbation presentation were analysed. Overall, there was no significant change between the stable, exacerbation and exacerbation recovery states, but the small number of patients significantly limited the analysis of recovery samples by exacerbation pathogens, and statistical methods could not be applied and the study was underpowered to detect any differences. However, in the 4 patients with HRV detected at exacerbation, 3 showed a subsequent decrease in the phagocytic capacity at 2-weeks, and thus the depression in macrophage phagocytosis may not be seen until this time point. Therefore further study in a larger dataset is needed to investigate this potential effect.

The results shown herein demonstrate considerable inter-patient variability in macrophage phagocytosis at exacerbation and could not account for the variability seen in airway pathogens. The ECLIPSE cohort demonstrated that exacerbation frequency remains stable in approximately 70% of patients (Hurst et al., 2010) and thus the significant relationship seen between stable MDM phagocytosis and exacerbation frequency, described in chapter 7, is likely to represent an intrinsic defect within the monocyte. Whether this defect is modified during exacerbation, when alveolar macrophages are exposed to changing inflammatory stimuli within the lung microenvironment is unknown, as due to problems with the collection of alveolar macrophages at exacerbation, such studies have not been feasible to perform. The MDMs used in this study are cultured *ex vivo* in the presence of GM-CSF for 12 days after collection. This generates the more pro-inflammatory macrophage phenotype thought to be representative of the macrophages found in the lungs of COPD patients. However, it is increasingly recognised that macrophage plasticity occurs and therefore

the local environment may contribute to a change in macrophage phenotype either to one more or less able to phagocytose bacteria (Lee, 2012). This could potentially account for intra-patient variability in exacerbation characteristics, not only in the pathogens isolated, but the durations and severity. Therefore, further research examining repeated exacerbations within the same individual as well as investigating changes in macrophage phenotype is required.

Medications such as corticosteroids and antibiotics, are commonly used in the treatment of exacerbations, have been shown to affect macrophage phagocytosis in stable disease, as discussed in detail in Chapter 7 (Hodge et al., 2008; Taylor et al., 2010; Zetterlund et al., 1998). Therefore, it is pertinent that the sampling of patients at exacerbation onset occurred before the addition of any systemic corticosteroids and antibiotics, and no changes were made to their regular inhaled medication, with the exception of increased short-acting □2-agonist use. Therefore, it is unlikely that the timing of the samples would have any effect on the results presented. In addition, patients presented early in the course of their exacerbation, with the median time from exacerbation onset to sampling at exacerbation presentation being 3 days. Therefore, the variability seen is unlikely to be due to some patients having prolonged exacerbation symptoms prior to the time of sampling.

The main limitation in this part of the study was the number of patients included in analyses, particularly when bacterial and viral pathogens were studied, either at exacerbation only, or a change in bacterial load between stable and exacerbation states. The overall prevalence of bacteria at exacerbation was high, with 83% prevalence in the overall 47 patients, 77% prevalence in the 30 patients with paired

stable and exacerbation states. However, when changes in bacterial load between the paired samples were considered, only 2/3 of this sample set had concurrent paired sputum samples. Obtaining paired sputum samples from patients at both visits was difficult as a considerable number of patients had provided a sample at one of the visits, and either failed to produce on subsequent induction during a visit, or declined induction, which was more common at exacerbation due to concurrent dyspnoea. In addition, human rhinovirus prevalence was significantly lower in the dataset, with only 27% of exacerbation visits being positive, compared to recently published work from our department which demonstrated a 53.3% prevalence rate, although this study did include repeat exacerbations from the same individual (George et al., 2014). However, for the first time in this dataset, MDMs have been cultured at exacerbation and examined in this degree of detail, with patient symptoms, exacerbation duration and severity available, in addition to a significant proportion of concurrent microbiology and virology obtained. Furthermore, both cross-sectional data was collected at exacerbation and also paired changes between stable and exacerbation visits were included, with concurrent microbiology and virology obtained.

