A Bench to Bedside Investigation into Defective Innate Immunity in Chronic Obstructive Pulmonary Disease

A Thesis Submitted to Imperial College London for the Degree of Doctor of Philosophy

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Abstract

Chronic obstructive pulmonary disease (COPD) is characterised by inflammation that is unresponsive to corticosteroids. Innate immunity appears to be defective and may contribute to this inflammatory pathophysiology. This is exemplified by upregulated inflammatory mediators and ineffective bacterial phagocytosis by alveolar macrophages (AM). This thesis compares phagocytosis by macrophages from non-smokers, smokers and COPD patients. The mechanisms underpinning defective bacterial phagocytosis are investigated and the potential anti-inflammatory effects of novel immunomodulatory compounds explored.

Correlations were observed between the phagocytosis of paired AM and monocyte-derived macrophages (MDM) from COPD patients and controls. MDM were therefore adopted as a model. Defective bacterial phagocytosis was confirmed in MDM, relative to non-smoking controls and when using *H. influenzae* and *S. pneumoniae*. The magnitude of this defect did not correlate with FEV₁, symptom-burden or exacerbation frequency, but defective bacterial phagocytosis was associated with less stable microtubule polymers.

Solithromycin is a novel macrolide antibiotic with immunomodulatory properties and a low potential for microbial resistance. Although it was unable to augment defective bacterial phagocytosis, it did inhibit LPS-stimulated TNF-α release by MDM. This property was shared by several analogues, some of which were inactive against common respiratory pathogens (*i.e.* pure immunomodulators). The anti-inflammatory effects of oral solithromycin were therefore studied *in vivo*, during a randomised, placebo-controlled, clinical trial. To aid this investigation, a novel sampling technique was first developed, using synthetic absorptive matrix to sample the nasal epithelial lining fluid as a less invasive surrogate measure of bronchial inflammation. However, treatment with 400 mg of solithromycin for 28 days proved hepatotoxic in COPD and the trial was terminated early.

Bacterial phagocytosis is defective in COPD. It might arise from unstable microtubule polymers and permit colonisation with pathogenic bacteria. It therefore represents a novel anti-inflammatory target. Although solithromycin is immunomodulatory, its therapeutic application is limited in COPD by hepatotoxicity.
Declaration of originality

I confirm that I have performed all of the experiments and analysis reported in this thesis, unless otherwise stated.

I developed the study protocols and applied for Research Ethics Committee (REC) and Medicines and Healthcare products Regulatory Agency (MHRA) approval. I screened and recruited all of the subjects for the individual studies. I collected all of the clinical data and undertook all clinical procedures, including spirometry, impulse oscillometry, skin prick testing, venepuncture, sputum induction, nasosorption and bronchoscopy (bronchoalveolar lavage and bronchosorption). Some clinical samples were processed by laboratory technicians prior to experiments. The same technicians were also blinded and therefore asked to perform the differential cell counts (Section 2.2.8.2). Quantitative polymerase chain reactions (Section 2.2.15) were undertaken by the microbiology department at the Royal Brompton and Harefield Hospital Trust. I undertook all of the laboratory experiments (unless otherwise stated) and analysed all of the data.

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Acknowledgements

First and foremost, I would like to thank my supervisors Professors Louise Donnelly and Peter Barnes for all their support, invaluable guidance and the opportunity to undertake this PhD. It has been an absolute privilege to work within your section of the NHLI. I would also like to thank the NIHR and Cempra Pharmaceuticals for funding my research.

I would like to extend my gratitude to all of my collaborators, without whom this PhD would never have been possible. In particular, Will Man and his team at Harefield (Jane, Claire, Sam, Sarah, Alex and Ian) for accepting me as an honorary member of the Muscle Lab, and Trevor Hansel and Onn Min Kon for their support with all things ‘sorption’. Newara Ramadan and the microbiology team at the Royal Brompton also deserve special mention, for their invaluable help with the qPCR. I am also grateful for Sally and JP’s commitment to recruiting subjects.

Thank you to all my friends and colleagues for making these last few years incredibly enjoyable. Ceri, Adam, Peter, Tash, Jess, Amy, Michelle, Kylie, Meg, Sam, Gurpreet, Jacquie, Rebecca, Jonathan, Tank and Duncan, thanks for putting up with me and for being there when the chips were down. Burger week and camping will never be forgotten! Peter, Ceri and Rebecca, thanks for teaching me literally everything. You were always available and incredibly patient.

Last but not least, I would like to thank my wife Ani for her endless support over the last 4 years. Her encouragement and wise words have been instrumental in completing this thesis.

Abstracts and publications

Data from this thesis (Chapter 6) have been presented at the European Respiratory Society (ERS) Annual Congress, London (oral presentation on Sunday 4th September 2016):

**CM Batista, MR McIntosh, TT Hansel, LE Donnelly, PJ Barnes. Elevated concentrations of CXCL8 in the nasal mucosal lining fluid of COPD patients as an accessible surrogate measure of bronchial levels. European Respiratory Journal. 2016; 48 (Supplement 60): page 491.**
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<tr>
<td>Ac-T</td>
<td>Acetylated tubulin</td>
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<td>AE</td>
<td>Adverse event</td>
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<tr>
<td>ALP</td>
<td>Alanine phosphatase</td>
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<td>Alanine transaminase</td>
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<td>Activator protein</td>
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<td>BAL(F)</td>
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<td>Brain heart infusion</td>
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<td>British Lung Foundation</td>
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<td>BMI</td>
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<td>BSA</td>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<td>CFU</td>
<td>Colony forming units</td>
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<tr>
<td>CXCR</td>
<td>Chemokine (C-X-C motif) receptor</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
</tbody>
</table>
DoH  Department of Health
DPB  Diffuse panbronchiolitis
D-PBS  Dulbecco’s phosphate buffered saline
DPX  Distyrene, tricresyl phosphate and xylene (mounting agent)
DTT  Dithiothreitol
EC_{50}  Half maximal effective concentration
ECL  Enhanced chemiluminescence
eCRF  electronic Case report form
EDTA  Ethylene diamine tetra-acetic acid
ELF  Epithelial lining fluid
ELISA  Enzyme linked immunosorbent assay
ERS  European Respiratory Society
FcR  Fc receptor
FCS  Foetal calf serum
FDA  Food and Drug Administration
FEEL  Fasciclin, EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor
FEF  Forced expiratory flow
FEV_{1}  Forced expiratory volume in 1 second
FVC  Forced vital capacity
GDP  Guanosine diphosphate
GM-CSF  Granulocyte macrophage-colony stimulating factor
GOLD  Global initiative for Obstructive Lung Diseases
GTP  Guanosine-5’-triphosphate
HBSS  Hanks’ balanced salt solution
HDAC  Histone deacetylase
HI  *Haemophilus influenzae*
HIV  Human immunodeficiency virus
HMG-CoA  Hydroxymethylglutaryl-CoA
HRP  Horseradish peroxidise
ICAM  Intercellular adhesion molecule
ICRRU  Imperial Clinical Respiratory Research Unit
ICS  Inhaled corticosteroid
IFN  Interferon
Ig  Immunoglobulin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>Interstitial macrophage</td>
</tr>
<tr>
<td>IOS</td>
<td>Impulse Oscillometry</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine activation motifs</td>
</tr>
<tr>
<td>JRCO</td>
<td>Joint Research &amp; Compliance Office</td>
</tr>
<tr>
<td>LABA</td>
<td>Long-acting β-agonists</td>
</tr>
<tr>
<td>LAMA</td>
<td>Long-acting muscarinic antagonists</td>
</tr>
<tr>
<td>LOS</td>
<td>Lipooligosaccharide</td>
</tr>
<tr>
<td>LOX-1</td>
<td>Lectin-like oxidized low-density lipoprotein receptor-1</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
</tr>
<tr>
<td>LTB₄</td>
<td>Leukotriene B₄</td>
</tr>
<tr>
<td>LTOT</td>
<td>Long term oxygen therapy</td>
</tr>
<tr>
<td>LVR(S)</td>
<td>Lung volume reduction (surgery)</td>
</tr>
<tr>
<td>MAP</td>
<td>Microtubule associated protein</td>
</tr>
<tr>
<td>MARCO</td>
<td>Macrophage receptor with collagenous structure</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factors</td>
</tr>
<tr>
<td>MDM</td>
<td>Monocyte-derived macrophage</td>
</tr>
<tr>
<td>MHRA</td>
<td>Medicines and Healthcare products Regulatory Agency</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mMRC</td>
<td>modified Medical Research Council</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino) propanesulfonic acid</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MR</td>
<td>Mannose receptor</td>
</tr>
<tr>
<td>MSD</td>
<td>MesoScale Discovery</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule organising centre</td>
</tr>
<tr>
<td>MTT</td>
<td>Methylthiazolydiphenyl-tetrazolium bromide</td>
</tr>
<tr>
<td>NAATs</td>
<td>Nucleic acid amplification tests</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
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<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NC</td>
<td>Not calculable</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NE</td>
<td>Neutrophil elastase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>NHLI</td>
<td>National Heart &amp; Lung Institute</td>
</tr>
<tr>
<td>NHS</td>
<td>National Health Service</td>
</tr>
<tr>
<td>NICE</td>
<td>National Institute for Health and Care Excellence</td>
</tr>
<tr>
<td>NIV</td>
<td>Non-invasive ventilation</td>
</tr>
<tr>
<td>NTHi</td>
<td>Non-typeable <em>Haemophilus influenzae</em></td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>ONS</td>
<td>Office for National Statistics</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphoinositide</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetics</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>pMDI</td>
<td>pressurised Metered dose inhaler</td>
</tr>
<tr>
<td>PPB</td>
<td>Potentially pathogenic bacteria</td>
</tr>
<tr>
<td>PR</td>
<td>Pulmonary rehabilitation</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern-recognition receptor</td>
</tr>
<tr>
<td>PSOX</td>
<td>phosphatidylserine and oxidized lipoprotein</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative Polymerase chain reaction</td>
</tr>
<tr>
<td>R₅</td>
<td>Resistance at 5 Hz</td>
</tr>
<tr>
<td>R₂₀</td>
<td>Resistance at 20 Hz</td>
</tr>
<tr>
<td>R₅₋₂₀</td>
<td>Resistance at 5 Hz minus resistance at 20 Hz</td>
</tr>
<tr>
<td>RBHT</td>
<td>Royal Brompton &amp; Harefield Foundation Trust</td>
</tr>
<tr>
<td>rBRU</td>
<td>Respiratory Biomedical Research Unit</td>
</tr>
<tr>
<td>RF</td>
<td>Resonant frequency</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence units</td>
</tr>
<tr>
<td>RIIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SABA</td>
<td>Short acting β-agonist</td>
</tr>
</tbody>
</table>
CHAPTER 1

Introduction
1.1. **Chronic obstructive pulmonary disease**

1.1.1. **Definition**

Chronic obstructive pulmonary disease (COPD) is a common, debilitating and life-threatening respiratory condition. It is best described by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) 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 co-morbidities contribute to the overall severity in individual patients’ (GOLD, 2015).

COPD is a source of much morbidity and typically manifests with chronic respiratory symptoms, such as progressive dyspnoea, sputum production, cough, wheeze and chest tightness (Kessler *et al.*, 2011). Unlike asthma, these symptoms display little day-to-day variation (NICE, 2010). Moreover, periods of relative clinical stability are often punctuated by sudden episodes of deterioration, termed acute exacerbations. Many patients also experience non-respiratory symptoms, such as fatigue, anorexia, weight loss, low mood and anxiety (Schols *et al.*, 1993, Hanania *et al.*, 2011).

1.1.2. **Diagnosis and disease severity**

There is no single diagnostic test for COPD. In the United Kingdom (UK), a diagnosis requires compatible symptoms, an appropriate age (usually > 35 years) and exposure to recognised risk factors (Section 1.1.5) (NICE, 2010). Persistent airflow limitation must also be present (NICE, 2010, GOLD, 2017). This is best demonstrated using spirometry (Section 1.1.7.1) and is defined as a post-bronchodilator forced expiratory volume in one second (FEV$_1$) / forced vital capacity (FVC) ratio < 0.7 (GOLD, 2017). Patients can be further stratified into four stages of disease,
according to the degree of airflow limitation. This is determined by the FEV\textsubscript{1} % predicted, \textit{i.e.} FEV\textsubscript{1} corrected for age, ethnicity, gender and height (Table 1.1) (GOLD, 2017).

<table>
<thead>
<tr>
<th>Stage</th>
<th>FEV\textsubscript{1}/FVC ratio</th>
<th>FEV\textsubscript{1} % predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOLD I (Mild)</td>
<td>&lt; 0.7</td>
<td>FEV\textsubscript{1} ≥ 80%</td>
</tr>
<tr>
<td>GOLD 2 (Moderate)</td>
<td>&lt; 0.7</td>
<td>50% ≤ FEV\textsubscript{1} &lt; 80%</td>
</tr>
<tr>
<td>GOLD 3 (Severe)</td>
<td>&lt; 0.7</td>
<td>30% ≤ FEV\textsubscript{1} &lt; 50%</td>
</tr>
<tr>
<td>GOLD 4 (Very severe)</td>
<td>&lt; 0.7</td>
<td>FEV\textsubscript{1} &lt; 30%</td>
</tr>
</tbody>
</table>

\textit{Table 1.1. Classification of airflow limitation in COPD.} COPD is characterised by persistent airflow limitation (FEV\textsubscript{1}/FVC < 0.7) and is stratified according to its severity, as determined by FEV\textsubscript{1} % predicted. Adapted from GOLD (2017).

Spirometry alone cannot comprehensibly assess the severity of COPD; a highly heterogenous disease with both pulmonary and non-pulmonary manifestations. Indeed, FEV\textsubscript{1} % predicted and GOLD stage (Table 1.1) are only weak predictors of symptom burden and disability (Jones, 2009, Agusti, 2007). Spirometry also fails to capture the importance of exacerbations to the overall symptom-burden and outcome (Section 1.4). Although patients with the worst airflow obstruction are more prone to exacerbations (Hurst \textit{et al.}, 2010, Decramer \textit{et al.}, 2009, Jenkins \textit{et al.}, 2009), the best predictor of recurrent exacerbations is a history of previously treated events (Hurst \textit{et al.}, 2010). GOLD (2015) have therefore proposed a more comprehensive assessment of disease severity (Figure 1.1). It incorporates FEV\textsubscript{1} % predicted, records exacerbation frequency and assesses symptom burden, using the modified Medical Research Council (mMRC) dyspnoea scale to determine the severity of breathlessness (Appendix A) and
the COPD assessment test (CAT) to provide a more detailed evaluation of a wider range of symptoms (Appendix B). This multidimensional assessment stratifies patients into 4 distinct groups (A, B, C or D), which better reflects the condition’s complexity and helps to personalise management.

![Diagram](https://via.placeholder.com/500)

**Figure 1.1. The combined COPD assessment.** Symptoms are first assessed using the CAT or mMRC, and patients categorised to groups A/C (less symptoms) or B/D (more symptoms). Next, an assessment of exacerbation risk is performed, using either spirometry (GOLD 1-4) or history of exacerbations in the preceding year (greatest risk taken forward). Patients are thus stratified into Group A (less symptoms, low exacerbation risk), Group B (more symptoms, low exacerbation risk), Group C (less symptoms, high exacerbation risk) or Group D (more symptoms, high exacerbation risk). Y = year. Adapted from GOLD (2015).

### 1.1.3. Epidemiology

COPD is a major global health concern. The World Health Organisation (WHO) estimate that 210 million people (3.5% of the global population) are affected, of whom 65 million (30%) are thought to have moderate, severe or very severe disease (WHO, 2015). COPD is also associated with high rates of mortality. In 2012, approximately 3 million people died from the condition,
which equates to 5.6% of all global deaths for that year and identifies COPD as the third commonest cause of death (WHO, 2014). This global burden is also projected to increase over the coming decades, as a result of continued exposure to risk factors and an ageing population (Lopez et al., 2006).

The prevalence of COPD and its associated mortality vary significantly between countries and regions. In the UK, it is estimated that 3 million people (13% of the population aged ≥ 35 years) are affected and that most cases of COPD (2.2 million) are undiagnosed (Shahab et al., 2006, CHAI, 2006). It is the fifth leading cause of death in Britain and kills approximately 30,000 annually; a figure that is comparable to lung cancer (ONS, 2013). Indeed, the predicted 5-year survival from the point of diagnosis can be as low as 30% for those patients with more severe airflow obstruction (Soriano et al., 2000). The prevalence, incidence and mortality are highest in the north of England and Scotland, which correlates with historically higher rates of smoking in these poorer socioeconomic areas (BLF, 2016). However, for reasons which are unclear, London has significantly more COPD-related hospital admissions.

1.1.4. Socioeconomic impact

One must also consider the staggering socioeconomic footprint of COPD. Department of Health (DoH) figures suggest that the National Health Service (NHS) spends over £800 million annually on COPD (DoH, 2005). The severity and chronicity of respiratory symptoms are also frequently disabling and this places notable limitations upon basic daily activities (Rennard et al., 2002). As such, COPD indirectly costs the British economy £2.7 billion from lost productivity and working days (DoH, 2005).
1.1.5. Risk factors

1.1.5.1. Tobacco smoking

Tobacco smoke is the commonest risk factor for COPD. In the UK, at least 80% of cases are attributable to smoking cigarettes, pipes or cigars (NICE, 2010). Tobacco exposure results in more rapid declines in FEV1 (Fletcher and Peto, 1977) and radiological features of COPD (Kirchner et al., 2012). However, less than half of smokers develop the condition (Lundback et al., 2003), suggesting an inherent individual susceptibility with possible roles for genetic, epigenetic or host factors (yet to be identified).

1.1.5.2. Biomass fuel

Indoor air pollution remains an important risk factor for COPD. Globally, it is estimated that 50% of homes (3 billion people) are exposed to smoke from the burning of biomass fuel (Salvi and Barnes, 2010). This is a particular problem in rural areas of developing countries, where biomass is burnt in enclosed and poorly-ventilated areas. Women and small children typically receive the greatest exposure.

1.1.5.3. Outdoor air pollution

The inhalation of noxious substances and particles within outdoor air pollution may also lead to the development of COPD (Ko and Hui, 2012). Moreover, outdoor pollutants are important triggers for acute exacerbations (Wedzicha and Seemungal, 2007).