In COPD, alveolar macrophages are chronically activated releasing a large number of inflammatory mediators, including CXCL8 and TNF□, in response to inflammatory stimuli such as cigarette smoke and pathogens. CXCL8 plays a major role in neutrophil chemotaxis and TNF□ is a powerful pro-inflammatory cytokine with pleiotropic properties, contributing to the inflammatory process important in the pathophysiology of both stable and exacerbated COPD (Aaron et al., 2001; Berkow et al., 1987; Yang et al., 2006).

In this study, a significant difference in both CXCL8 and TNF release was seen in response to MDM incubation with all three prey used, with both bacteria resulting in a significantly greater response compared to the controls or beads. Although there was a trend toward a species-specific inflammatory effect, with higher CXCL8 and TNF in response to *H. influenzae* than *S. pneumoniae* compared to basal levels or following incubation with inert beads, this did not reach statistical significance. As shown in Chapter 4, COPD patients with evidence of airway bacteria during stable state have higher levels of airway inflammation than patients without any evidence of bacteria (Banerjee et al., 2004; Hill et al., 2000; Patel et al., 2002). This is thought to contribute to the vicious cycle of worsening airway inflammation and increased frequency of acute exacerbations associated with stable state bacterial presence (Sethi and Murphy, 2008). In addition, species-specific effects on airway inflammation in stable COPD have been previously described (Hill et al., 2000; Marin et al., 2012), with *H. influenzae* resulting in greater inflammation than other pathogens (as described in chapter 4). The outer membrane protein P6 of *H. influenzae* has been shown to be a potent and selective inducer of both CXCL8 and TNF from MDMs cultured from healthy patients (Berenson et al., 2005b). Thus, it is feasible that the lack of a species-specific effect may be due to the relatively small number of patients used for the cytokine analysis, as well as the fact that only inflammatory responses from macrophages were measured, whereas in sputum analyses, the measured inflammatory mediators are secreted by a plethora of airway inflammatory cells, which may contribute to a more marked differential cytokine response.

There was no significant effect of concurrent inhaled corticosteroid use on MDM activation following bacterial incubation and subsequent release of CXCL8 and

TNF□. Corticosteroid insensitivity is a well-recognised feature of COPD, with little impact of inhaled corticosteroids on airway inflammatory cells or inflammatory mediators observed in both *in vivo* and *ex vivo* studies (Bourbeau et al., 2007; Culpitt et al., 1999; Culpitt et al., 2003; Keatings et al., 1997). It is likely that several cellular and molecular mechanisms contribute to the development of corticosteroid insensitivity in COPD, including genetic susceptibility, defective glucocorticoid receptor binding and abnormal histone acetylation (Barnes, 2013). The latter mechanism may be an important mechanism of corticosteroid insensitivity in alveolar macrophages. Histone acetylation suppresses the activation of numerous inflammatory genes through the recruitment of histone deacetylase 2 (HDAC2). A reduction of HDAC2 expression, believed to be secondary to oxidative stress, is observed in alveolar macrophages from patients with COPD (Barnes et al., 2004; Ito et al., 2005). Indeed Chana and colleagues identified a specific, more mature macrophage population isolated from lung resection tissue, which conferred insensitivity to corticosteroids selective for lipopolysaccharide (LPS)-stimulated CXCL8 and TNF□ release (Chana et al., 2014). In this macrophage population, corticosteroids also reduced the anti-inflammatory IL-10 cytokine, suggesting that corticosteroids cause an imbalance of pro-inflammatory and anti-inflammatory mediators.

Although the inability of concurrent corticosteroids to reduce MDM activation in this study is similar to previous reports, the results are limited by the relatively small sample size. Cytokine measurements were taken from patients who all had at least one sampled exacerbation for paired stable and exacerbation MDM activation analysis, and the median exacerbation frequency in these patients was 2.5 events per year.

Therefore, as these patients represent frequent exacerbators, as per recommended guidelines (Vestbo et al., 2013), 32/37 patients were using concurrent inhaled corticosteroids, and the dose was high at 2000 μg beclomethasone equivalent, and so if any significant effect of inhaled corticosteroid were present, this would be unlikely to be identified in the group without concurrent treatment. In addition, the duration of inhaled corticosteroid use prior to sampling was not determined. This has shown to be an important factor in the risk of pneumonia in COPD patients using inhaled corticosteroids (Suissa et al., 2013), and thus may exert some influence on the cytokine release from MDMs.

As previously discussed, exacerbations are key events for both COPD patients and healthcare providers. Macrophage phagocytosis was unable to provide a plausible biological mechanism for the heterogeneity associated with exacerbations. However, previous work has suggested that pathogen specific macrophage activation may confer exacerbation susceptibility. Berenson and colleagues compared the inflammatory responses of alveolar macrophages collected from stable COPD patients, who were either exacerbation prone (defined as at least one exacerbation during a one-year follow-up period) or non-exacerbation prone (exacerbation-free during follow-up) (Berenson et al., 2014). Alveolar macrophages from exacerbation prone patients released less inflammatory cytokines, including CXCL8 and TNF□, than non-exacerbation prone patients. This was evident following activation with either *H. influenzae* or *M. catarrhalis*, but not with *S. pneumoniae* and is likely a consequence of altered TLR signalling.

In the present study, there was no significant difference between the cytokines release following incubation with either *H. influenzae* or *S. pneumoniae* between frequent and infrequent exacerbators. Previous work has demonstrated that TLR2 and TLR4 expression is not significantly altered on MDMs compared to MDMs cultured from non-smokers and healthy smokers (Taylor et al., 2010). However, in this study, no comparison was made between the TLR expression on MDMs and alveolar macrophages, and thus, if any differences did exist, this may explain the difference in the results shown. As in the study by Berenson and colleagues (Berenson et al., 2014), the data presented herein also measured the cytokine release in stable patients. However, an annual exacerbation frequency was used to define the two groups as either frequent or infrequent exacerbators (□2 exacerbations/year, <2 exacerbations/year respectively), rather than defining patients as 'exacerbator-prone' as in the Berenson study. All patients included in this study had an exacerbation and therefore could not be defined as 'non-exacerbation prone', but rather as infrequent exacerbators, and therefore the groups used are not comparable. However, it is interesting that in the study by Berenson and colleagues (Berenson et al., 2014), the corresponding BAL was cultured for pathogens to determine colonisation status and only 3% of all included patients had evidence of a pathogen (all *H. influenzae*), which is considerably lower than previous reported rates of colonisation in BAL samples (Cabello et al., 1997; Sethi et al., 2006; Soler et al., 1999), and thus this may alter the risk of subsequent exacerbation.

At exacerbation, there was a differential change in cytokine release from stable state in response to bacteria, with CXCL8 significantly increasing in response to both bacteria, but TNF□ increased only in response to *S. pneumoniae*. Studies have

demonstrated that airway inflammation increases at exacerbation (Bhowmik, 2000; Hurst et al., 2006a; Hurst et al., 2006b; Papi et al., 2006; Wedzicha et al., 2000b), and alveolar macrophages are likely to play an important role in this heightened inflammatory response. Oxidative stress is a key mechanism for the activation of macrophages via the nuclear factor (NF)-□B pathway (Pappas et al., 2013; Santus et al., 2014). At exacerbation, oxidative stress has been shown to increase (Drost et al., 2005; Footitt et al., 2015), and therefore this could result in the up-regulation of airway inflammation through further macrophage activation. Lee and colleagues demonstrated that in stable COPD the NF-□B repressing factor (NRF), which normally acts to negatively control the NF-□B pathway, is reduced in PBMCs, as a result of increased intracellular oxidative stress, increasing subsequent CXCL8 production (Lee et al., 2012). Thus, it is plausible that at exacerbation when oxidative stress is higher, alveolar recruitment of monocytes from circulating PBMCs with reduced NRF expression occurs, and thus CXCL8 is increased irrespective of the stimulating bacterial pathogen. Alternatively, there may be a differential activation of monocyte populations between stable or exacerbation state. Unfortunately, cytokine release from recovery (2-week) MDMs were not analysed in this study, and thus, no information can be gained as to whether this change seen at exacerbation improves as exacerbation recovery occurs. Interestingly, the effect of exacerbations on MDM cytokine release was observed despite the 12-14 days needed to culture the MDMs from PBMCs *ex vivo* following collection at exacerbation presentation, suggesting this may be due to an intrinsic alteration within the PBMCs themselves, and could potentially be a novel target for reducing airway inflammation during exacerbation, although further work is needed to fully elucidate this mechanism.