1.1.5.4. Other risk factors

There are a number of other risk factors for COPD. These include advancing age, female gender, lower socioeconomic status, low birth weight or prematurity, severe childhood respiratory infection, smoking marijuana, α1-anti-trypsin deficiency and occupational exposure (e.g. organic/inorganic dusts, chemicals and fumes) (GOLD, 2017).
1.1.6. Pathophysiology

The progressive airflow obstruction that characterises COPD arises from three pathophysiological processes, namely obstructive bronchiolitis, chronic bronchitis and emphysema (Figure 1.2). All result from a heightened and persistent inflammatory response to inhaled noxious substances and/or particles, such as those contained within tobacco smoke. They typically occur in combination but their overall contribution to the disease process may differ between patients. This gives rise to clinical heterogeneity and different COPD phenotypes, such as the ‘chronic-bronchitic’ or ‘emphysema-predominant’ patient (Siafakas et al., 2017).

1.1.6.1. Obstructive bronchiolitis (small airways disease)

The small airways are the major site of obstruction in COPD (Hogg et al., 2004). They are located distally (i.e. from the 4th-14th division of the tracheobronchial tree), non-cartilaginous, less than 2mm in diameter and largely compromised of bronchioles. Their involvement in COPD often precedes the development of emphysema (McDonough et al., 2011) and features of obstructive bronchiolitis are even identified in patients with mild disease (Galbán et al., 2012). Obstruction arises from remodelling and repair, which results in fibrosis with airway thickening and narrowing (Hogg, 2004). This is further compounded by mucous hypersecretion, which occludes the airway (Hogg et al., 2007). The end result is airway closure, alveolar air trapping and dynamic hyperinflation (Barnes, 2016).

1.1.6.2. Chronic bronchitis

Chronic bronchitis is traditionally defined in clinical terms, as the presence of cough with sputum production for at least three months of two or more consecutive years (CGS, 1959). Pathologically, it represents an imbalance between mucus secretion and its clearance by mucociliary transport. Histologically, goblet cell hyperplasia, mucus hypersecretion, changes
in the mucin composition of sputum, disruption of the epithelial cell layer and cilia dysfunction, have all been reported (Ramos et al., 2014). The net result is an accumulation of sputum within the airway lumen with subsequent airflow obstruction ('mucous plugging'). This promotes bacterial growth, colonisation and recurrent infections (Jansen et al., 1995).

1.1.6.3. Emphysema

Emphysema is a pathological process whereby the lung parenchyma, including the alveoli, are destroyed by an imbalance in protease and anti-protease activity (Barnes et al., 2003). The increased recruitment of activated neutrophils, macrophages and T-lymphocytes (Section 1.2) results in an increase in elastolytic enzymes such as neutrophil elastase, matrix-metalloproteinases (MMPs), cathepsins and caspases (Owen, 2008). This is not matched by anti-proteases and the resultant imbalance disrupts the attachments that hold open the alveoli, with a loss of elastic recoil, airway closure during expiration, gas trapping and hyperinflation (Barnes, 2004b). Lost alveoli also impact upon gas exchange, with reductions in gas transfer often preceding changes in FEV\textsubscript{1} by many years (Gould et al., 1991).
**Figure 1.2. COPD Pathophysiology.** COPD encompasses obstructive bronchiolitis (small airway remodelling and fibrosis), chronic bronchitis (mucous hypersecretion) and emphysema (parenchymal and alveolar destruction). They typically occur in combination but their overall contribution to COPD may differ between patients. The underlying aetiology is inflammation following the inhalation of noxious substances and particles. Adapted from Tubby et al. (2011).

1.1.7. Measuring airflow obstruction

1.1.7.1. Spirometry

Airflow obstruction is typically assessed by spirometry. A forced expiratory manoeuvre measures the volume of air expelled from the lungs during the first second ( Forced Expiratory Volume in 1 sec (FEV₁)) and the total volume expelled in its entirety ( Forced Vital Capacity (FVC)). Airflow obstruction is diagnosed when there is a reduction in FEV₁ that results in a FEV₁ to FVC ratio (FEV₁/FVC) less than 0.7. FEV₁ however, mainly reflects large airway obstruction
and is only a surrogate for small airway disease. Indeed, a significant amount of smaller airway obstruction must accumulate before reductions in FEV₁ are apparent (McNulty and Usmani, 2014). Forced expiratory flow (FEF), specifically the mid-portion of a flow-volume loop (between 25 and 75% of the FVC (FEF₂₅-₇₅)), can provide more information on small airway pathology (Mcfadden and Linden, 1972). It is not a sensitive measure however, and spirometry, in general, is effort dependent. Spirometry is also frequently combined with plethysmography and measures of carbon monoxide diffusion, which assess gas trapping (hyperinflation) and gas exchange, respectively. They require more equipment, cannot be performed at the bedside, and are both costly and time consuming, relative to spirometry.

1.1.7.2. Impulse Oscillometry

Impulse Oscillometry (IOS) is a more sensitive tool for the detection of small airways obstruction (McNulty and Usmani, 2014, Brashier and Salvi, 2015) and has been used in COPD before (Piorunek et al., 2015). A loudspeaker generates impulses at multiple frequencies, which are superimposed upon normal tidal breathing. As such, it is effort independent. Higher frequencies (e.g. 20 Hz) are easily absorbed and travel shorter distances. Hence, they reflect the larger airways. Lower frequencies however (e.g. 5 Hz), penetrate further and therefore represent the whole respiratory tract. A transducer measures pressure and flow at the mouth, allowing calculations of resistance to 20 Hz (R₂₀) and 5 Hz (R₅). Subtracting R₂₀ from R₅ (i.e. R₅-₂₀) provides a measure of small airway resistance (Figure 1.3). Like spirometry, IOS can be performed as a bedside test.
Figure 1.3. Impulse Oscillometry (IOS). IOS analyses tidal breathing using sound waves of different frequencies. Lower frequencies (e.g. 5 Hz) penetrate further into the lung, while higher frequencies (e.g. 20 Hz) are easily absorbed. Pressure and flow are measured at the mouth and used to calculate resistance at 20 Hz (R_{20}) and 5 Hz (R_{5}). Small airway resistance is then calculated by subtracting R_{20} from R_{5} (R_{5-20}). Adapted from Brashier and Salvi (2015).

1.2. Cellular and molecular mechanisms of inflammation in COPD

COPD is characterised by chronic inflammation of the airways and lung parenchyma (Barnes, 2016). This involves infiltration by activated leukocytes from both the innate (macrophages and neutrophils) and adaptive (CD8+ cytotoxic T cells) immune systems, which are linked by the activity of dendritic cells. There are also roles for structural cells, such as epithelial cells and fibroblasts. The coordinated release of a complex network of inflammatory mediators enables further cellular recruitment, activation and survival (Chung, 2001, Barnes, 2004b). The actions of specific cells and inflammatory mediators are discussed Sections 1.2.1 and 1.2.2, and summarised in Table 1.2 and Figure 1.4.
1.2.1. The role of immune cells

1.2.1.1. Macrophages

Macrophages orchestrate chronic inflammation in COPD (Barnes, 2004a). Their numbers are substantially increased within the alveoli and lung parenchyma of COPD patients (Retamales et al., 2001), they localise to sites of pulmonary inflammation (Meshi et al., 2002) and close associations are observed between macrophage counts and the severity of both airflow obstruction (Di Stefano et al., 1998) and emphysema (Finkelstein et al., 1995). Their accumulation within the lungs predominantly results from an increased recruitment of circulating monocyte precursors, in response to an upregulation of chemoattractants, such as CCL2 and CXCL1 (Traves et al., 2002). Reduced mucociliary clearance (Barnes, 2004a) and enhanced macrophage proliferation and/or survival (Tomita et al., 2002) may also contribute.

Macrophages are activated in COPD through exposure to a variety of stimuli, including tobacco smoke, bacteria / bacterial products (e.g. LPS) and inflammatory mediators (e.g. IL-1β) (Vlahos and Bozinovski, 2014). Upon activation, they release a plethora of cytokines, chemokines and growth factors, which are important in the induction and maintenance of pulmonary inflammation, repair and remodelling (Figure 1.4). The individual roles of specific mediators are discussed in Section 1.2.2 and summarised by Table 1.2. In short, there is an upregulation of TNF-α (Soler et al., 1999), CXCL8 (Keatings et al., 1996), IL-6 (Ravi et al., 2014), CXCL1, CCL2 (Traves et al., 2002), leukotriene (LT)B4 (Montuschi et al., 2003) and transforming growth factor (TGF)-β (Vignola et al., 1996). Activated macrophages are also capable of releasing elastolytic enzymes, such as MMP-9 and MMP-2 (Russell et al., 2002).

1.2.1.2. Neutrophils

Elevated numbers of neutrophils are found in the lungs of COPD patients, mainly within the bronchial epithelium and airway lumen (Lacoste et al., 1993, Keatings and Barnes, 1997). They
have migrated into the respiratory tract in response to increased levels of chemoattractants, such as CXCL8 (Keatings et al., 1996), LTB₄ (Montuschi et al., 2003), CXCL1 (Traves et al., 2002) and CXCL5 (Qiu et al., 2003). The recruited neutrophils are activated (Keatings and Barnes, 1997) and degranulate to release serine proteases (e.g. neutrophil elastase, cathepsin G and proteinase-3) and MMPs, which contribute to emphysema through parenchymal and alveolar degradation (Owen, 2008). The serine proteases also stimulate submucosal glands and goblet cells (Sommerhoff et al., 1990), thereby increasing mucus production and sputum viscosity. Neutrophils are important in the pathogenesis of COPD and their numbers within bronchial biopsies correlate with disease severity (Di Stefano et al., 1998).

1.2.1.3. Epithelial cells

The airway epithelium incorporates ciliated cells, mucus-secreting cells (goblet or club) and basal cells, all of which are activated in COPD by inhaled toxic substances, such as those contained within tobacco smoke. This results in the release of inflammatory mediators, such as TNF-α, IL-1β, IL-6, granulocyte-macrophage colony stimulating factor (GM-CSF) and CXCL8 (Mio et al., 1997, Masubuchi et al., 1998, Rusznak et al., 2000, Fuke et al., 2004, Thorley and Tetley, 2007). Small airway epithelial cells are also an important source of TGF-β, a potent fibrogenic factor that promotes obstructive bronchiolitis by inducing peribronchiolar fibrosis (Takizawa et al., 2001). Histopathological changes also occur within the airway epithelium, including basal and goblet cell hyperplasia with subsequent mucus hypersecretion (Schamberger et al., 2015) and squamous metaplasia, which may predispose to bronchial carcinoma (Papi et al., 2004).

1.2.1.4. T-lymphocytes

There is an increased number of T-lymphocytes within the lung parenchyma and airways of COPD patients, with the greatest expansion observed in the CD8+ cytotoxic population.
(Grumelli et al., 2004). It is thought that these cells play a significant role in the pathophysiology of COPD, as their numbers within bronchial biopsies and lung resection tissue inversely correlate with both FEV₁ and GOLD stage (O'Shaughnessy et al., 1997, Saetta et al., 1998, Freeman et al., 2010). It is highly likely that they are responsible for cytolysis and apoptosis, and contribute to emphysema via an increased expression and release of perforins, granzyme B, and TNF-α (Majo et al., 2001, Chrysofakis et al., 2004, Hodge et al., 2006a).

1.2.1.5. Eosinophils

Eosinophils are the predominant leukocyte involved in the pathophysiology of asthma but their role in COPD is less clear. Sputum eosinophilia has been observed in as many as 20%–40% of stable COPD patients (Saha and Brightling, 2006). However, this could simply reflect coexisting asthma and/or overlap, i.e. the so-called asthma-COPD overlap syndrome (ACOS) (Barnes, 2016). Regardless, eosinophilic inflammation is a good predictor of a clinical response to inhaled corticosteroids in COPD (Section 1.3.1.3) (Pascoe et al., 2015, Pavord et al., 2016).

1.2.1.6. Dendritic cells

Dendritic cells are antigen-presenting cells that form an important link between the innate and adaptive immune responses. Cigarette smoke promotes their survival and elevated numbers of activated dendritic cells have been observed within the lungs of COPD patients (Demedts et al., 2007, Vassallo et al., 2010, Van Pottelberge et al., 2010). They may indirectly contribute to the pathophysiology of COPD by activating other key leukocytes, such as macrophages, neutrophils and lymphocytes (Barnes, 2016). Indeed, markers of cell maturity correlate with airflow obstruction (Freeman et al., 2009).
1.2.2. The role of inflammatory mediators

The aforementioned cells release a complex network of inflammatory mediators, many of which are upregulated in COPD and play crucial roles in the condition’s pathogenesis. Over 50 such mediators have now been identified (Barnes, 2009). They are pro- or anti-inflammatory (or a combination of both), and broadly include lymphokines (released by T-cells), growth factors (promote cell survival and changes in airway or parenchymal structure) and chemokines (induce chemotaxis). Their actions frequently overlap or oppose, there is functional redundancy and the roles of many are yet to be fully determined (Chung, 2001). This altered inflammatory gene expression is in response to inhaled noxious stimuli, which activate transcription factors within leukocytes, such as nuclear factor kB (NF-kB) and activated protein-1 (AP-1) (Di Stefano et al., 2002, Barnes, 2006).

It is beyond the scope of this PhD to discuss each individual mediator. However, some of the most commonly-implicated are listed in Table 1.2. CXCL8 and TNF-α are two highly-researched pro-inflammatory mediators with important roles in COPD. They have been studied within this thesis and therefore warrant specific attention (Sections 1.2.2.1 and 1.2.2.2).
<table>
<thead>
<tr>
<th>Mediator</th>
<th>Cellular Source(s)</th>
<th>Proposed effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL8</td>
<td>Multiple leukocytes (predominantly macrophages)</td>
<td>Neutrophil chemotaxis and activation</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Multiple leukocytes and structural cells (predominantly macrophages)</td>
<td>Activates the expression of multiple genes (inflammatory amplification)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Most leukocytes and structural cells</td>
<td>Activates the expression of multiple genes (inflammatory amplification) Lymphocyte growth factor</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Monocytes, macrophages and fibroblasts</td>
<td>Activates macrophages</td>
</tr>
<tr>
<td>CCL2</td>
<td>Macrophages, T-lymphocytes and epithelial cells</td>
<td>Monocyte chemoattractant</td>
</tr>
<tr>
<td>CXCL1</td>
<td>Macrophages, neutrophils and epithelial cells</td>
<td>Monocyte and neutrophil chemoattractant</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Macrophages, epithelial cells and T-lymphocytes</td>
<td>Leukocyte differentiation and survival</td>
</tr>
<tr>
<td>LTB₄</td>
<td>Macrophages and neutrophils</td>
<td>Neutrophil chemoattractant</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Epithelial cells and macrophages</td>
<td>Airway remodelling</td>
</tr>
<tr>
<td>MMPs</td>
<td>Macrophages and neutrophils</td>
<td>Degradation of extracellular matrix</td>
</tr>
<tr>
<td>Neutrophil Elastase</td>
<td>Neutrophils</td>
<td>Degradation of extracellular matrix</td>
</tr>
<tr>
<td>MPO</td>
<td>Neutrophils</td>
<td>Contributes to oxidative stress</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Activates the expression of multiple inflammatory genes</td>
</tr>
</tbody>
</table>

Table 1.2. Pro-inflammatory mediators in COPD. This table lists pro-inflammatory mediators that are commonly reported to be upregulated in COPD. It also details the likely cellular sources and proposed effects. Adapted from Chung (2001) and Barnes (2009).

1.2.2.1. Chemokine (C-X-C motif) ligand 8 (CXCL8)

CXCL8 is secreted by macrophages, T-cells, epithelial cells and neutrophils in response to a number of stimuli, including cigarette smoke, bacterial products (e.g. LPS), viruses, hypoxia, reactive oxygen species (ROS) and pro-inflammatory cytokines, such as IL-1β or TNF-α (Mukaida, 2003). Concentrations are primarily regulated by NF-κB (Kunsch and Rosen, 1993), which is activated in COPD (Di Stefano et al., 2002). As such, CXCL8 is overexpressed with elevated levels within sputum (Keatings et al., 1996, Yamamoto et al., 1997) and bronchoalveolar lavage (BAL) (Nocker et al., 1996, Soler et al., 1999).

CXCL8 is a potent chemokine that is responsible for recruiting and activating neutrophils. The increased levels observed in COPD not only correlate with a higher proportion of resident...
pulmonary neutrophils (Keatings et al., 1996, Yamamoto et al., 1997) and products of their activation (e.g. neutrophil elastase) (Yamamoto et al., 1997, Grootendorst et al., 2007) but also the severity of airflow obstruction (Yamamoto et al., 1997) and emphysema on CT imaging (Parr et al., 2006). CXCL8 promotes neutrophil degranulation and chemotaxis via CXCR1 and CXCR2 receptors, respectively (Mukaida, 2003).

1.2.2.2. Tumour necrosis factor-α (TNF-α)

Concentrations of TNF-α are elevated within induced sputum (Keatings et al., 1996), BAL (Soler et al., 1999) and bronchial biopsies (Mueller et al., 1996) from stable COPD patients. The primary source is macrophages that have been stimulated via activation of toll-like receptors (TLR) (Chung, 2001). This results in the nuclear translocation of NF-κB and AP-1 with subsequent overexpression of TNF-α (Lucas and Maes, 2013). Neutrophils, T-cells, epithelial cells, fibroblasts and airway smooth muscle cells may also contribute (Mukhopadhyay et al., 2006).

TNF-α amplifies inflammation in COPD by potently activating NF-κB and AP-1 within neutrophils, epithelial cells and macrophages (Baldwin, 1996). This induces the expression of inflammatory genes with a broad range of effects that are likely to be important in the pathogenesis of COPD, including the upregulation of chemokines (e.g. CXCL8 and CCL2) (Matera et al., 2010), MMPs (Li et al., 2006), extracellular matrix glycoproteins (Harkonen et al., 1995), endothelial adhesion molecules (e.g. ICAM-1) (Mackay et al., 1993) and mucin (Levine et al., 1995, Lora et al., 2005).
Figure 1.4. Cells and inflammatory mediators involved in the pathophysiology of COPD. Stimuli, such as cigarette smoke, activate epithelial cells and leukocytes, which release various inflammatory mediators. This results in emphysema, obstructive bronchiolitis and chronic bronchitis. --- represents monocyte recruitment and differentiation.

1.2.2.3. Measuring inflammatory mediators in COPD

The accurate quantification of pulmonary inflammatory mediators can provide a better understanding of the pathogenesis of COPD and its response to intervention (Barnes et al., 2006). They may also act as biomarkers, with the potential to personalise the assessment and
treatment of this highly heterogeneous condition (Agusti et al., 2016). Traditionally, induced sputum and BAL have been used to sample the bronchial epithelial lining fluid (ELF) for inflammatory mediators. However, both techniques have limitations. Induced sputum can be contaminated with saliva and predominantly samples the proximal, rather than distal airways (Holz et al., 1998), which are the primary site of inflammation in COPD. Although BAL is more representative of lower airway inflammation, bronchoscopy is invasive and the recovery of lavage fluid (BALF) can be highly variable and occasionally very poor (Klech and Hutter, 1990). BAL also results in an unknown dilution factor with many mediators falling below the detection limits of subsequent analytical assays (Rennard et al., 1998, Kavuru et al., 1999). The detection of inflammatory mediators within exhaled breath condensates has also been trialled in COPD (Gessner et al., 2005). However, most cytokines, chemokines and enzymes cannot be quantified reliably (Effros et al., 2005). Novel sampling techniques are therefore required and should be urgently developed and considered for any investigation into the immune pathogenesis of COPD.

1.2.3. The role of oxidative stress

Oxidative stress occurs when ROS (free radicals) overwhelm available antioxidant defences (Kirkham and Barnes, 2013). Within the lung, ROS are either exogenous (acquired via inhalation) or endogenous (produced by cellular mitochondrial respiration) (Domej et al., 2014). In COPD, oxidative stress arises from the chronic inhalation of tobacco smoke (or polluted air), which contains many different oxidants and free radicals (> 10^{15} molecules per ‘puff’) (Church and Pryor, 1985). However, it is also supplemented by the overproduction of endogenous ROS from cells that are activated during inflammation, such as airway epithelial cells, neutrophils and macrophages (Ludwig and Hoidal, 1982, Rahman and MacNee, 1996). Specific examples include the superoxide anion, peroxynitrite, hydroxyl radical and
isoprostanes (Domej et al., 2014). This is further compounded by dysfunctional antioxidant mechanisms, which would otherwise counteract ROS, such as superoxide dismutase (SOD), glutathione and catalase (Domej et al., 2014). Oxidative stress plays an important role in the inflammatory pathogenesis of COPD. Of note, it activates NF-κB, inhibits the action of antiproteases and contributes to corticosteroid resistance by suppressing the activity of histone deacetylase 2 (HDAC2) (Rahman and Adcock, 2006, Kirkham and Barnes, 2013, Domej et al., 2014). The role of HDAC2 in corticosteroid resistance is detailed in Section 1.3.1.3.

1.2.4. The role of bacteria

1.2.4.1. Detecting airway bacteria

The detection of bacteria within the airways of COPD patients has conventionally required the routine microbiological culture of respiratory tract samples, such as induced sputum or BALF. Standard culture techniques however, are unable to reliably detect bacteria at concentrations of less than $10^2$ colony forming units/ml, nor accurately reproduce the lung habitat. Moreover, it is estimated that over 70% of bacterial species present on mucosal surfaces cannot be cultured using currently-available methods (Marsland and Gollwitzer, 2014). The advent of nucleic acid amplification tests (NAATs), including real-time quantitative polymerase chain reaction (qPCR), has enabled the more accurate detection and quantification of bacteria. In COPD, qPCR is highly sensitive and rapidly detects more bacterial species within respiratory samples than microbiological culture, with limited cross-reactivity (Curran et al., 2007).

1.2.4.2. Bacterial colonisation in COPD

Unless infected, the lower respiratory tract has traditionally been considered sterile. NAATs however, have revealed that even healthy lungs harbour a microbiota (Marsland et al., 2013, Marsland and Gollwitzer, 2014, Sze et al., 2014). It is likely that the upper airways continually supply the lungs with a variety of non-pathogenic and pathogenic bacterial species via
inhalation and/or micro-aspiration, but competent host defences prevent potentially pathogenic bacteria (PPB) from persisting (King et al., 2013).

COPD is associated with incompetent host defences that favour the survival of PPB within the lungs (Section 1.5). Indeed, a large study involving the routine microbiological culture of bronchoscopic samples from over 100 stable, non-exacerbating COPD patients, revealed that as many as 30% will harbour PPB in their lower respiratory tract at concentrations $\geq 10^2$ colony forming units/ml (Rosell et al., 2005). This was in stark contrast to a group of healthy controls ($n = 70$), which were matched for age, gender and smoking history, of whom only 4% were culture positive for PPB (also $\geq 10^2$ colony forming units/ml). The bacteria that were most commonly isolated in COPD were *H. influenzae* (17%), *S. pneumoniae* (9%) and *M. catarrhalis* (4%) (Rosell et al., 2005) and their presence within respiratory tract samples from stable patients is termed bacterial colonisation, rather than infection; the latter being associated with greater concentrations of PPB and more overt, acute deteriorations in respiratory symptoms. These findings have also been replicated in subsequent studies, with similar proportions of stable COPD patients reported to be colonised (Sethi et al., 2006).

More recently, studies incorporating NAATs have demonstrated that COPD may be associated with an increase in total bacterial load, less bacterial diversity and an outgrowth of certain species, including PPB (Hilty et al., 2010, Erb-Downward et al., 2011, Pragman et al., 2012, Sze et al., 2012). As such, it has been proposed that COPD may actually be accompanied by a more generalised change in the lung microbiota. This is termed bacterial dysbiosis and the findings from the above studies are summarised in Figure 1.5. However, all of these studies were small ($n \leq 10$) and the data should be interpreted with caution, as NAATs (e.g. qPCR) are unable to determine the viability of microorganisms, *i.e.* differentiate between live and dead bacteria.
Figure 1.5. The airway microbiome in health and COPD. The healthy lungs harbour a diverse and well-balanced microbiota. In COPD, there is a dysbiosis, with an increased bacterial load, less diversity and an outgrowth of certain phyla and species. This includes PPB, such as *H. influenzae*, *S. pneumoniae*, *M. catarrhalis*, *H. parainfluenzae* and *P. aeruginosa*. These bacterial species are more commonly cultured from COPD airways (i.e. are colonising) and perpetuate inflammation. Adapted from Marsland and Gollwitzer (2014).

Colonisation with PPB, and perhaps airway dysbiosis in general, contributes to the inflammatory pathogenesis of COPD. When compared to non-colonised patients, even those with low PPB loads have amplified markers of pulmonary inflammation, including airway neutrophilia, CXCL8, LTβ4, TNF-α, IL-1β, IL-6, myeloperoxidase (MPO), neutrophil elastase and active MMP-9 (Hill *et al.*, 2000, Banerjee *et al.*, 2004b, Sethi *et al.*, 2006, Marin *et al.*, 2010, Marin *et al.*, 2012, Singh *et al.*, 2014). The two most commonly colonising PPB are *H. influenzae* and *S. pneumoniae*. The mechanisms through which they promote inflammation are described in Sections 1.2.4.3 and 1.2.4.4. Furthermore, colonisation with PPB may promote disease progression. Accelerated declines in lung function (Wilkinson *et al.*, 2003), poorer health status (Banerjee *et al.*, 2004, Marin *et al.*, 2012) and more frequent exacerbations (Patel *et al.*, 2002) have all been observed in colonised patients.
1.2.4.3. *Haemophilus influenzae*

*Haemophilus influenzae* is a Gram-negative coccobacillus of the Pasteurellaceae family. Strains are categorised according to the presence (typeable) or absence of a tough polysaccharide capsule (non-typeable (NTHi)). It is the most commonly isolated PPB in COPD. Using standard bacterial culture, 17% of patients are colonised with *H. influenzae* (Rosell et al., 2005) and it is associated with 30% of all acute exacerbations (Sethi and Murphy, 2008).

*H. influenzae* is a potential inflammatory stimulus for immune cells residing within the lungs. As such, it may play an important role in the pathogenesis of COPD. Indeed, some studies have suggested that it is the most ‘pro-inflammatory’ of all the colonising PPB, with the greatest concentrations of inflammatory mediators identified in patients harbouring *H. influenzae* (Hill et al., 2000, Marin et al., 2012, Singh et al., 2014). As a Gram-negative bacterium, it contains lipopolysaccharide (LPS) within its cell wall, or in the case of NTHi, a related molecule called lipoooligosaccharide (LOS) (Alexander and Rietschel, 2001). LPS and LOS potently stimulate TLR expressed by macrophages and neutrophils, which results in cellular activation and the expression of pro-inflammatory genes via NF-κB (Pålsson-McDermott and O'Neill, 2004). Other outer membrane proteins, such as P2 and P6, are also recognised by TLRs and capable of NF-κB activation (Berenson et al., 2005). Moreover, *H. influenzae* induces mucus hypersecretion (Adler et al., 1986), inhibits ciliary function (Denny, 1974, Wilson et al., 1985) and promotes the release of IL-6, CXCL8 and TNF-α from airway epithelium (Khair et al., 1994).

1.2.4.4. *Streptococcus pneumoniae*

*Streptococcus pneumoniae* is a Gram-positive, α-hemolytic diplococcus, that is encapsulated in polysaccharides and belongs to the Streptococcaceae family. It is a commensal and resides asymptptomatically in the sinuses and nasal cavities of healthy carriers. In susceptible individuals however, it is capable of causing pulmonary infection, including pneumonia. After *H. infe...
influenzae, it is the second most commonly isolated PPB in COPD. Standard bacterial culture has estimated that at least 9% of patients are colonised (Rosell et al., 2005) and 15% of all acute exacerbations are associated with *S. pneumoniae* (Sethi and Murphy, 2008).

Like *H. influenzae*, *S. pneumoniae* may also provide an inflammatory stimulus for leukocytes and airway epithelial cells within the lungs of COPD patients. Although a Gram-positive bacterium and therefore without LPS, TLRs are still activated by cell wall components, such as peptidoglycan, lipoteichoic acid and pneumolysin. This results in NF-κB activation and an upregulation of CXCL8, TNF-α, IL-1β and IL-6 (Tomlinson et al., 2014). Furthermore, *S. pneumoniae* is capable of inducing mucus hypersecretion (Adler et al., 1986).

1.2.5. **Extrapulmonary inflammation in COPD**

1.2.5.1. **Systemic inflammation and sequelae**

Stable COPD is associated with systemic inflammation that is related to the severity of lung disease (Su et al., 2016, Gan et al., 2004). There are an increased number of circulating activated leukocytes (Noguera et al., 2001, Agusti et al., 2012), greater concentrations of acute phase proteins (Mannino et al., 2003) and upregulated levels of serum inflammatory mediators (Garcia-Rio et al., 2010). This includes CXCL8 (Spruit et al., 2003) and TNF-α (Di Francia et al., 1994), which have both been previously discussed (Sections 1.2.2.1 and 1.2.2.2). The origins of this systemic inflammatory response are unclear. Theories include ‘overspill’ from the lungs into the circulation (Sinden and Stockley, 2010), vascular endothelial injury and oxidative stress that arises from exposure to components of tobacco smoke (Agusti, 2007), hypoxia (Kent et al., 2011) and hyperinflation (Gatta et al., 2011).

There are many extrapulmonary manifestations of COPD, including skeletal muscle weakness, cachexia, endothelial dysfunction (e.g. hypertension, coronary heart disease and heart failure),
insulin resistance, osteoporosis and depression (Barnes and Celli, 2009). Some of these co-existing conditions may arise from risk factors that are shared with COPD, or result from specific treatments (e.g. corticosteroids). However, it is likely that systemic inflammation also contributes to their pathophysiology. For example, the elevated levels of serum CXCL8 and TNF-α have been implicated in the development of muscle weakness and cachexia (Di Francia et al., 1994, de Godoy et al., 1996, Spruit et al., 2003), while TNF-α has been shown to induce the apoptosis of skeletal muscle (Dirks and Leeuwenburgh, 2006).

1.2.5.2. Sinonasal inflammation and sequelae

It is not widely appreciated, but COPD is associated with inflammation of the upper respiratory tract, including the nasal cavity and sinuses (Hurst, 2010). Although this inflammatory response remains poorly characterised, studies have identified an increased number of inflammatory cells and mediators within sinonasal samples from COPD patients. For example, Vachier et al. (2004) found an increased number of neutrophils, macrophages and CD8+ lymphocytes within the nasal mucosa of COPD patients who were currently smoking (n = 14), relative to both healthy non-smoking controls (n = 7) and current smokers without COPD (n = 7), who were matched for cigarette smoke exposure. In accordance with these findings, Hurst et al. (2005) reported an approximate 2.7-fold increase in the concentrations of CXCL8 within the nasal wash of 35 ex-smoking COPD patients, when compared to 12 control subjects, some of whom were ex-smokers. This increased concentration of CXCL8 might be representative of a greater number of activated macrophages and may facilitate neutrophil recruitment. Elevated concentrations of eotaxin, GM-CSF and IFN-γ have also been reported in the nasal lavage fluid of COPD patients, relative to control subjects that were matched for age and cigarette smoking (Hens et al., 2008).
Although it is postulated that sinonasal inflammation in COPD results from direct exposure of the sinonasal epithelium to tobacco smoke (Hurst, 2010), the exact mechanism still remains unknown. It could equally be indirectly acquired from the lower airways, which are in close proximity. Moreover, many of the quoted studies included patients and/or controls with known sinonasal pathologies and/or infrequently reported on the incidence of such conditions. Regardless, this inflammatory process is still thought to play a pivotal role in the apparent increased incidence of sinonasal symptoms in COPD and associated upper airway pathology, as frequently reported by large cohort studies (Roberts et al., 2003, Hakansson et al., 2012).

1.3. Management of stable COPD

1.3.1. Pharmacotherapy

There are no disease-modifying medications that are capable of slowing the progression of COPD or improving survival (Gross and Barnes, 2017). Current pharmacotherapy can only prevent and/or alleviate symptoms. Certain drugs may also reduce the frequency of exacerbations, albeit modestly (Qureshi et al., 2014). Broadly, COPD medications are administered orally (Section 1.3.1.2) or via inhalers (Section 1.3.1.1). Special mention must also be made of corticosteroids, which can be administered via either route, but only benefit a small subset of stable patients and are potentially harmful (Section 1.3.1.3).

1.3.1.1. Inhaled treatments

Inhaled drugs belong to one on three classes; β₂-agonists, muscarinic antagonists (M₃) or corticosteroids (discussed in Section 1.3.1.3). Many inhalers now combine two classes of drug, and inhalers incorporating all three are in clinical development (Montuschi et al., 2016). β₂-agonists and muscarinic antagonists are bronchodilators. Stimulation of β₂-adrenergic receptors and antagonism of muscarinic acetylcholine receptors, independently result in the
relaxation of bronchial smooth muscle (Cazzola et al., 2012). Muscarinic antagonists may also act as anti-inflammatories by reducing neutrophil chemotaxis, cytokine expression, mucus gland hypertrophy and sputum production (Alagha et al., 2014).

β₂-agonists and muscarinic antagonists are either short-acting (SABA or SAMA) or long-acting (LABA or LAMA). Common examples include salbutamol or terbutaline (SABAs), ipratropium (SAMA), salmeterol, formoterol, indacaterol, vilanterol or olodaterol (LABAs) and tiotropium, glycopyrronium, umeclidinium or aclidinium (LAMAs). SABAs/SAMAs are prescribed to relieve symptoms, while LAMAs/LABAs are used to ‘prevent’ (or at least reduce) symptoms. LABAs and LAMAs can improve FEV₁, exacerbation rates and associated hospitalisation, but have no effect on the rate of lung function decline or mortality (Karner et al., 2012, Kew et al., 2013). Moreover, their effectiveness can be hindered by poor inhaler technique, patient compliance or adverse side-effects (Sulaiman et al., 2017).

1.3.1.2. Oral treatments

Currently available oral medication belong to one of the following classes: mucolytics (carbocisteine), antioxidants (N-acetylcysteine), methylxanthines (theophylline), selective phosphodiesterase-4 (PDE4) inhibitors (roflumilast) and macrolide antibiotics. The role of macrolide antibiotics is investigated within this thesis and therefore warrants separate discussion (Section 1.3.1.4). Continuous oral corticosteroids are also occasionally prescribed. However, current guidance advises against this approach, as there is no evidence of benefit and long-term use is associated with multiple side-effects (GOLD, 2017).

Carbocisteine is a mucoactive drug that reduces sputum viscosity (Braga et al., 1989), promotes mucociliary clearance (Braga et al., 1990) and scavenges ROS (Pinamonti et al., 2001). N-acetylcysteine (NAC) is anti-oxidant that contains a free thiol group, which is capable
of ‘de-toxifying’ ROS (Moldeus et al., 1986). Both carbocisteine and NAC reduce the frequency of COPD exacerbations (Cazzola et al., 2015, Poole et al., 2015).

The phosphodiesterases (PDE) are a series of enzymes that are important in cell signalling. Their inhibition causes a reduction in the degradation of secondary messengers, such as cAMP. Within airway smooth muscle, this ultimately results in bronchodilation (Rabe et al., 1995). PDE are widely expressed however, including within macrophages, neutrophils, lymphocytes, goblet cells and epithelial cells. As such, PDE inhibition also results in a variety of anti-inflammatory effects, including reductions in CXCL8, TNF-α, LTB₄, ROS and MMP-9 (Currie et al., 2008). Available PDE inhibitors are either non-selective (methylxanthines (theophylline)) or selective for PDE4 (roflumilast). The principle effect of theophylline is bronchodilation (Ram et al., 2002), while roflumilast is predominantly anti-inflammatory and has been shown to prevent exacerbations (Chong et al., 2013). Both theophylline and roflumilast however, are limited by a substantial side-effect profile and are often poorly tolerated or unsuitable for many COPD patients (Chong et al., 2013).

1.3.1.3. Corticosteroids and corticosteroid-resistance in COPD

Corticosteroids influence inflammatory gene expression. They upregulate a variety of anti-inflammatory proteins (‘transactivation’) while simultaneously downregulating proinflammatory genes (‘transrepression’). Short courses of oral corticosteroids (e.g. 40 mg of prednisolone for 5-7 days) play an important role in the management of acute exacerbations. They shorten recovery time and reduce the length of hospitalisation (Walters et al., 2014). However, despite stable COPD being associated with chronic inflammation, oral corticosteroids are ineffective as a maintenance treatment (Walters et al., 2005). Their long-term use can also result in a number of adverse effects. As such, they are not recommended for stable patients.
Inhaled corticosteroids (ICS), such as beclomethasone, budesonide, mometasone and fluticasone, are used in stable COPD. However, when administered alone, or more commonly in combination with either LABAs, LAMAs or both, they fail to slow the decline of FEV\(_1\) or improve survival (Nannini \textit{et al.}, 2007, Yang \textit{et al.}, 2007). Furthermore, ICS use is associated with a number of adverse effects (Barnes, 2010), including an increased risk of pneumonia (Drummond \textit{et al.}, 2008). As such, many advocate the withdrawal of ICS from patients who do not derive clinical benefit.