Therefore, in conclusion, macrophage phagocytosis at exacerbation does not appear to alter or reflect the underlying aetiology of the exacerbation or its characteristics. In addition, macrophage activation increases at exacerbation, although a differential change in cytokine release is seen. Thus it is likely that the relationship between MDM phagocytosis and exacerbation frequency in stable disease is due to an intrinsic monocyte defect that is not modulated at exacerbation, and an intrinsic monocyte defect may also confer the differential cytokine response. A further understanding of these mechanisms may help to provide novel targets for the management of exacerbations in COPD.

Chapter 9:

Discussion

9.1 Summary

The overall aim of this study was to investigate the relationship between lower airway bacterial colonisation, chronic inflammation and clinical outcomes in COPD. The data presented herein used limited qPCR to identify *H. influenzae, S. pneumoniae* and *M. catarrhalis* in sputum samples, and has been shown to have a high diagnostic yield in comparison to both qualitative and quantitative bacterial culture. By using this technique to study airway bacteria in COPD, two common themes have been highlighted in the data analyses presented in this thesis. Firstly, in stable COPD, total airway bacterial load, rather than solely bacterial presence, influences the pathogenesis and natural history of the disease. Secondly, a species-specific effect in the role bacteria play in COPD pathogenesis was observed, which was largely driven by the effects of *H. influenzae*. When MDMs were used to explore the mechanisms of bacterial colonisation, increasing exacerbation frequency was significantly associated with the worsening ability of MDMs to phagocytose *H. influenzae*, although this could not be explained by a relationship with bacterial colonisation status or load. Furthermore, the impaired MDM phagocytosis did not alter at exacerbation or was affected by airway pathogens detected at exacerbations, suggesting an intrinsic monocyte defect.

A significant relationship between total airway bacterial load and airway inflammation was seen, although importantly as demonstrated in chapter 4, an apparent inflammatory threshold in bacterial load was observed, where airway inflammation was significantly higher in those patients with high total bacterial loads compared to those patients without evidence of airway bacteria or those with lower loads. Therefore detection of an airway pathogen alone may not be a sufficient to

cause a heightened inflammatory response or change in clinical outcomes in patients with COPD. The use of this bacterial load threshold was further investigated in chapter 5 to determine whether high bacterial loads at stable state influence clinical outcomes including exacerbation susceptibility and characteristics of the exacerbation. Patients with high bacterial loads had a shorter time to next exacerbation over both 90-days and 180-days post stable visit compared to patients with lower bacterial loads, and there was a trend towards higher symptom scores at exacerbation in patients with high bacterial loads, although no effect was seen on exacerbation duration or prospective exacerbation frequency.

These findings support that the proposal that the presence of airway bacteria in COPD above a load-threshold should be defined as ‘chronic infection’ rather than ‘bacterial colonisation’, which is more suggestive of a benign process. It is now acknowledged that the tracheobronchial tree and lung parenchyma in healthy adults are not sterile, as previously believed, but a core microbiome exists, which include potentially pathogic micro-organisms, albeit at low loads (Erb-Downward et al., 2011). At these lower bacterial loads, the pathological and clinical consequences of heightened airway inflammation and effects on exacerbations are not observed, and therefore may be described as ‘colonisation’, whereas at higher bacterial loads, these pathological and clinical adverse outcomes are evident, and hence ‘chronic infection’ is more appropriate.

A bacterial species-specific effect was observed throughout the data presented, which was largely driven by the effects of *H. influenzae*, often the most prevalent airway bacterial pathogen cultured in stable COPD patients (Marin et al., 2012; Patel et al.,

2002; Sethi et al., 2006; Wilkinson et al., 2003). As shown in chapter 4, *H. influenzae* presence at stable state was associated with significantly higher airway inflammatory response and importantly, this was at all given pathogen loads, further adding to previous reports of heightened airway inflammation associated with *H. influenzae* (Hill et al., 2000; Marin et al., 2012). Furthermore, changes in *H. influenzae* load between paired stable and exacerbation states was significantly associated with increasing airway CXCL8 and IL-1□(chapter 4), and when multiple linear regression analysis was used to assess the independent effects of bacteria and HRV at exacerbation in the exploratory biomarker dataset (chapter 6), increases in *H. influenzae* load at exacerbation were associated with significantly higher levels of airway IL-1□ although significantly lower levels of airway Flt-1 and ICAM-1. However, interestingly, although *H. influenzae* presence has been shown to be associated with a heightened inflammatory response at both stable and paired exacerbation states, no significant species-specific effect was observed on time to next exacerbation or exacerbation characteristics, including symptom scores (chapter 5).

Exacerbations are heterogeneous events, involving complex interactions between the host, potential pathogens and environmental factors that contribute to worsening respiratory symptoms (Wedzicha and Seemungal, 2007). Thus, it may be hypothesised, that although *H. influenzae* is associated with a heightened inflammatory response at stable state, its ability to persist in airways by evading mucosal immunity (King, 2012) results in a persistent heightened airway inflammatory response, rather than one that waxes and wanes, which may be associated with airway pathogens, such as *S. pneumoniae* and *M. catarrhalis* which are only intermittently present. This persistent inflammation may induce a state of

tolerance, so that further insults at exacerbation may only be considered a normal or slight variation in the patients' symptoms. Furthermore, as shown in chapter 4, airway inflammation correlates poorly with patient reported outcomes, and thus other factors may influence symptoms and thus exacerbations by definition.

This was the first study to examine the impaired macrophage phagocytic response observed in COPD and its detailed relationship to airway pathogens at both stable and exacerbation states, using MDMs to model alveolar macrophages. In chapter 7, no relationship was seen between impaired phagocytosis and clinically relevant demographics, including age and disease severity in stable state. However, impaired phagocytosis to *H. influenzae* was associated with increasing exacerbation frequency, although this could not be explained by a relationship between phagocytosis and airway bacterial presence or load in sputum. However, sputum is measured because it is an accessible sample, both in the stable state and at exacerbation, but whether this accurately reflects the presence of bacteria in the periphery is not known. Nevertheless, no changes were seen in phagocytic ability at exacerbation presentation (chapter 8) and therefore this could not provide a biological explanation for the variation in pathological aetiology seen with exacerbation. These findings suggests that impaired phagocytosis in COPD is due to an intrinsic monocyte defect, which confers susceptibility to exacerbations but does influence exacerbation characteristics. Furthermore, as shown in the cytokine release data in chapter 8, differential activation of MDMs occurs and this may represent macrophages developing from different monocyte precursors at exacerbation.

Therefore, in conclusion, the findings from this thesis have important implications in the management of COPD to modifying exacerbation risk. The evidence provided should encourage clinical trials to investigate the use of prophylactic antibiotics specifically for COPD patients with high bacterial loads and those at risk of *H. influenzae* infection at both stable and exacerbated states, and the future development of novel treatments such as specific anti-cytokine agents or those designed to improve macrophage phagocytosis, thereby improving the clinical outcomes for patients with COPD.

9.2 Recommended Future Study Design

There are a number of questions arising from work presented in this thesis and the following could be performed to address these:

- The total airway bacterial-load threshold described was determined using previous culture-based data, as no direct comparison between traditional culture-based and molecular diagnostic microbiological techniques had been performed. Therefore, this phenomenon needs further investigation in larger cohorts and with different molecular techniques, particularly as high throughput sequencing is readily becoming a more affordable, and thus available research tool. However, at present, these techniques are not quantitative and should be used in conjunction with more quantitative assays. Furthermore, due to low numbers of patients with mono-microbial isolation at stable state, individual thresholds for the different species should be studied, as it is likely that a different threshold may be seen for each species.