The ineffective nature of both ICS and oral prednisolone in stable COPD may, in part, be explained by an underlying resistance to the anti-inflammatory effects of corticosteroids (Barnes, 2010). Indeed, both treatments are unable to suppress many of the pulmonary inflammatory indices associated with COPD (Keatings \textit{et al.}, 1997). Such corticosteroid-resistance is thought to result from oxidative stress within macrophages and other leukocytes, which inhibits the activity of HDAC2, an important histone deacetylase that would otherwise mediate corticosteroid-induced transrepression (Ito \textit{et al.}, 2004, Ito \textit{et al.}, 2005, Ito \textit{et al.}, 2006). It is important to recognise however, that some COPD patients will benefit from ICS, including those with an eosinophilic inflammatory phenotype (Section 1.2.1.5) (Pascoe \textit{et al.}, 2015, Pavord \textit{et al.}, 2016).

\subsection*{1.3.1.4. Macrolide antibiotics}

Macrolide antibiotics are broad spectrum and bacteriostatic. They have a large macrocyclic lactone ring with either 14 (\textit{e.g.} erythromycin and clarithromycin) or 15 members (\textit{e.g.} azithromycin) (Figure 1.7). This inhibits protein synthesis by binding to the 50s subunit of the bacterial ribosome (Zuckerman, 2004). Macrolides also concentrate within leukocytes, which transport them to the site of infection (Rodvold \textit{et al.}, 1997). Short courses are therefore
commonly prescribed for various bacterial infections, including those associated with COPD exacerbations.

The long-term use of macrolides has also consistently demonstrated clinical benefits for stable COPD patients. Specifically, the addition of daily azithromycin (250 mg orally) to a standard treatment regimen has been shown to reduce the frequency of exacerbations and improve quality of life during a large randomised placebo-controlled trial involving over 1500 stable COPD patients and conducted over 1 year (Albert et al., 2011). These findings have been replicated in a number of other clinical trials. Indeed, in a meta-analysis of 6 such randomised placebo-controlled trials by Donath et al. (2013), macrolide therapy (either azithromycin, clarithromycin or erythromycin for at least 3 months) was associated with a 37% and 21% relative risk reductions in exacerbations and hospitalisation, respectively. Although this meta-analysis pooled over 700 patients into each treatment arm, these studies were clearly dissimilar, as different macrolides, different doses, dosing frequencies and treatment durations (3 to 12 months) were adopted. Moreover, no study explored the mechanism behind the reduction in exacerbations. Regardless, long-term macrolide therapy is still a recognised supplementary treatment for COPD (GOLD, 2017). In the UK, oral azithromycin is often prescribed for ‘stable’ patients who exacerbate frequently or display a particular ‘inflammatory clinical phenotype’ that is unresponsive to standard treatment. Typically, a dose of 250 mg thrice weekly is used.

Although clinical benefits from long-term macrolide treatment may result from airway sterilisation and/or changes in the lung microbiota (Murphy et al., 2010), macrolides are also capable of immunomodulation that is independent of their antibiotic action. This is best highlighted by the related non-antibiotic macrolides, tacrolimus and sirolimus. Both contain macrocyclic lactone rings (23 and 31-membered respectively) and are clinically-important
immunosuppressants that are used to prevent organ rejection following transplantation (Karam and Wali, 2015). Macrolide antibiotics themselves are also important immunomodulators. They successfully treat the inflammatory lung condition, diffuse panbronchiolitis (DPB), where they significantly improve symptoms, lung function and survival (Kudoh et al., 1998). These effects are independent of their antimicrobial properties, as they are achieved at doses lower than the minimum inhibitory concentrations (MIC), despite high rates of bacterial-resistance to macrolides and with bacteria persisting in the airways (Kanoh and Rubin, 2010). Instead, macrolides are thought to mediate effects through reductions in neutrophil chemotaxis (Oda et al., 1994), the downregulation of adhesion molecules (Kawasaki et al., 1998) and by inhibiting the expression of multiple inflammatory mediators (Oda et al., 1995, Sakito et al., 1996, Tamaoki et al., 2004). Numerous other effects on a variety of leukocytes and structural cells have also been reported in vitro and following oral administration (Kanoh and Rubin, 2010). It is possible that these actions contribute to a net anti-inflammatory effect in COPD. This is summarised by Figure 1.6 and discussed in more detail in subsequent chapters. Macrolides can downregulate inflammatory gene expression, through the inhibition of NF-κB and AP-1 (Desaki et al., 2000, Kikuchi et al., 2002, Cheung et al., 2010, Li et al., 2012, Kobayashi et al., 2013b). They may also augment bacterial phagocytosis by macrophages (Hodge and Reynolds, 2012). Both of these actions are explored by this thesis.
Figure 1.6. **Macrolide antibiotics and immunomodulation.** Macrolides are immunomodulators, with multiple effects on macrophages, neutrophils, epithelial cells, lymphocytes and fibroblasts. The net effect is anti-inflammatory and this may benefit COPD patients clinically. This figure has been constructed after a review of the literature, including *in vitro* experiments, animal studies and human trials involving patients with chronic inflammatory lung conditions.

### 1.3.1.5. Ketolides and fluoroketolides

Commercially-available macrolide antibiotics bind to a single bacterial ribosomal site (‘P-site’). As such, macrolide-resistance is generated with relative ease through methylation or mutation of this solitary target (Leclercq and Courvalin, 2002). It is therefore unsurprising that chronic macrolide use is associated with notably high rates of microbial resistance (Serisier, 2013, Li et
and it is frequently argued that their long-term use is best avoided in COPD (Crosbie and Woodhead, 2013).

Ketolides are semi-synthetic erythromycin derivatives (Figure 1.7). They include the drugs telithromycin and cethromycin, which are both capable of binding to at least 2 ribosomal sites (Hansen et al., 1999). As such, bacterial resistance to ketolides would require more extensive ribosomal modification and might therefore be expected to occur less frequently. Solithromycin is a novel ketolide. It differs structurally from telithromycin in that it contains a fluorine group within the macrolactone ring, i.e. it is a fluoroketolide (Figure 1.7) (Fernandes et al., 2016). Solithromycin binds to at least three ribosomal sites (Llano-Sotelo et al., 2010) and therefore has an even lower potential for microbial resistance than macrolides and other ketolides (McGhee et al., 2010). Moreover, ketolides and fluoroketolides still possess immunomodulatory/anti-inflammatory properties that are similar to earlier generations of macrolide (Kobayashi et al., 2013b, Kobayashi et al., 2013a). Although there is a potential role for solithromycin in COPD, this is yet to be explored in any detail and is a particular focus of this thesis (Chapter 5 and Chapter 7).

1.3.2. Non-pharmacological interventions

Non-pharmacological interventions are also available for stable COPD patients. These include smoking cessation, vaccination against pneumococcus and influenza, pulmonary rehabilitation (PR), long-term oxygen therapy (LTOT) for hypoxaemic patients (PaO2 ≤ 7.3 KPa) (Cranston et al., 2005), lung volume reduction (LVR) via surgery (Tiong et al., 2006) or endobronchial valves (Kemp and Shah, 2016), domiciliary non-invasive ventilation (NIV) for chronic hypercapnia (Murphy et al., 2017) and lung transplantation.
Figure 1.7. The chemical structures of macrolide antibiotics. Macrolide antibiotics have macyclic lactone rings. Erythromycin is a first-generation macrolide. Clarithromycin and azithromycin are second generation macrolides. Telithromycin is a ketolide. Solithromycin is a fluoroketolide. Azithromycin is the only compound with a 15-membered macrocyclic ring. Erythromycin, clarithromycin, telithromycin and solithromycin all contain 14 members.
1.4. Exacerbations of COPD

1.4.1. Defining an exacerbation

An exacerbation of COPD is described by GOLD (2015) as ‘an acute event characterised by a worsening of respiratory symptoms that is beyond normal day-to-day variations and leads to a change in medication’. They can also be defined as ‘a deterioration in symptoms, sustained for at least two consecutive days, with worsening in two or more of three major symptoms (increased dyspnoea, sputum purulence or sputum volume), or any one major symptom together with one minor symptom (increased cough, wheeze, fever, nasal discharge or sore throat)’ (Seemungal et al., 1998). These criteria have been used to define acute exacerbations in this thesis (Section 2.2.4).

Exacerbations can range in severity. They may be ‘mild’ (treated with short-acting bronchodilators only), ‘moderate’ (treated with short-acting bronchodilators and antibiotics, and/or corticosteroids) or ‘severe’ (requiring a hospital attendance or admission) (GOLD, 2017). Severe exacerbations can be life-threatening and result in respiratory failure, requiring ventilatory support.

In the UK, exacerbations are the second commonest cause of emergency hospital admission, with each episode costing the NHS £1500 on average (CHAI, 2006). They are also associated with significant morbidity and mortality. Approximately 8% of patients admitted to hospital with an exacerbation will die during that admission, while a further 25% will succumb during the following year (Alrawi et al., 2010). As many as 14% of patients will remain symptomatic a month after exacerbation onset, whilst symptoms may be even more protracted and/or never return to baseline in a small proportion of patients (4%) (Seemungal et al., 2000).
1.4.2. Causes of exacerbations

There are several triggers for an acute exacerbation. Each may precipitate an exacerbation individually, or in combination. They include viruses (most commonly rhinovirus, influenza, parainfluenza, coronavirus, adenovirus and respiratory syncytial virus), bacteria (most commonly *H. influenzae, S. pneumoniae, M. catarrhalis* and *P. aeruginosa*) and environmental pollutants, such as nitrogen oxides and particulate matter (Wedzicha and Seemungal, 2007, Sethi and Murphy, 2008). Gastroesophageal reflux and micro-aspiration may also act as triggers in some patients (Rascon-Aguilar et al., 2006, Terada et al., 2008).

Most COPD exacerbations (approximately 75-80%) are ‘infective’ in origin and therefore initiated by, or associated with, the presence of pulmonary pathogens (Sethi and Murphy, 2008). Bacterial infection accounts for approximately 50% of all exacerbations (Sethi and Murphy, 2008). This can result from the acquisition of a new bacterial strain (Sethi et al., 2012) or arise from the growth of a pre-existing PPB (Patel et al., 2002, Sethi et al., 2007). Indeed, similar PPBs are isolated during clinical stability (colonisation) and acute exacerbations, of which *H. influenzae* and *S. pneumoniae* are the two most common (Monso et al., 1995, Rosell et al., 2005). Bacterial and viral co-infection also occurs, with the latter predisposing to the former. The exact reason is unclear. However, viral infection may impair macrophage phagocytosis (Oliver et al., 2008, Finney et al., 2016) and upregulate the expression of intracellular adhesion molecule (ICAM)-1, thereby promoting the adherence and invasion of epithelial cells by bacteria (Avadhanula et al., 2006).

1.4.3. Exacerbations and inflammation

Regardless of the underlying aetiology, pulmonary inflammation is significantly amplified during COPD exacerbations, (Sapey and Stockley, 2006). Elevated numbers of leukocytes (most
notably neutrophils), increased concentrations of CXCL8, TNF-α, LTB₄, neutrophil elastase and MPO, as well as enhanced MMP activity, have all been observed (Crooks et al., 2000, Aaron et al., 2001, Gompertz et al., 2001, Hurst et al., 2006a, Papakonstantinou et al., 2015). Markers of oxidative stress (e.g. isoprostanes) may also rise during an exacerbation (Biernacki et al., 2003). Acutely, this pulmonary inflammatory response causes airway oedema, bronchospasm, increased sputum production and mucous plugging, which result in a temporary deterioration in airflow obstruction and dynamic hyperinflation (Wedzicha and Seemungal, 2007). However, regular exacerbations may also promote chronic pulmonary inflammation and COPD progression. Indeed, those patients who exacerbate most frequently may have a worse prognosis (Section 1.4.4).

Systemic and sinonasal inflammation are also upregulated during acute exacerbations. Serum concentrations of IL-6 and the acute-phase proteins, C-reactive protein (CRP) and fibrinogen, are all elevated during exacerbations (Wedzicha et al., 2000, Hurst et al., 2005). There are also increased leukocyte counts and greater concentrations of CXCL8, IL-6 and MPO within the nasal lavage fluid of exacerbating patients (Hurst et al., 2005, Mallia et al., 2006).

1.4.4. **Frequent Exacerbators**

Most COPD patients will exacerbate infrequently. However, there is a subgroup who will experience ≥ 2 exacerbations per year. These patients are termed ‘frequent exacerbators’ and belong to the ‘frequent exacerbator phenotype’ (Wedzicha et al., 2013). Several studies have reported that such patients have amplified pulmonary inflammation and a poorer prognosis, relative to infrequent exacerbators. Indeed, higher concentrations of both IL-6 and CXCL8 (approximately five-fold) have been identified in stable frequent exacerbators (n = 23) when compared to otherwise-matched infrequent exacerbators (n = 21) (Bhowmik et al., 2000). Large prospective cohort studies, that have followed up hundreds of COPD patients over
several years, have also revealed that frequent exacerbators have faster declines in FEV₁ (Donaldson et al., 2002), more frequent hospitalisation (Garcia-Aymerich et al., 2001), increased mortality (Soler-Cataluna et al., 2005) and a worse quality of life (Seemungal et al., 1998), including an increased likelihood of becoming housebound (Donaldson et al., 2005). It is therefore of paramount importance to identify these patients and target them with specific therapies capable of preventing exacerbations.

1.5. Pulmonary host defences in health and COPD

The lungs are continually exposed to microorganisms, including PPB. They have therefore evolved a multifaceted defence, designed to prevent infection by eliminating and clearing pathogens. These pulmonary defences include physical barriers and involve cells of the innate and adaptive immune responses. Each is discussed below. These host defences are defective in COPD and this is a likely mechanism through which PPB colonise the airways (Section 1.2.4.2).

1.5.1. Barrier immunity

Sneezing and coughing expel inhaled particles and microorganisms from the respiratory tract. This supplemented by the mucociliary apparatus, which comprises mucus and cilia that trap and continually ‘sweep’ any inhaled matter or microbes towards the pharynx and eventual clearance via the digestive tract (Knowles and Boucher, 2002). The ELF and alveoli also contain a number of soluble constituents, which are released by epithelial cells and leukocytes. These eliminate or neutralise bacteria and their products. They include lysozyme, defensins and cathelicidins (bind and disrupt bacterial membranes), lactoferrin (chelate iron and inhibit bacterial metabolism), colectins (e.g. mannose-binding or surfactant proteins) and complement (Chaudhuri and Sabroe, 2008). Bacterial invasion is also prevented by the
formation of cellular junctional complexes, which maintain epithelial integrity and limit paracellular permeability (Ganesan et al., 2013).

COPD is associated with defects in barrier immunity. Mucociliary transport is impaired by mucous hypersecretion, an increase in mucous viscosity (Ramos et al., 2014), fewer cilia (Tamashiro et al., 2009), cilia shortening (Leopold et al., 2009) and reduced ciliary beating (Yaghi et al., 2012). There are also fewer epithelial secretory cells (Lumsden et al., 1984) with an overall lower concentration of bactericidal constituents within ELF (Parameswaran et al., 2011). Furthermore, epithelial integrity is compromised by the disruption of cell junctions (Schamberger et al., 2015).

1.5.2. Adaptive immunity

Adaptive immunity is characterised by a highly antigen-specific response that is delayed (*i.e.* after hours or days) but provides ‘immunological memory’ with long-lasting protection, such that subsequent encounters with the same pathogen will provoke more rapid and enhanced responses (Dempsey et al., 2003). It includes both humoral (immunoglobulins produced by B lymphocytes) and cell-mediated components (T lymphocytes). Both B and T lymphocytes are derived from the same multipotent hematopoietic stem cells, with T cell progenitors migrating from the bone marrow to the thymus prior to differentiation.

In health, adaptive immunity distinguishes foreign antigens from ‘self’ (Dempsey et al., 2003). Upon exposure to foreign antigens, B cells are activated, proliferate and differentiate into plasma cells, whilst releasing immunoglobulins (IgG, IgM, IgA and IgE) to neutralise, agglutinate, precipitate, opsonise or identify pathogens for destruction via complement or phagocytosis. T cells differentiate into either T helper cells (CD4+ or regulator cells) or cytotoxic T cells (CD8+ lymphocytes). T helper cells release cytokines which regulate immunity, while
cytotoxic lymphocytes eliminate infected cells by releasing cytotoxins and/or inducing apoptosis. Following an infection, a proportion of B and T lymphocytes will persist as memory cells. A more detailed description of adaptive immunity is beyond the scope of this thesis.

Defects in adaptive immunity have been reported in COPD. These could play a role in permitting colonisation and frequent pulmonary infection. Abe et al. (2002) observed a diminished proliferation of lymphocytes in response to NTHi antigens. This defect was also associated with an increased occurrence of NHTi-induced exacerbations (Abe et al., 2002). T helper cell responses to H. influenzae are also blunted in COPD (Knobloch et al., 2011) and there are reports of lower IgA levels (Polosukhin et al., 2011), including H. influenzae-specific IgA (Murphy et al., 2011, Millares et al., 2012), of which the lowest concentrations were found in COPD patients colonised with H. influenzae (Millares et al., 2012).

1.5.3. **Innate immunity**

Innate immune responses are immediate and generic (*i.e.* not pathogen-specific). They are therefore fundamental in providing instant protection against any newly-invading pathogen. Within the lungs, innate immunity broadly involves the activity of macrophages, neutrophils, natural killer cells, dendritic cells and complement (Dempsey et al., 2003, Martin and Frevert, 2005, Shaykhiev and Crystal, 2013). Phagocytosis by macrophages and neutrophils is the primary means by which pathogens are cleared. Whereas macrophages are resident to the lung, neutrophils must first be recruited by chemoattractants (Section 1.2.1.2). Both of these leukocytes are also capable of a ‘respiratory burst’, which releases oxidising agents that destroy microbes (Shaykhiev and Crystal, 2013). This is supplemented by complement, which opsonises pathogens (*i.e.* marks them for phagocytosis) and directly disturbs bacterial cell walls through the formation of a membrane attack complex. The role of natural killer cells is analogous to that of cytotoxic T lymphocytes; they target infected cells in an antigen non-
specific manner by releasing of cytotoxins (Shaykhiev and Crystal, 2013). Dendritic cells specialise in antigen presentation. They extend cytoplasmic projects to sample luminal antigens before migrating to local lymphoid tissue and presenting processed antigens to lymphocytes (Van Pottelberge et al., 2009). They are therefore an important link between innate and adaptive immunity.

Innate immunity is defective in COPD. Neutrophil chemotaxis is less accurate (Yoshikawa et al., 2007, Sapey et al., 2011), while phagocytosis by the same cells is attenuated (Fietta et al., 1988, Stringer et al., 2007). Cigarette smoke also impairs innate immune responses, such as the neutrophil oxidative burst (Zappacosta et al., 2005), the cytotoxic activity of natural killer cells (Mian et al., 2008) or antigen-presenting (Robbins et al., 2004) and efferocytosis by dendritic cells (Nouri-Shirazi and Guinet, 2003). Significantly fewer dendritic cells are also found in the bronchial epithelium of COPD patients who smoke and these numbers normalise upon smoking cessation (Rogers et al., 2008). Moreover, the dendritic cell population is less mature in COPD (Van Pottelberge et al., 2010).

Macrophages are a key component of pulmonary innate immunity. They are professional phagocytes and clear invading microorganisms (Murray and Wynn, 2011). Their numbers are increased in COPD (Barnes, 2004b), yet they are unable to prevent colonisation with PPB or COPD exacerbations, which are frequently triggered by bacteria. One explanation might be that macrophage phagocytosis is defective in COPD (Taylor et al., 2010). This is a specific focus of this thesis and will now be discussed (Section 1.6).

1.6. Pulmonary macrophages

Pulmonary macrophages primarily originate from circulating bone-marrow derived monocyes, which upon activation, have migrated into the lungs and differentiated (Gordon et
al., 1992, Wynn et al., 2013). They are heterogeneous with at least two subtypes identified in human lungs; alveolar macrophages (AM) and interstitial macrophages (IM) (Bharat et al., 2016). AM are immediately exposed to inhaled pathogens and widely accepted to principally function as phagocytes, while IM are more capable of antigen presentation and inflammatory regulation (Cai et al., 2014). AM constitute over 90% of the pulmonary macrophage population (van oud Alblas and van Furth, 1979) and are the focus of this thesis.

1.6.1. Alveolar macrophages

AM account for approximately 90-95% of all airway leukocytes (Harris et al., 1970). They are long-lived and can survive within the alveolus for several months or years (Thomas et al., 1976). They are the principle phagocyte, and constantly survey their environment for any inhaled pathogens or particulate matter (Martin and Frevert, 2005). They can also initiate and maintain inflammation by releasing a variety of inflammatory mediators (Section 1.2.2) and orchestrate inflammatory resolution by clearing apoptotic and necrotic cells via efferocytosis, thereby preventing secondary necrosis (Martin et al., 2014, Hochreiter-Hufford and Ravichandran, 2013).

1.6.1.1. Alveolar macrophages phenotype

Macrophages, AM included, are highly heterogeneous, with different characteristics being observed among cells from the same tissue (Sica and Mantovani, 2012). Traditionally, such phenotypic variety has been described as either M1 (classically-activated) or M2 (alternatively-activated) macrophages (Murray and Wynn, 2011). While M1 macrophages are thought to enhance the pro-inflammatory environment through the release of TNF-α, CXCL8, MMPs and ROS, M2 macrophages play a role in inflammatory resolution, through enhanced phagocytosis and the release of IL-10 (Murray and Wynn, 2011). However, it is now recognised that such phenotypic variability is not simply confined to a two-cell model, and M1/M2 macrophages in
fact represent the polarised end of a spectrum containing numerous subtypes, each with a distinct mode of induction and cellular function, e.g. M1, M2a (wound healing), M2b (tumour-associated), M2c (regulatory), M4 (atherogenic) and Mhem (Atheroprotective) (Mosser and Edwards, 2008). Macrophages are also considered to be highly plastic, i.e. readily capable of switching phenotype according to the requirement of the tissue (Porcheray et al., 2005, Galli et al., 2011).

To date, only two studies have specifically examined the phenotype of AM isolated from the BALF of healthy human lungs, and they disagree on the markers best placed to define the M1 and M2 phenotypes (Shaykhiev et al., 2009, Pechkovsky et al., 2010). As such, there is currently no general consensus over which AM phenotype predominates in health, and further research is certainly warranted.

1.6.1.2. Alveolar macrophages in COPD

The literature strongly implies that AM are important in the pathogenesis of COPD. Not only is the AM population substantially expanded, with an approximate 15-fold increase in macrophages found within the alveolar space of histological specimens from COPD patients (Retamales et al., 2001), but AM numbers have also been shown to correlate with the degree of emphysema on CT imaging (Retamales et al., 2001) and the severity of airflow obstruction (FEV1 % predicted) (Di Stefano et al., 1998). AM are activated in COPD via exposure to a variety of different stimuli (Section 1.2.1.1). This is evidenced by increased concentrations of AM-derived proteins within airway samples (i.e. BALF and sputum). These include CXCL8 (Nocker et al., 1996), TNF-α (Soler et al., 1999) and MMPs (Finlay et al., 1997), which are all considered to be important in the condition’s pathogenesis (Table 1.2). Moreover, bacterial phagocytosis (Taylor et al., 2010) and efferocytosis (Hodge et al., 2003) are defective in AM isolated from BALF. The former is pertinent to this thesis and its implications are discussed in Section 1.6.4.
It is currently unknown whether pro-inflammatory AM responses arise solely from continued cellular activation in COPD, or whether there is a fundamental change in AM phenotype within the alveoli of patients (*e.g.* a predominance of M1- / M2-like AM or a COPD-specific phenotype). It has also been proposed that AM might lack plasticity in COPD and are therefore unable to switch from a pro- to anti-inflammatory phenotype once the inflammatory stimulus is removed (Chana *et al.*, 2012), thereby perpetuating inflammation.

1.6.1.3. **Monocyte-derived macrophages (alveolar macrophage model)**

Studying AM function requires the isolation of cells from respiratory tract samples, such as BALF or induced sputum. These sampling techniques do however have several inherent limitations, as discussed elsewhere in this thesis (see **Section 1.2.2.3** and **Chapter 3**). For example, bronchoscopy is invasive and can result in complications (Peacock *et al.*, 1994, Du Rand *et al.*, 2013), while induced sputum may contain higher proportions of neutrophils and fewer macrophages (Maestrelli *et al.*, 1995, Holz *et al.*, 1998).

Such limitations have led to the development of surrogates. The most common is an *in vitro* model, which uses monocytes isolated from peripheral blood to generate monocyte-derived macrophages (MDM). The process mimics monocyte differentiation *in vivo*, by exposing cells in culture to colony-stimulating factors (CSF) such as granulocyte macrophage-CSF (GM-CSF) or macrophage-CSF (M-CSF) (Burgess and Metcalf, 1980). Alternative cytokines do not generate MDM, but in the case of IL-4 may promote differentiation into monocyte-derived dendritic cells (Xia and Kao, 2002). Human monocytes cultured in the presence of GM-CSF will differentiate into a highly pure MDM monolayer (**Figure 1.8**) with a consistent and stable phenotype that shares several morphological and functional similarities with AM (Akagawa *et al.*, 2006, Winkler *et al.*, 2008). This includes the internalisation of particles and bacteria by phagocytosis (Taylor *et al.*, 2010). The MDM model is reviewed further in **Chapter 3**.
**Figure 1.8. Light microscopy of MDM and AM:** (A) Monocytes (B) Monocytes and MDM after culture in GM-CSF for 6 days (C) MDM monolayer after 12 days of culture in GM-CSF (D) Alveolar macrophages isolated from BALF. Monocytes were isolated from the peripheral blood of a non-smoker and cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM. AM were isolated from the BALF of the same non-smoking volunteer. Cells were viewed on an inverted light microscope (x 40 objective lens). Scale bar = 50 μm.

### 1.6.2. Macrophage Phagocytosis

Phagocytosis is a complex series of events, through which particles or pathogens larger than 0.5 μM in diameter are engulfed (Aderem and Underhill, 1999). Many cells are capable of phagocytosis but AM and neutrophils are professional airway phagocytes, tasked with bacterial clearance (Rabinovitch, 1995). The process is initiated through particle recognition by cell surface receptors, with or without prior opsonisation. This in turn generates signals for the rearrangement of the actin cytoskeleton to enfold the prey within actin-rich membrane extensions. These extensions pull the particle or pathogen into the cell and form the phagosome, which matures, acidifies and fuses with lysosomes to create a phagolysome,
within which prey are destroyed by exposure to ROS and degradative enzymes (Figure 1.9) (Aderem and Underhill, 1999).

1.6.2.1. Receptors and particle recognition

One of the complexities of phagocytosis is the diversity and interaction of receptors. Particles are frequently recognised by more than one receptor, and these may have dual functions (e.g. adhesion and internalisation) and/or be capable of cross-talk or synergy (Aderem and Underhill, 1999). Pathogens are distinguished from host cells by phagocytic receptors that recognise conserved motifs not found in higher eukaryotes. These targets are called ‘pathogen-associated molecular patterns’ (PAMPs) and the receptors termed ‘pattern-recognition receptors’ (PRRs) (Janeway, 1992). PAMPs include mannans in yeast cell walls, formylated peptides in bacteria, LPS on Gram-negative bacteria and lipoteichoic acid (LTA) on Gram-positive bacteria. Recognition may also require prior opsonisation with components such as mannose binding protein (binds mannans), surfactant protein A (binds carbohydrates), complement or immunoglobulins (Aderem, 2003). Receptors specifically requiring initial opsonisation include the C1q receptor (mannose binding protein), surfactant-associated protein A receptor 210, complement receptors (CRs) and a family of Fc receptors (FcR) (Section 1.6.2.2) (Aderem, 2003).

Inhaled particles and pathogens are far less likely to be opsonised due to the relative lack of serum in the lung. As such, their internalisation is predominantly mediated by receptors involved in the direct recognition of non-opsonised PAMPs. These include CD206 (mannose receptor), CD14, TLRs and a host of scavenger receptors (e.g. SR-A, CD36, CD163 and macrophage receptor with collagenous structure (MARCO)) (Aderem and Underhill, 1999). Scavenger receptors (SR) mediate non-opsonised phagocytosis by macrophages. They also act as co-receptors for TLR, resulting in pro-inflammatory cytokine responses to various PAMPs.
MARCO is the dominant SR expressed by human AM and is largely responsible for the phagocytosis of non-opsonised inert particles, Gram-positive and Gram-negative bacteria (Palecanda et al., 1999, Arredouani et al., 2004, Arredouani et al., 2005). Other SR are expressed however, and these possess overlapping ligand specificity. It is therefore likely that some inhaled particles will be internalised by simultaneously binding multiple alternative SR (Sulahian et al., 2008). The phagocytic prey used in this PhD are non-opsonised, in order to mimic the predominant pathogen-AM interaction within the lung (i.e. phagocytosis mediated by SR, primarily MARCO).

1.6.2.2. Internalisation by FcR and complement receptors

Current knowledge of macrophage phagocytosis is largely derived from studying FcR and CR-mediated phagocytosis. As such, both will be briefly discussed. The Fc-R are a family of receptors capable of binding to the generic Fc domain of IgG (Fcγ-R), IgA (Fcα-R) or IgE (Fcε-R). Macrophages express high levels of Fcγ-R, including Fcγ-RI (CD64), Fcγ-RII (CD32) and Fcγ-RIII (CD16) (Guilliams et al., 2014). These receptors have cytoplasmic tails containing immunoreceptor tyrosine activation motifs (ITAMs). Binding to IgG results in Fc-R cross-linking and tyrosine phosphorylation of ITAMs. This triggers particle internalisation and phagosome formation by activating downstream effectors such as phosphoinositide (PI)3-kinase, the rho family of GTPases, protein kinase (PK) C and motor proteins (myosins) (Aderem and Underhill, 1999).

There are several differences between the internalisation of prey by Fc-R and CR. During FcR-mediated phagocytosis, macrophages extend pseudopods to reach around prey, before fusing and drawing the particle into the cell (Allen and Aderem, 1996). In contrast, prey sink into the cell during CR-mediated phagocytosis, with minimal, if any, pseudopodia formation (Allen and Aderem, 1996). Although PKC plays a role in the internalisation of prey by both Fc-R and CR,
the inhibition of tyrosine kinase only blocks FcR-mediated phagocytosis (Allen and Aderem, 1996). Furthermore, only FcR-mediated phagocytosis stimulates the release of pro-inflammatory molecules, such as ROS (Wright and Silverstein, 1983, Aderem et al., 1985). Finally, and of particular relevance to this thesis, inhibiting microtubules affects the uptake of prey that have been opsonised by complement, but not IgG (Section 1.7) (Newman et al., 1991).

1.6.2.3. Internalisation by scavenger receptors

As discussed in Section 1.6.2.1, SR mediate the phagocytosis of non-opsonised pathogens by macrophages. They are PRRs and recognise PAMPs such as bacterial LPS and LTA (Areschoug and Gordon, 2009). They also act as co-receptors for TLRs, thereby stimulating pro-inflammatory responses (Peiser and Gordon, 2001). SR are classified according to their structure into 10 different classes (A-J) (Zani et al., 2015). MARCO belongs to class A and is the most highly expressed on human AM. As detailed above, it is the primary receptor for the phagocytosis of unopsonised particles and bacteria (Palecanda et al., 1999, Arredouani et al., 2004, Arredouani et al., 2005). Other SR from classes A (SR-AI and SR-AII), B (CD36 and SR-BI), E (LOX-1), F (SREC-1), G (PSOX) and H (FEEL1 and FEEL2) are also capable of mediating bacterial uptake (Areschoug and Gordon, 2009). SR downstream signalling is similar to that of FcR or CR. Indeed, the inhibition of tyrosine kinase, PKC and PI-3 kinase, all reduce the internalisation of unopsonised prey (Sulahian et al., 2008). Furthermore, in a similar manner to CR-mediated phagocytosis, SR require an intact microtubule network to facilitate the uptake of prey (Newman et al., 1991, Allen and Aderem, 1996, Sulahian et al., 2008).
1.6.3. Phagocytosis assays

Phagocytosis by AM can be quantified using labelled prey and fluorometry (Taylor et al., 2010, Berenson et al., 2013) or flow cytometry (Hodge and Reynolds, 2012). Similar assays have also been performed using MDM (Taylor et al., 2010, Hodge and Reynolds, 2012). Previous work from the laboratory has demonstrated that particles and bacteria are internalised by MDM during fluorometric assays and electron micrographs have demonstrated that bacterial uptake occurs via phagocytosis (Figure 1.10) (Taylor, 2009, Taylor et al., 2010, Thomas, 2012). These conditions and assays have been optimised for both AM and MDM, with respect to fluorescent-labelling of bacteria, macrophage numbers, concentrations of prey and incubation times (Taylor, 2009, Thomas, 2012). These assay conditions are used in this thesis (Section 2.2.11).
Figure 1.10. Electron and confocal microscopy of MDM phagocytosis (*H. influenzae*). Electron microscopy is used to visualise *H. influenzae* phagocytosis by MDM, including phagocytic cup formation (A), encapsulation by pseudopodia (B) and transportation through the cell cytoplasm within phagosomes (C). (D) Confocal microscopy demonstrates that fluorescently-labelled *H. influenzae* are internalised by MDM (representative orthogonal Z-stack views). Adapted from Taylor *et al.* (2010).

### 1.6.4. Macrophage phagocytosis in COPD

Several studies have demonstrated that phagocytosis by AM is defective in COPD. This includes the phagocytosis of PPB, such as *H. influenzae* (Berenson *et al.*, 2006, Marti-Lliteras *et al.*, 2009), *S. pneumoniae* (Taylor *et al.*, 2010) and *M. catarrhalis* (Berenson *et al.*, 2013), as well as pathogenically less relevant *E. coli* (Taylor *et al.*, 2010, Hodge and Reynolds, 2012) and fungi such as *A. fumigatus* (Belchamber *et al.*, 2016) and *C. albicans* (Vecchiarelli *et al.*, 1991, Ferrara *et al.*, 1996). In all of the studies investigating bacterial phagocytosis, AM were isolated from BALF and at least 15 COPD patients were recruited (range n = 15 – 64), with an approximate 50% reduction in bacterial phagocytosis. The patients typically had mild to moderate airflow
obstruction and were either ex-smokers or matched for smoking status (and/or pack years) with the control group(s). Moreover, defective bacterial phagocytosis is replicated in MDM (Taylor et al., 2010, Hodge and Reynolds, 2012) and appears to be independent of the adopted experimental methodology, i.e. observed with fluorometry and adherent cells (Taylor et al., 2010, Berenson et al., 2013) or with flow cytometry and non-adherent macrophages (Hodge and Reynolds, 2012).

Defective bacterial phagocytosis may be an important mechanism through which PPB are able to colonise COPD airways and perpetuate inflammation (Section 1.2.4.2). Indeed, correlations have previously been described between bacterial phagocytosis by AM (H. influenzae and M. catarrhalis) and markers of COPD severity (FEV₁ % predicted) (Berenson et al., 2013). It is therefore possible that targeting this defect pharmacologically might modify the pathophysiology of COPD. However, the mechanism(s) underpinning defective bacterial phagocytosis are unknown and must first be identified. The defect(s) is most likely to lie downstream of receptor expression. In a study by Taylor et al. (2010), MDM from COPD patients (n = 16) internalised less E. coli, H. influenzae and S. pneumoniae than both smokers (n = 13) and non-smokers (n = 13), despite expressing equivalent levels of TLR2, TLR4, CD163, CD36, CD14, CD206 and the dominant scavenger receptor, MARCO, all of which were quantified by flow cytometry. Microtubules also play an important role in AM phagocytosis, yet their function is yet to be investigated in COPD. This is a focus of this PhD (Chapter 4).