- COPD patients with high bacterial load at stable state may represent a clinical phenotype with an increased susceptibility to acute exacerbations. qPCR analysis of a stable sputum sample to determine bacterial load could be used as inclusion criteria for entry into future randomised controlled trials of prophylactic antibiotics in stable COPD, as work from this thesis suggests that these patients may have the largest benefit. Detection of bacteria from a single sputum sample at stability should be sufficient for enrolment, as this is a good predictor of future bacterial detection.

- The dynamics of the presence of airway bacteria during stable disease need further exploration in larger cohorts and over a longer period of time. The interplay of numerous factors, including exacerbation history, aetiology, antibiotic therapies and patient demographics are all likely to contribute to this phenomenon. Furthermore, detailed microbiome analysis of sputum samples could be used to analyse microbial richness and diversity in a significantly larger number of samples than bronchoscopic sampling allows, including longitudinal sampling. This would enable further study of microbial diversity and composition to determine whether this is influenced by stable state clinical characteristics, or by clinical state and whether there is evidence of temporal variability. Such a study would be more feasible now that high throughput sequencing techniques have become more cost-effective and bioinformatic analysis has improved.

- Airway inflammation is a key pathophysiological process, and IL-1□ has been shown to increase in response to higher bacterial loads at both stable and

exacerbated COPD. Therefore, randomised controlled studies of IL-1□ antagonists may enable development of alternative agents to treat COPD.

- The relationship between the macrophage phagocytosis and airway bacteria at both stable and exacerbation states should be further studied, in particular increasing the number of patients analysed to determine the relationship between the ability of MDMs to phagocytose the same bacteria as detected in sputum. In this thesis, only one exacerbation per patient was included. As exacerbations are heterogeneous events, even within the same individual, it would be interesting to study repeated exacerbation events within the same individual, and whether differences in MDM phagocytosis could be associated with specific exacerbation characteristics. Further comparison of this relationship should be carried out using BAL to obtain both alveolar macrophages and concurrent airway samples, although this would not be possible during naturally occurring exacerbations. An alternative would be the examine sputum macrophages and therefore it could be possible to establish assays using these cells.

- Limited data presented in this thesis suggested a possible decrease in MDM phagocytic activity 2-weeks after exacerbation with HRV. This needs further work to increase patient numbers to determine whether secondary bacterial infection following HRV infection results from HRV further impairing the ability of macrophages to phagocytose bacteria, and whether this is specific to HRV or whether other respiratory viruses elicit similar effects. The possibility of future, collaborative work with Mallia and colleagues would enable

sampling of not only MDMs, but also concurrent alveolar macrophages in their experimental HRV infection model of exacerbations.

- The MDMs used in this thesis were cultured in the presence of GM-CSF to obtain a more pro-inflammatory macrophage. However, as macrophage plasticity has been observed, experiments should be repeated with different macrophage phenotypes and relate these to both stable and exacerbation characteristics, with the possibility that manipulating the macrophage phenotype could improve phagocytosis and the clearance of pathogens.

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Appendix

- Example of the London COPD Cohort diary card

NAME	
Study Number	

THE LONDON COPD STUDY

April 2014	NEXT APPOINTMENT
WORSENING SYMPTOMS? CALL US	/ /14 . am

DATE	1 tues	2 wed	3 thur	4 fri	5 sat	6 sun	7 mon	8 tues	9 wed	10 thur	11 fri
Peak Flow											
CHANGE in Symptoms											
CHANGE in Treatment											
Hours out of the home											

DATE	12 sat	13 sun	14 mon	15 tues	16 wed	17 thur	18 fri	19 sat	20 sun	21 mon	22 tues
Peak Flow											
CHANGE in Symptoms											
CHANGE in Treatment											
Hours out of the home											

DATE	23 wed	24 thur	25 fri	26 sat	27 sun	28 mon	29 tues	30 wed
Peak Flow								
CHANGE in Symptoms								
CHANGE in Treatment								
Hours out of the home								

- The BronkoTest® chart