1.7. Microtubules

Microtubules are filamentous organelles, which facilitate a variety of cellular functions, including phagocytosis. They also maintain overall cell structure, promote migration (e.g. chemotaxis), cilia beating, intracellular transport, mitosis and meiosis (Nogales, 2000). They
are essential for the uptake of non-opsonised prey by AM, which is primarily mediated by SR (Newman et al., 1991, Allen and Aderem, 1996, Sulahian et al., 2008). Indeed, microtubules are involved in conformational changes to phagocytosis receptors, receptor mobility or clustering, particle internalisation, membrane trafficking and phagosome / lysosome formation and migration (Niedergang and Chavrier, 2004, Harrison and Grinstein, 2002, Swanson et al., 1987).

1.7.1. Microtubule structure and dynamics

Microtubules are formed from the polymerisation of soluble α- and β-tubulin heterodimers, that are assembled into filaments of 25 nm in diameter, arranged around a hollow core (Walczak, 2000). The resulting polymer is dynamic and exists in a state of ‘dynamic instability’, i.e. continually polymerising or depolymerising according to cellular requirements (Nogales, 2000). Microtubules are inherently polar structures with fast-growing (plus) and slow-growing (minus) ends; the latter embedded in the juxtanuclear microtubule organising centre (MTOC) (Joshi, 1998). They also associate with a number of microtubule-associated proteins (MAPs) (Hirokawa, 1994), which regulate microtubule dynamics and function. These include motor proteins called kinesins and dyneins, which use energy from the hydrolysis of ATP to transport cargo towards the plus and minus ends of the microtubule, respectively (Nogales, 2000). Cargo include vesicles, organelles and proteins, as well as internalised bacteria contained within phagosomes or phagolysosomes (Rai et al., 2016).

Polymerisation requires α- and β-tubulin to be added to the growing end of a microtubule. Both are bound to guanosine triphosphate (GTP). This forms a GTP-tubulin cap at the tip of the filament, which prevents the polymer from unravelling and enables continued growth by allowing the addition of further tubulin subunits (Walczak, 2000). Tubulin however, possess intrinsic GTPase activity and therefore hydrolyses GTP to guanosine diphosphate (GDP)
(Hyman et al., 1992, Nogales et al., 1998, Roychowdhury et al., 1999). This is triggered once the tubulin has docked with the microtubule and it subsequently alters the kinetics of assembly, as GDP-tubulin is more prone to disassociation from the polymer (Weisenberg et al., 1976). Although GDP-tubulin at the centre of a microtubule cannot spontaneously disassociate, once hydrolysis has ‘caught up’ with the GTP cap, the polymer will rapidly depolymerise and shrink (Margolin et al., 2012). This process is termed microtubule catastrophe. Microtubule structure and dynamics are summarised in Figure 1.11.

1.7.1.1. Stable and dynamic microtubule polymers

Two subpopulations of microtubules exist within any cell, namely dynamic and stable. They are defined by differences in their stability, i.e. the rate at which the polymer is assembled and disassembled (Schulze and Kirschner, 1987). Dynamic microtubules have a brief half-life, in the order of minutes, and are easily disassembled by pharmacological agents or cold (Bulinski and Gundersen, 1991, Schulze and Kirschner, 1987). In contrast, stable microtubules can remain as polymers for several hours (Infante et al., 2000) with stability modulated by the addition of various MAPs (Hirokawa, 1994). While the dynamic state facilitates cell motility and mitosis, stable microtubules are required for effective phagocytosis (Peachman et al., 2004).

Stable microtubules are sufficiently long-lived to enable the accumulation of post-translational modifications to their tubulin subunits (Janke and Bulinski, 2011). This includes tyrosination, glutamylation and glycylation (Song and Brady, 2014). A modification that is pertinent to this thesis is the addition of an acetyl group to lysine-40 of α-tubulin, which occurs on the inside of the hollow microtubule core. Under conditions that promote depolymerisation, microtubules containing acetylated tubulin are significantly more resistant to disassembly (Piperno et al., 1987, Matsuyama et al., 2002, Xie et al., 2010). α-tubulin acetylation is therefore a marker of polymer stability. It is undertaken by α-tubulin acetyltransferase-1 (αTAT1), which has a
catalytic preference for polymerised microtubules over monomeric tubulin (Shida et al., 2010). An αTAT1 mutant with no acetyltransferase activity has also been shown to promote microtubule stability (Kalebic et al., 2013). As such, the importance of αTAT1 in achieving microtubule stability is not simply confined to its enzymatic activity *per se*. The acetylation of α-tubulin is reversible. HDAC6 and sirtuin type 2 (SIRT2) are histone deacetylases that are also capable of acting on non-histone substrates (Schemies et al., 2009). They both deacetylate α-tubulin (Hubbert et al., 2002, North et al., 2003).

### 1.7.2. Pharmacological agents can influence microtubule dynamics

Several pharmacological agents are capable of influencing microtubule dynamics. They are therefore used *in vitro* to study polymer assembly and disassembly, or administered to patients as chemotherapy to treat cancer (*i.e.* uncontrolled mitosis) (Stanton et al., 2011). Broadly, they are categorised as either ‘microtubule stabilisers’ (*e.g.* taxanes and epothilones) or ‘microtubule destabilisers’ (*e.g.* vinca alkaloids and colchicine). Nocodazole is one such agent. It was originally developed as an anti-neoplastic drug but never achieved therapeutic application. It is however, still widely used to study microtubule-dependent processes *in vitro* because of its ability to specifically bind tubulin (Jordan and Wilson, 1998). Its effect on microtubule dynamics will now be discussed.

#### 1.7.2.1. Nocodazole

The effect of nocodazole on microtubule dynamics is highly concentration-dependent. At nanomolar concentrations (4 – 400 nM), it inhibits dynamic instability, thereby inducing a ‘paused state’ amongst polymers with slower rates of both elongation and shortening (Vasquez et al., 1997). This action has proved particularly helpful in arresting and synchronising cell division cycles for study. At higher concentrations (micromolar range) nocodazole has been shown to rapidly depolymerise microtubules and inhibit phagocytosis (De Brabander et al., 2013).
1976, Cannon and Swanson, 1992). This action is achieved by increasing intrinsic GTPase activity to thereby induce polymer ‘catastrophe’ (Lin and Hamel, 1981, Mejillano et al., 1996).

**Figure 1.11. Microtubule structure and dynamics.** Microtubules are assembled from α- and β-tubulin heterodimers (bound to GTP), which polymerise into filaments with a hollow core and GTP-cap. They continually polymerise and depolymerise (‘dynamic instability’). They have fast-growing (+) and slow-growing (−) ends. The latter is embedded in the microtubule organising centre (MTOC). Intrinsic GTPase activity hydrolyses GTP to GDP, and this results in microtubule catastrophe. Two subpopulations exist (dynamic and stable). Stable microtubules are resistant to disassembly and long-lived. They associate with microtubule-associated proteins (MAPs) and undergo post-translational modifications, such as reversible acetylation (Ac). Acetylation is undertaken by α-tubulin acetyltransferase-1 (αTAT1), while deacetylation is performed histone deacetylase-6 or sirtuin type 2 (SIRT2). Stable microtubules are effective at mediating phagocytosis (e.g. providing the necessary tracks for phagolysosome migration).
1.7.3. Microtubule dynamics in COPD

As discussed in Section 1.6.4, it is currently unknown why bacterial phagocytosis by AM is defective in COPD. Current evidence suggests that any defect is most likely to lie downstream of receptor expression (Taylor et al., 2010). It is therefore possible that defects in microtubule arrangements might contribute and that the microtubule subpopulation within AM is skewed towards a predominantly dynamic group. These microtubules may be less resistant to pharmacological disruption, contain less acetylated tubulin and be less effective at mediating bacterial phagocytosis (Peachman et al., 2004). This is yet to be researched and is a specific focus of this PhD (Chapter 4).

1.8. Hypothesis

The hypothesis for this thesis is: ‘Bacterial phagocytosis by alveolar macrophages is defective in COPD, results from unstable microtubule polymers and can be augmented by solithromycin, a novel macrolide with immunomodulatory properties’.

1.9. Aims

The following aims were adopted for Chapters 3 – 7 to investigate the above hypothesis:

- **Chapter 3**: Undertake phagocytosis assays with paired AM and MDM, to determine whether MDM can provide a surrogate measure of AM phagocytosis and therefore act as a reliable model.

- **Chapter 4**: Undertake phagocytosis assays with MDM to determine whether bacterial phagocytosis is defective in COPD and correlates with markers of disease severity. Examine the stability of microtubule polymers in macrophages. Specifically, to determine whether an unstable population predominates in COPD patients and might account for defective bacterial phagocytosis.
• **Chapter 5**: Use MDM to investigate the immunomodulatory effects of solithromycin and its analogues, including any ability to augment defective bacterial phagocytosis in COPD.

• **Chapter 6**: Develop an alternative to BAL, which is less invasive and could be used to sample inflammatory mediators in COPD, including during therapy with a novel anti-inflammatory drug (Chapter 7).

• **Chapter 7**: Undertake a clinical trial in COPD to evaluate the anti-inflammatory effects of solithromycin.
CHAPTER 2

Materials and Methods
2.1. Materials

Listed below are the materials used in this thesis and the corresponding supplier. Unless otherwise stated, all medication were purchased from the pharmacy at the Royal Brompton & Harefield NHS Foundation Trust (RBHT). The solithromycin capsules (200 mg) used for the CE01-204 clinical trial (Chapter 7) were supplied by Cempra Pharmaceuticals, Chapel Hill, USA.

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<td>New England Biolabs, Herts, UK</td>
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<td>Anti-rabbit HRP-labelled IgG (goat)</td>
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2.2. Methods

2.2.1. Subject selection

Healthy non-smoking and smoking volunteers without COPD were recruited by advertisement through the National Heart and Lung Institute (NHLI), Imperial College London. COPD patients were recruited from RBHT, London. All participants gave written informed consent, as approved by National Research Ethics Committees; Westminster, London (Reference 14/LO/2066) for the CE01-204 solithromycin clinical trial and Bloomsbury, London (Reference 13/LO/1403) for all other participants and procedures. NHS permission was granted by the RBHT research office. Clinical procedures were undertaken at RBHT facilities, including the asthma lab, respiratory Biomedical Research Unit (rBRU), Lind ward bronchoscopy suite and the muscle lab at Harefield hospital. Potential subjects were screened by Dr Craig Batista and research nurse Sally Meah, in order to determine suitability for inclusion.

2.2.1.1. Non-smoking volunteers

The non-smoking volunteers had never smoked. Other inclusion criteria were: age 40-80 years, no history of respiratory disease or co-morbidity, no regular medications, normal spirometry as predicted for sex, age and height (Section 2.2.2.1) and no evidence of atopy, as determined by skin prick testing to common aero-allergens (Section 2.2.2.3).

2.2.1.2. Smoking volunteers

A smoker was defined as a subject who was currently smoking with a smoking history of > 10 pack years (one pack year being equivalent to smoking one pack of 20 cigarettes per day for one year). The inclusion criteria for smoking volunteers were: age 40-80 years, no history of respiratory disease or co-morbidity, no regular medications, normal spirometry as predicted for sex, age and height (Section 2.2.2.1), no airway reversibility to bronchodilators (Section
2.2.2.2) and no evidence of atopy, as determined by skin prick testing to common aero-allergens (Section 2.2.2.3).

2.2.1.3. COPD patients

A COPD patient was defined according to the GOLD classification (GOLD, 2015) as a subject with a smoking history of > 10 pack years (or appropriate occupational or industrial dust exposure) and spirometry demonstrating persistent airflow obstruction, specifically a FEV₁ < 80% of the predicted value (based on sex, age and height) and a FEV₁/FVC ratio < 0.7 after bronchodilator use (Sections 2.2.2.1 and 2.2.2.2). COPD patients were included if aged 40-80 years, there was no history of any other disease or major co-morbidity, no airway reversibility to bronchodilators (Section 2.2.2.2) and no evidence of atopy, as determined by skin prick testing to common aero-allergens (Section 2.2.2.3). They could be current or ex-smokers. The recruitment and specific inclusion / exclusion criteria used to screen COPD patients for the CE01-204 solithromycin trial are discussed in Chapter 7.

2.2.1.4. Exclusion criteria

Subjects from all three groups were excluded if they met any of the following exclusion criteria at screening, or during participation: inability to give informed consent, pregnancy, breastfeeding, any major co-morbidity (including commencement of new medication) and any infection (including upper and lower respiratory tract and / or the use of antibiotics or steroids within the last 6 weeks). Patients with severe COPD (FEV₁ < 50 %) have a higher risk of bronchoscopy complications and are less likely to provide an adequate BAL sample (Section 2.2.5.4). Therefore, such patients did not undergo bronchoscopy.
2.2.2. **Clinical measurements**

2.2.2.1. **Spirometry**

Spirometry was performed in accordance with British Thoracic Society guidance (BTS, 2005) and using a Pneumotrac® spirometer (Vitalograph, Buckinghamshire, UK). Three reproducible attempts were recorded, allowing 1 min for the patient to recover between each manoeuvre. The best values for \( \text{FEV}_1 \) and \( \text{FVC} \) have been reported. Both \( \text{FEV}_1 \) and \( \text{FVC} \) have been expressed as either absolute values (litres) or as a percentage of a value predicted for the subjects’ age, sex, height and ethnicity. These predicted values have been calculated by the spirometer’s Spirotrac® software, which uses the European Respiratory Society 1993 criteria (ERS, 1993).

2.2.2.2. **Reversibility testing**

Reversibility was performed according to guidelines from the BTS and Scottish Intercollegiate Guidelines Network (BTS/SIGN, 2012). Subjects performed baseline spirometry (Section 2.2.2.1) prior to the administration of 2.5 mg of nebulised salbutamol. Spirometry was repeated after 20 min and post-bronchodilator values recorded. A significant response (i.e. airway reversibility) was defined as a percentage change > 15% when compared to baseline \( \text{FEV}_1 \), that also exceeded 200 ml in absolute terms.

2.2.2.3. **Skin prick testing**

Skin prick testing to common aero-allergens was performed as previously described (Heinzerling et al., 2013). Single drops of each allergen solution, in addition to positive and negative controls (histamine and saline, respectively), were placed at least 2 cm apart on the subject’s inner forearm. Individual lancets were then used to pierce the skin through each drop. Any surplus fluid was carefully removed by blotting with tissue to avoid any cross contamination. The forearm was inspected after 15 – 20 min and the test confirmed as valid if the negative control was < 3 mm and the positive control ≥ 3 mm. A positive test was recorded.
when the longest diameter of any wheal measured ≥ 3 mm. The following allergens were tested on each occasion: house dust mite (*Dermatophagoides pteronyssinus*), cat, dog, aspergillus, grass and tree pollen.

**2.2.2.4. Impulse Oscillometry**

Impulse oscillometry (IOS) was performed using a JAEGER® Vyntus™ IOS system and SentrySuite® software (CareFusion, UK) (Figure 2.1). Measures of $R_5$, $R_{20}$, $X_5$ (reactance at 5 Hz), $AX$ (area of reactance) and $RF$ (resonant frequency) were calculated by the software during 30 s of tidal breathing. $R_5-R_{20}$ was manually calculated by subtracting $R_{20}$ from $R_5$. Measurements were performed using nose-clips, bacterial filters, tongue-deflector mouthpieces and with the neck in a neutral position with the cheeks held firmly (Figure 2.1). Three reproducible attempts were performed and the mean value for each parameter was recorded, as is conventional for IOS. IOS was always undertaken prior to spirometry to avoid the possible recruitment of collapsed airways during the forced manoeuvre. A comparison of results between non-smokers and COPD patients, and correlations with spirometry values can be viewed in Appendix C.
2.2.3.  Symptom assessments

2.2.3.1. COPD assessment test

All COPD patients were asked to complete a COPD assessment test (CAT). An example of the questionnaire can be viewed in Appendix B.

2.2.3.2. Modified medical research council dyspnoea scale

All COPD patients reported on their degree of breathlessness according to the modified Medical Research Council (mMRC) dyspnoea scale. An example of this scale can be viewed in Appendix A.

2.2.3.3. Combined GOLD assessment

Spirometry (Section 2.2.2.1), CAT scores (Sections 2.2.3.1), mMRC scores (Section 2.2.3.2) and exacerbation frequency (Sections 2.2.4) were used to stratify the COPD patients according to the combined GOLD assessment. A summary can be found in Figure 1.1. In short, patients were categorised into ‘group A’ (less symptoms and low risk of exacerbation), ‘group B’ (more...
symptoms and low risk of exacerbation), ‘group C’ (less symptoms and high risk of exacerbation) or ‘group D’ (more symptoms and high risk of exacerbation).

2.2.4. COPD exacerbations

A COPD exacerbation was defined as a worsening of symptoms, sustained for ≥ 2 days; specifically, a deterioration in 2 major symptoms (dyspnoea, sputum purulence or sputum volume), or 1 major and 1 minor symptom (cough, wheeze, fever, nasal discharge or sore throat) (Seemungal et al., 1998). The number of exacerbations that were experienced by each patient during the preceding year were recorded and was used to divide the COPD cohort at baseline into infrequent or frequent exacerbators accordingly (< 2 or ≥ 2 exacerbations). In the main, COPD patients who had exacerbated recently (within 6 weeks) were excluded from providing clinical samples. The only exception was during the collection of nasal ELF by nasosorption (Chapter 7).

2.2.5. Sampling methods

2.2.5.1. Venepuncture

Subjects donated 60 ml of whole blood for the generation of MDM. Syringes (3 x 20 ml) were first prepared by the addition of 500 μl of 0.5M ethylenediaminetetraacetic acid (EDTA). Venepuncture was then performed with either a 21-gauge butterfly needle or 20-gauge intravenous cannula (Section 2.2.5.4). Venepuncture was also used to collect venous blood for the CE01-204 clinical trial (clinical tests, serum biomarkers and pharmacokinetics) (Section 7.4.6.1 and 7.4.7.4). A standard technique, 21-gauge butterfly needle and appropriate vacutainer tubes (Becton Dickinson medical technology, USA) were used.
2.2.5.2. Sputum induction

Sputum induction was undertaken as described previously (Pavord et al., 1997). An UltraNeb U3000 ultrasonic nebuliser (DeVilbiss Healthcare Ltd, UK) and 7% (v/v) hypertonic saline were used. Different concentrations of hypertonic saline (3%, 4% and 5% (v/v)) were prepared for nebulisation by dilution in sterile water. Hypertonic saline can induce bronchoconstriction. Therefore, spirometry was performed before and after the brief administration of a test dose of 7 ml 3% (v/v) hypertonic saline. The induction protocol was terminated if a fall in FEV₁ > 20% was observed, relative to baseline. In such circumstances, subjects immediately received a bronchodilator (salbutamol pMDI or nebule) and samples were not collected.

Subjects with a fall in FEV₁ < 20% continued with the protocol. Those with a drop between 10% and 20% cautiously received 3% saline (v/v), while those without a drop in FEV₁ (or < 10%) were asked to repeat this process but using test doses of 4% (v/v) and 5% (v/v) saline. Nebulisation cycles (4 x 5 min) were then undertaken at the maximum safe dose of hypertonic saline. After each cycle, the subject was encouraged to cough deeply and perform active cycle of breathing techniques to aid expectoration. They were allowed to expectorate at any point during or after the induction period. Prior to collecting the sputum into a sterile container, subjects blew their nose and rinsed their mouth in an attempt to avoid contamination from the upper respiratory tract. The induction protocol was terminated early if the clinician felt that the patient had become notably unwell or if the FEV₁ fell by > 20%. All samples were immediately stored on ice and processed within 2 h of collection.

2.2.5.3. Nasosorption

The nasal ELF was sampled using the Nasosorption™ FX·i device (Hunt Developments, UK). It contains a strip of SAM measuring 7 x 24 mm (Figure 2.2). The subject was positioned upright with their head extended backwards. Devices were then passed into each nostril under direct
vision, with the length of the SAM applied laterally against the anterior portion of the inferior turbinate. The devices were held in place for 2 min by asking the subject to lightly pinch their nose, before being carefully recovered. Samples were kept on ice and immediately processed (Section 2.2.7.2). Both nostrils were sampled simultaneously and the resulting ELF was pooled in order to limit any variability that could arise from a nasal cycle (Alam et al., 1992).

Figure 2.2. Nasosorption. (A) Nasosorption™ FX-i device (B) Nasosorption™ FX-i device sited in the nasal cavity (C) Costar Spin-X filter cup and Eppendorf tube (D) SAM portion of Nasosorption™ FX-i device is cut and immersed in assay buffer prior to centrifugation.

2.2.5.4. Bronchoscopy (bronchosorption and BAL)

Bronchoscopy was performed by Dr Craig Batista using an Olympus BF-1T260 flexible bronchoscope. Subjects were intravenously cannulated and 60 ml of whole blood was drawn as described in Section 2.2.5.1, in order to isolate monocytes and obtain paired MDM (Section 2.2.6). In COPD patients, paired nasosorption samples were also obtained immediately before bronchoscopy (Section 2.2.5.3).

Subjects received 2 – 5 mg midazolam and 0.1 – 0.4 mg alfentanil intravenously to consciously sedate and suppress cough for the procedure. The upper respiratory tract was then anaesthetised by the local application of 4% (w/v) xylocaine. The bronchoscope was inserted
through the oropharynx and 2% (w/v) lignocaine was instilled via the operating channel onto the vocal cords, trachea and lower respiratory tract. Visual inspection excluded the presence of any macroscopic pathology prior to sampling by bronchosorption and BAL, in that order.

The Bronchosorption™ FX SAM device (Hunt Developments, UK) was used to sample the bronchial ELF of the right middle lobe. It contains a thin strip of SAM measuring 2.7 cm in length, capable of passing through a bronchoscope operating channel ≥ 2.6 mm (Figure 2.3). The tip of the bronchoscope was first wedged into a sub-segmental bronchus, before the device was passed down the operating channel and the SAM extended under direct vision (Figure 2.4). The full length of the SAM was applied against the wall of the bronchus to allow the absorption of ELF. After 30 s, the SAM was retracted and the device removed. Samples were kept on ice and immediately processed (Section 2.2.7.2).

BAL of the right middle lobe was performed following bronchosorption. The position of the bronchoscope was maintained (i.e. wedged in the right middle lobe). 50 ml of 0.9% (w/v) saline was instilled through the operating channel and any returning fluid was aspirated. This was repeated 4 times (total instilled volume of 0.9% (w/v) saline was 200 ml). The BALF sample was kept on ice and immediately processed (Section 2.2.6.4).
2.2.6. Cell isolation and culture

2.2.6.1. Peripheral blood mononuclear cells

PBMC were isolated from 60 ml of whole blood, which was obtained by venepuncture (Section 2.2.5.1). 20 ml aliquots of blood were first mixed with 10 ml 6% (w/v) dextran and 20 ml Hank’s balanced salt solution (HBSS) in 3 separate Falcon tubes. The mixtures were gently inverted.
prior to incubation at room temperature for 20 min to allow erythrocyte sedimentation. The
top leukocyte-rich layer was then transferred to 3 clean tubes, adjusted to a volume of 50 ml
with Hank’s balanced salt solution (HBSS) and centrifuged for 10 min (350 x g, room
temperature).

The resulting cell pellet contains a mixture of leukocytes. Therefore, discontinuous Percoll
gradients (GE Healthcare, USA) were used to separate the peripheral blood mononuclear cells
(PBMC) from the polymorphonuclear cell fraction. A 100% (v/v) Percoll solution was first
prepared using 20 ml of Percoll and 2 ml of 9% (w/v) saline. From this solution, Percoll gradients
of 81% (v/v), 67% (v/v) and 55% (v/v) were prepared with 0.9% (w/v) saline. Gradient columns
were next assembled by transferring 4 ml of 81% (v/v) Percoll into a 15 ml Falcon tube and
overlaying this solution with 4 ml of 67% (v/v) Percoll. The leukocyte pellet was re-suspended
in 4 ml of the 55% (v/v) Percoll solution and layered on top of the gradient column. The cell
fractions were separated by density using centrifugation at 750 x g for 30 min at room
temperature (Figure 2.5).

The PBMC fraction was harvested from the 55% (v/v) - 67% (v/v) Percoll interface (Figure 2.5)
and transferred to a single clean 50 ml Falcon tube. The volume was adjusted to 50 ml with
HBSS and the cells were washed (centrifugation at 1500 x g for 5 min). The PBMC pellet was
re-suspended in 50 ml HBSS and cell counts were performed using a Neubauer
haemocytometer after staining with 1:1 Kimura stain (0.05% (v/v) toluidine blue, 0.03% (v/v)
light green, 10% (v/v) saponin, 0.7M phosphate buffer). The PBMC were finally re-suspended
in complete media (Roswell Park Memorial Institute (RPMI) 1640 medium, 10% (v/v) foetal
bovine serum, 10 mg/ml (1% (v/v)) penicillin/streptomycin and 2mM (1% (v/v)) L-glutamine) at
a concentration of 4x10⁶ cells/ml.
Figure 2.5. Isolation of PBMC using discontinuous Percoll gradients. 67% (ν/ν) Percoll was layered onto 81% (ν/ν) Percoll in a 15ml falcon tube. A leukocyte cell pellet was then resuspended in 55% (ν/ν) Percoll and layered on top. The columns were centrifuged at 750 x g for 30 min and PBMC were extracted from the 55% - 67% (ν/ν) interface.

2.2.6.2. Monocytes

Monocytes were separated from PBMC using adherence. PBMC were first isolated from whole blood as described in Section 2.2.6.1. The cell suspensions were seeded into either 96 or 24 well tissue culture plates at 100 μl/well (1x10⁵ monocytes/well) or 500 μl/well (5x10⁵ monocytes/well) respectively. Black 96 well plates were used for phagocytosis assays. Plates were incubated at 37°C and 5% (ν/ν) CO₂ for 2h to allow monocyte adherence. The complete media and any non-adherent cells were then aspirated from the wells.

2.2.6.3. Monocyte-derived macrophages

Monocytes were first isolated from PBMC, as described in Sections 2.2.6.1 and 2.2.6.2. After aspirating the complete media and non-adherent cells, fresh complete media containing GM-CSF (2ng/ml) was immediately added and the cells were incubated at 37°C and 5% (ν/ν) CO₂ for 12 days, replacing the media on days 4 and 8.
2.2.6.4. Alveolar macrophages

AM were isolated from BALF obtained during flexible bronchoscopy (Section 2.2.5.4). Isolation took place within 30 min of bronchoscopy and under sterile conditions. The BALF was first passed through 70 μm filters into 50 ml Falcon tubes. The volume was then adjusted with D-PBS prior to centrifugation at 500 x g for 10 min. The resulting supernatant was aspirated and the cell pellet re-suspended in 1ml complete media, which had been supplemented with 1% (v/v) amphotericin B.

Differential cell counts were performed using a haemocytometer after staining 10 µl of cell suspension with 10 µl Kimura stain (i.e. 1:1). Cell viability was also assessed by mixing a further 10 µl of cells with 20 µl of 4% (v/v) trypan blue and 30 µl media, before counting the number of live and dead cells on a haemocytometer. Samples with <70% viability were discarded. For all other samples, the number of viable macrophages were calculated (i.e. the proportion of live cells). The volume of cell suspension was then adjusted with more complete media to a final concentration of 1 x 10⁶ viable macrophages / ml. These cells were seeded into black 96 well tissue culture plates at 100 μl/well (1 x 10⁵ macrophages/well). Plates were incubated overnight at 37°C and 5% (v/v) CO₂ to allow the macrophages to adhere.

2.2.7. Processing clinical samples

2.2.7.1. Induced Sputum

Sputum was obtained by induction with hypertonic saline (Section 2.2.5.2). Plugs were removed from the whole sample, weighed and mixed with an equal volume of D-PBS. Dithiothreitol (DTT) was then added to give a final concentration of 0.05% (w/v). The resulting mixture was rocked on a bench rocker at room temperature for 30 min, before filtration through a 70 μm nylon gauze and centrifugation at 300 x g for 5 min at room temperature. Supernatants were then aspirated and stored at -80°C. The remaining cell pellet was
resuspended in 10 ml D-PBS and differential cell counts were performed as described in

Section 2.2.8.2. To ensure that samples were representative of the lower respiratory tract, any
that contained squamous epithelial cells > 40% of the total cell count were discarded, as is
conventional (Sohani et al., 2011). Cell suspensions were adjusted to 50 ml with D-PBS for one
final wash (300 x g for 5 min). Cell pellets were then resuspended in D-PBS (3 x 10^5 total cell / ml) for cytospins, staining and light microscopy (Section 2.2.8.2).

2.2.7.2. Synthetic absorptive matrix

Nasosorption or bronchosorption was performed as described in Sections 2.2.5.3 and 2.2.5.4. Once the device was recovered, the SAM strip was immediately cut free and immersed in 200 µl of pre-chilled assay buffer (D-PBS with 1% (v/v) BSA, 0.05 % (v/v) Tween®-20 and 0.05% (w/v) sodium azide). This had been pipetted into the filter cup of an Eppendorf tube (Costar Spin-X®, cellulose acetate with pore size 0.22 µm, Sigma-Aldrich, UK) (Figure 2.2). The samples were then immediately centrifuged at 16,000 x g for 10 minutes (cooled to 4 °C) and the eluates were stored at -80°C prior to ELISA.

2.2.8. Microscopy of sputum leukocytes

2.2.8.1. Cytospins

Induced sputum samples were processed as described in Section 2.2.7.1. Cell suspensions containing 3 x 10^5 total cells / ml of D-PBS were used for cytospins. 100 µl of cell suspension was transferred into a cytospin funnel, which was attached to a filter and microscope slide. Cells were then centrifuged at 650 x g for 7 min. Slides were allowed to air-dry, before being stored at -80°C prior to staining and microscopy (Section 2.2.8.2).
2.2.8.2. Quick-Diff staining

Microscopy slides were first prepared as described in Section 2.2.8.1. These were then stained using the REASTAIN Quick-Diff kit, which allows for differential staining of cellular components of leukocytes. Staining was performed as described in the manufacturer’s handbook. In brief, each slide was immersed for 4 s in fixative (100% (v/v) methanol), 6 s in Red solution (12% (w/v) Eosine Yellow, 0.1% (w/v) sodium azide) and 10 s in Blue solution (0.09% (w/v) Azur II, 5% (v/v) Glycerol, 0.1% (w/v) sodium azide), before being washed repeatedly in distilled water to remove any excess stain. Slides were air dried and mounted with DPX medium and a cover slip. Cells were then viewed under a light microscope and differential cell counts were performed. Differential cell counts were performed by a blinded laboratory technician, who counted a minimum of 200 cells on each slide. Five slides were counted and the mean recorded. The intra-assay coefficient of variability (CV) i.e. the mean CV between replicate slides was < 10% for all of the differential cell counts presented within this thesis.

2.2.9. Preparation of heat-killed and fluorescently-labelled bacteria

2.2.9.1. Culture of Haemophilus influenzae

Non-typeable H. influenzae strain 1479 (isolated from a COPD patient during exacerbation) was streaked onto Columbia agar supplemented with bovine hemin and NAD+ (10μg/μl), and incubated for 24 h at 37°C and 5% (v/v) CO₂. 20 ml of brain heart infusion (BHI) broth with bovine hemin and NAD+ (10μg/μl) was then inoculated with a single bacterial colony and incubated on a shaker incubator at 37°C (200 rpm). Measures of optical density (OD) were used to determine the bacterial growth rate. Each hour, 500 μl of H. influenzae were removed from the broth and transferred to a cuvette and read at λ600nm in a spectrophotometer. Serial dilutions of bacteria were also plated onto BHI plates and incubated for 12h at 37°C and 5% (v/v) CO₂. Colony forming units (CFU) were counted the following day and CFU/ml were
calculated ($\text{CFU/ml} = (\text{colony number} \times \text{dilution factor}) / \text{volume plated (ml)})$. This was used to derive a growth curve by plotting $\text{CFU/ml} (y)$ versus $\text{OD} (x)$ (Figure 2.6).

The now turbid BHI broth could then be divided evenly into fresh 50ml Falcon tubes. 2ml of turbid broth was added to each tube and supplemented with 18ml of fresh BHI. This was incubated on a shaker at 37°C (200 rpm) until a given OD, which corresponded to a CFU/ml of $5 \times 10^9$. The live $H. \text{influenzae}$ was then harvested by centrifugation ($1600 \times g$ for 15 min), washed repeatedly and resuspended in D-PBS. Before being used in phagocytosis assays, these bacteria were heat killed (Section 2.2.9.3) and fluorescently-labelled (Section 2.2.9.4).

**Figure 2.6. Growth curve for $H. \text{influenzae}$.** A single colony of $H. \text{influenzae}$ was inoculated into BHI broth and incubated at 37°C and 5% (v/v) $\text{CO}_2$. Each hour, 500 $\mu$l aliquots were removed and the optical density (OD) was read at $\lambda_{600 \text{nm}}$ in a spectrophotometer. Serial dilutions were also plated and incubated for 12h (37°C and 5% (v/v) $\text{CO}_2$), before colony counting. CFU/ml were then calculated and plotted against OD. The linear portion of the curve (exponential growth) could be used to derive CFU/ml for a given OD.
2.2.9.2. Culture of *Streptococcus pneumoniae*

*S. pneumoniae* serotype 9V, strain 10692 (isolated from a COPD patient during exacerbation) was grown using similar methodology to that described for *H. influenzae* (Section 2.2.9.1). In summary, *S. pneumoniae* was cultured for 24 h on Columbia blood agar plates, before being inoculated into Todd-Hewitt broth containing 5% (v/v) yeast extract. As before, bacterial growth curves were derived by plotting CFU/ml (y) against OD (x) (Figure 2.7). *S. pneumoniae* was cultured to an OD that corresponded with a CFU/ml of $5 \times 10^9$. The live bacteria were subsequently harvested, heat killed (Section 2.2.9.3) and fluorescently-labelled (Section 2.2.9.4), prior to phagocytosis assays.

![Figure 2.7. Growth curve for *S. pneumoniae*.](image)

A single colony of *S. pneumoniae* was inoculated into Todd-Hewitt broth with 5% (v/v) yeast extract and incubated at 37°C 5% (v/v) CO$_2$. Each hour, 500 μl aliquots were removed and the optical density (OD) was read at λ600nm in a spectrophotometer. Serial dilutions were also plated and incubated for 12h (37°C and 5% (v/v) CO$_2$), before colony counting. CFU/ml were then calculated and plotted against OD. The linear portion of the curve (exponential growth) could be used to derive CFU/ml for a given OD.
2.2.9.3. Heat killing

*H. influenzae* and *S. pneumoniae* were heat killed by incubation at 70°C for 2 h. The bacteria were then washed twice by centrifugation at 300 x g for 10 min, the media was aspirated and the pellet re-suspended in fresh D-PBS. To ensure effective heat killing, 100 µl of bacterial suspension was streaking onto agar plates and incubated overnight at 37°C. A colony count of zero was considered indicative of heat killing.

2.2.9.4. Fluorescent labelling

Heat-killed bacteria were fluorescently labelled with Alexa-fluor 488 dye (excitation λ 480 nm and emission λ 520 nm), which was reconstituted in DMSO (1 mg / ml). 10 µl of Alexa-fluor 488 was added to 1 ml aliquots of bacterial suspension and rotated overnight in the dark, at room temperature. Bacteria were then repeatedly washed in D-PBS to remove any unbound dye, before being re-suspended in D-PBS and stored at -20°C.

2.2.10. Antibiotic susceptibility testing

2.2.10.1. Clinical bacterial isolates

Single isolates of live *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* were used for antibiotic susceptibility testing. They were obtained from the clinical microbiology laboratory at RBHT as pure bacterial growths that had been isolated from 3 separate COPD patients during exacerbation. They were harvested from the agar plates and suspended in 1 ml of glycerol, before being aliquoted (200 µl) and stored at -80°C. Each aliquot was later used to prepare bacterial lawn growths (Section 2.2.10.2) for Kirby-Bauer disc diffusion testing (Section 2.2.10.3).
2.2.10.2. Bacterial lawn growths

Bacterial lawn growths were used for antibiotic susceptibility testing. The aliquots of *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* that were prepared in Section 2.2.10.1, were defrosted and washed twice in D-PBS (300 x g for 2 min). The supernatant was discarded and the bacteria resuspended in 50 ml RPMI. 1 ml of this bacterial suspension was then dropped onto the centre of separate agar plates and spread using a swirling motion. Each plate was allowed to dry for 15 min, before the addition of antibiotic-impregnated discs (Section 2.2.10.3). Columbia blood agar plates were used (chocolate agar for *H. influenzae* and *M. catarrhalis*).

2.2.10.3. Kirby-Bauer disc diffusion

Antibiotic susceptibility testing was performed using the Kirby-Bauer disc diffusion method (Bauer et al., 1966). 6 mm discs of blotting paper were soaked for 5 seconds in varying concentrations of antibiotic (or vehicle control) before being immediately placed onto the agar plate containing bacteria (Section 2.2.10.2). The plates were subsequently incubated for 16h at 37°C and 5% (\(\text{v}/\text{v}\)) CO\(_2\). The zones of inhibition (diameter of inhibited bacterial growth) were then measured for each condition (at 3 points and the mean calculated).

2.2.11. Phagocytosis assays

2.2.11.1. Beads

Fluorescently-labelled carboxylate-modified polystyrene microspheres of 2.0 μm diameter and yellow-green fluorescence (ex λ505nm / em λ515nm, 4.5 x 10\(^9\) microspheres/ml) were purchased from Invitrogen (Life Technologies Corporation, UK). They were sonicated for 2 min to prevent particle aggregation before being diluted (11μl beads/ml of RPMI). This achieves a concentration of 50 x 10\(^6\) microspheres/ml, which has previously been shown to give optimum measurements for bead phagocytosis (data not shown) (Taylor et al., 2010).
2.2.11.2. Bacteria

Aliquots of *H. influenzae* and *S. pneumoniae* suspensions (Section 2.2.9) were brought to room temperature and sonicated for 2 min. Each was then diluted in RPMI prior to phagocytosis (100μl bacteria/ml). The resulting concentration was 5x10⁸ CFU/ml, which was chosen as it represents the levels of bacteria present in the airways of patients during clinically-relevant infective exacerbations of COPD (where clinically relevant infection occurs with >10⁷ CFU/ml) (Garcha *et al.*, 2012). It has also been shown to be the optimum concentration of bacteria for phagocytosis over 4 h (data not shown) (Taylor *et al.*, 2010).

2.2.11.3. Phagocytosis using fluorimetry

Suspensions of beads, or fluorescently labelled *H. influenzae* and *S. pneumoniae* were prepared using RPMI (Sections 2.2.11.1 and 2.2.11.2). 100μl of each were then added to separate wells of a black 96 well plate, containing either 1x10⁵ MDM or AM. Plates were then incubated at 37°C, 5% (v/v) CO₂ for 4h. After incubation, the cells were washed by adding 100μl/well of D-PBS and using a plate shaker for 2 min, in order to remove any unbound prey. 100μl of Trypan Blue 1% (v/v) was then added to each well for 1 min to quench the fluorescence of any extracellular particles. The Trypan blue was then removed and the fluorescence determined by fluorimetry using a BMG Fluostar plate reader (excitation λ480nm and emission λ520nm).

2.2.12. Cell viability

Media was aspirated from each well and replaced with 50 μl of methylthiazolyl diphenyl-tetrazolium bromide (MTT) solution (1mg/ml). Plates were then incubated at 37°C for 40 min, before the MTT was aspirated and the cells lysed by adding 50 μl of DMSO to each well. The plates were briefly shaken to obtain an even distribution of colour within the wells and absorbance was measured on a spectrophotometer at λ570 nm. Data were normalised to the
viability of control cells (e.g. untreated MDM) and presented as a percentage of this control, which was taken as 100%.

2.2.13. Western blotting

Western blotting was used to analyse proteins within the lysates of unstimulated MDM. Gel electrophoresis separated the proteins according to size, before a second electrical current was applied to transfer them onto a nitrocellulose membrane. This was probed for the protein of interest using a primary antibody. A HRP-conjugated secondary antibody was then added in order to detect the primary antibody. The amount of protein was visualised by chemiluminescence and exposure to photographic film. Densitometry of the corresponding protein band allowed concentrations of the protein to be calculated; semi-quantitatively and relative to a loading control.

2.2.13.1. Protein extraction

MDM were cultured for 12 days in 24-well plates, as described in Section 2.2.6.3. The cells and following reagents were then chilled to 4°C. Protein extraction was performed on ice. Each well was washed with 500 μl of D-PBS. After aspirating this D-PBS, the cells were then lysed by adding 25μl/well of RIPA lysis buffer (150mM NaCl, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 50mM Tris-HCL), which contained protease and phosphatase inhibitors. Cell scrapers were then used to mechanically disrupt the adherent cells. The resulting cell lysates were harvested and stored at -80°C in Eppendorf tubes.

2.2.13.2. Protein Assay

MDM lysates were thawed before centrifugation at 1600 x g for 2 min. Total protein concentrations within the resulting supernatants were then calculated using a BioRad protein assay. This ensured that an equal amount of protein from each sample was used for
electrophoresis. This protein assay was performed on ice. Each supernatant (1 μl) was added in triplicate to a clear 96-well plate. A standard curve was then constructed using 1mg/ml BSA diluted in D-PBS to concentrations ranging from 0.2-1mg/ml. Empty wells acted as 0 mg/ml of protein. Bradford protein assay solution was then diluted 1:5 in distilled water and 200 μl was added to each well (standards, empty wells and samples). The absorbance was read on a spectrophotometer at λ550nm and unknown protein concentrations were interpolated from the standard curve (Figure 2.8).

**Figure 2.8. Standard curve used to derive unknown protein concentrations.** BSA was diluted in D-PBS to given concentrations ranging from 0.2-1mg/ml. Bradford protein assay reagent was added and the absorbance read at λ550nm. Values were plotted to give a standard curve, from which unknown protein concentrations were interpolated. Level of sensitivity for standard curve is 0.2 mg/ml. Representative of n=5.
2.2.13.3. Gel Electrophoresis

Precast SDS-PAGE gels (4 - 12 % (w/v)) were used to separate proteins according to their molecular size. The gels were secured in a tank, along with MOPS running buffer (250mM MOPS, 250mM Tris, 5mM EDTA, 1% (w/v) SDS). Samples were prepared in Eppendorf tubes by dilution in a sample buffer (62.5mM Tris-HCl, 10% (v/v) glycerol, 1% (w/v) SDS, 1% (v/v) β-mercaptoethanol, 0.01% (w/v) bromphenol blue), such that each was made up to a final volume of 30 μl and contained an equal amount of protein (20 μg). These samples were then heated to 95°C for 5 min, in order to denature the proteins. They were thoroughly cooled and 25 μl was loaded into individual wells of the gel. 7.5μl of a molecular weight rainbow marker was then loaded into the final well. The gel tank was connected to a power source and the proteins were resolved for 2h at 120 mA and 120V.

2.2.13.4. Transfer of protein to nitrocellulose

Transfer cassettes were assembled by placing four sheets of chromatography paper (3MM, Whatman) between two sponge pads. All of the components had been pre-soaked in transfer buffer (0.191M glycine, 0.02M Tris-HCl, 20% (v/v) methanol). The gel was placed on a Hybond nitrocellulose membrane (also wet with transfer buffer) before being positioned between the filter paper. Once closed, the cassettes and an ice block were placed into the transfer tank, which was then filled with transfer buffer. Proteins were transferred from the gel to the nitrocellulose membrane with 200mA and 200V for 1 h.

2.2.13.5. Immunoblotting

Non-specific binding of the antibodies to the membrane was limited by blocking the membrane with 5% (w/v) milk, dissolved in TBS-Tween 20 (0.5M Tris base, 9% (w/v) NaCl, 0.5% (v/v) Tween 20). The membrane was washed in this solution for 1h at room temperature. The blocking solution was then replaced with fresh 5% milk (w/v) in TBS-Tween 20, containing the
primary antibody against the protein of interest. Incubation with the primary antibody was performed for 16 h at 4°C (on a rocker).

In order to remove any unbound antibody, the membrane was washed three times in TBS-Tween 20, by placing it on a rocker for 10 min at room temperature. The membrane was then incubated for 1 h at room temperature with fresh 5% (w/v) milk/TBS-Tween 20, containing an appropriate HRP-conjugated secondary antibody. As before, any unbound antibody was washed with TBS-Tween 20 and the membranes were blotted dry with filter paper. They were then incubated with 10 ml enhanced chemiluminescence (ECL) detection solution for 1 min (placed on a rocker at room temperature for 1 min), immediately removed, blotted dry and wrapped in cling film, before being exposed to Hyperfilm in a dark room, for an appropriate time. The films were developed using an AFP imaging developer. Protein bands were analysed using the UVP GelDoc-IT imaging system and Labworks software.

2.2.13.6. Striping and re-probing

In order to detect alternative proteins, membranes were first stripped of all antibodies by incubation in 15 ml stripping buffer (62.5 mM Tris-HCL, 2% (w/v) SDS, 100 mM β-mercaptoethanol) for 20 min at 50 °C. The membranes were then washed in TBS-Tween 20, before being blocked in fresh 5% milk (w/v) / TBS-Tween 20, as described above. They were then incubated with a second primary antibody, using the methodology above.

2.2.14. Enzyme-linked immunosorbent assay

2.2.14.1. Measurement of CXCL8

Measurement of CXCL8 was performed using ELISA according to the manufacturer’s instructions. A CXCL8 mouse anti-human capture antibody (Invitrogen, UK) was diluted in PBS to a concentration of 0.5 μg/ml and used to coat 96 well NUNC Maxisorp plates (100 μl / well).
The plates were incubated overnight at room temperature. The following morning, the antibody was removed and the plates were washed three times using a wash buffer (PBS with 0.05% (v/v) Tween®-20) before being blocked at room temperature (200 μl / well) with a buffer containing 1% (w/v) BSA, 5% (w/v) sucrose and 0.05% (w/v) sodium azide in PBS. After 2 h, the blocking buffer was removed and standards of human recombinant CXCL8 (R&D systems, UK) were added to appropriate wells (100 μl / well). The samples were then diluted with 0.5% (w/v) BSA in D-PBS, before being added to individual wells (100 μl / well) in triplicate. Individual dilution factors are specific to experiments and are discussed in subsequent chapters. The plates were then incubated with the standards and samples for 2 h at room temperature.

After incubation, the wells were emptied and washed three times with the wash buffer before a detection antibody (Invitrogen, UK) was added (100 μl / well) at a concentration of 0.4 μg / ml (in 0.5% (w/v) BSA). The plates were then incubated at room temperature for a further 2 h, after which they were washed again (three times) before the addition of 100 μl / well of 1 μg / ml (in 0.5% (w/v) BSA) streptavidin-horseradish peroxidase (HRP). The plates were then incubated for 30 min at room temperature, before being washed three times. 100μl of a substrate solution was subsequently added to each well (equal volumes of Colour Reagent A (H2O2) and Colour Reagent B (Tetramethylbenzidine) which had been mixed immediately before addition to the plates). The ensuing reaction was then stopped by 80 μl / well of 1M sulphuric acid.

The OD of each well was determined using a spectrophotometer. The plates were read at λ450nm, while the OD at λ650nm was subtracted to account for optical imperfections in the plate. The concentrations of CXCL8 contained within the samples were derived by interpolation from the standard curve (Figure 2.9). Each ELISA plate contained its own set of standards. The lower limit of detection (sensitivity) was 31.25 pg/ml.
Figure 2.9. Standard curve used to derive unknown concentrations of CXCL8. CXCL8 standard was diluted in PBS containing 0.5% (w/v) BSA to the given concentrations 31.25 - 2000 pg/ml. Plates were incubated with detection antibody (0.4 μg/ml) for 2 h prior to the addition of HRP for 30 min. Substrate solution was then added to each well and the reaction stopped by addition of 80 μl/well 1M sulphuric acid. The absorbance was read at λ450nm. OD was plotted to give a standard curve, from which unknown CXCL-8 concentrations were derived. Level of sensitivity for standard curve is 31.25 pg/ml. Representative of n=5.

2.2.14.2. Measurement of TNF-α

Measurement of TNF-α was performed using ELISA. The general methodology is described in Section 2.2.14.1. TNF-α mouse anti-human capture (2 μg/ml) and detection (0.1 μg/ml) antibodies were used (Invitrogen, UK). Standards were purchased from R&D systems (UK). Samples were diluted with 0.5% (w/v) BSA. However, individual dilution factors are discussed in subsequent chapters. The concentration of TNF-α contained within the samples was interpolated from the standard curve (Figure 2.10). The lower limit of detection (sensitivity) was 31.25 pg/ml.
Figure 2.10. **Standard curve used to derive unknown concentrations of TNF-α.** TNF-α standard was diluted in PBS containing 0.5% (w/v) BSA to the given concentrations 31.25 - 2000pg / ml. Plates were incubated with detection antibody (0.1 μg / ml) for 2 h prior to the addition of HRP for 30 min. Substrate solution was then added to each well and the reaction stopped by addition of 80 μl / well 1M sulphuric acid. The absorbance was read at λ450nm. OD was plotted to give a standard curve, from which unknown TNF-α concentrations were derived. Level of sensitivity for standard curve is 31.25 pg/ml. Representative of n=3.

### 2.2.14.3. Measurement of IL-6

Measurement of IL-6 was performed using ELISA. The general methodology is described in Section 2.2.14.1. IL-6 mouse anti-human capture (1 μg / ml) and detection (0.2 μg / ml) antibodies were used (Invitrogen, UK). Standards were purchased from R&D systems (UK). Samples were diluted with 0.5% (w/v) BSA. However, individual dilution factors are discussed in subsequent chapters. The concentrations of IL-6 contained within the samples were interpolated from the standard curve (Figure 2.11). The lower limit of detection (sensitivity) was 31.25 pg/ml.
Figure 2.11. Standard curve used to derive unknown concentrations of IL-6. IL-6 standard was diluted in PBS containing 0.5% (w/v) BSA to the given concentrations 31.25 - 2000 pg/ml. Plates were incubated with detection antibody (0.2 μg/ml) for 2 h prior to the addition of HRP for 30 min. Substrate solution was then added to each well and the reaction stopped by addition of 80 μl/well 1M sulphuric acid. The absorbance was read at λ450nm. OD was plotted to give a standard curve, from which unknown IL-6 concentrations were derived. Level of sensitivity for standard curve is 31.25 pg/ml. Representative of n=3.

2.2.15. Quantitative polymerase chain reaction

Real-time multiplex qPCR was undertaken by the RBHT microbiology department to detect *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in induced sputum samples. The commercially available Bacterial Pneumonia CAP kits were used, according to the manufacturer’s instructions (Fast Track Diagnostics, Luxembourg). These kits have been validated and confirmed to have no cross-reactivity with related oral or pulmonary bacterial species. They contain the primer and probe mixes for all three bacteria, positive controls (plasmid pool with *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*; each with $2.5 \times 10^5$ copies/ml), a negative control, internal control (*Streptococcus equi*), reverse transcriptase enzyme mix and buffer.
1ml of sputum plug was first homogenised by vortexing and rocking with glass beads, before DNA was extracted using the easyMAG® extraction kits, according to the manufacturer’s instructions (Biomérieux, USA). Subsequent qPCR assays were then performed on an Applied Biosystems® 7500 thermocycler (ThermoFisher, UK) using the Bacterial Pneumonia CAP kits. The minimum limit of detection was $10^4$ gene copies/ml.

**2.2.16. Statistical analysis**

A statistical analysis plan was designed separately for the CE01-204 clinical trial by Mr Winston Banya, a medical statistician (Chapter 7). All other data and experimental designs were discussed with the same statistician, who advised on the most appropriate statistical test (Chapters 3 – 6). Power calculations were performed by comparing two means, as previously described (Rosner, 2015), using a statistical power of 80%, significance levels of 5% and a 1:1 study group ratio. An additional 15% was added to the calculated sample size, to allow for the use of non-parametric tests, (Lehmann, 2006). Comparisons between groups (e.g. subject group or experimental condition) were conducted using either the Mann-Whitney U test (unpaired), Wilcoxon signed rank test (paired) or Kruskal-Wallace with Dunn’s post-hoc test (≥ 3 groups). Correlations were performed using a Spearman’s rank correlation coefficient. Data are expressed as mean ± SEM (demographic and normally distributed data) or median ± IQR (not normally distributed). A value of $p < 0.05$ was taken to be significant. All statistical analyses were performed using GraphPad Prism software (version 7.02). Wherever possible, the same colours and symbols have been used to represent the non-smokers ($\blacksquare$ / $\blacksquare$), smokers ($\blacklozenge$ / $\blacklozenge$) and COPD patients ($\bullet$ / $\square$) within the figures of this thesis.
Chapter 3

Using Alveolar Macrophages and Monocyte-Derived Macrophages to Study Phagocytosis in Health and COPD
3.1. Introduction

The alveolus is in continuity with the upper respiratory tract and therefore exposed to a diverse array of inhaled microbes and particulate matter. Innate immune cells reside within these airspaces, where they are strategically placed to interact with any inspired microorganisms or particles. AM account for the vast majority of these leukocytes (approximately 90-95%) (Harris et al., 1970). They are the primary sentinel, constantly surveying the alveolus, differentiating pathogens from self, and clearing any microbes or particles through phagocytosis (Martin and Frevert, 2005). An increased number of AM (5 – 10-fold) can be found in the airways of COPD patients (Barnes, 2004a). However, despite this increased presence, they are still unable to prevent the colonisation of the lower airways with PPB in over 30% of stable COPD patients. This is most commonly with *H. influenzae* (17%), *S. pneumoniae* (9%) and *M. catarrhalis* (4%) (Rosell et al., 2005).

The mechanisms underpinning bacterial colonisation in COPD are likely to be multifactorial and representative of the many defects that have been identified in both innate and adaptive immunity (Section 1.5). A failure to clear bacteria through phagocytosis may also contribute. A significant number of studies have demonstrated that bacterial phagocytosis by AM is defective in COPD. These include studies employing *E. coli* as prey (Taylor et al., 2010, Hodge and Reynolds, 2012), as well as others that have used known colonising bacteria and PPB, such *H. influenzae* (Berenson et al., 2006, Marti-Lliteras et al., 2009, Berenson et al., 2013), *S. pneumoniae* (Taylor et al., 2010) and *M. catarrhalis* (Berenson et al., 2013). Colonisation with PPB is not benign and even the presence of low bacterial loads in ‘stable’ patients are associated with raised markers of airway inflammation (Sethi et al., 2006), increased exacerbation frequency (Patel et al., 2002) and impaired health status (Banerjee et al., 2004b). The observation that FEV₁ % predicted correlates with the phagocytosis of both *H. influenzae*...
and M. catarrhalis, suggests that impaired PPB clearance may be important in the perpetuation of pulmonary inflammation, resultant tissue damage and COPD progression (Berenson et al., 2013). In contrast, the phagocytosis of inert particles by AM, such as polystyrene beads, has consistently been shown to be unaffected in COPD (Hodge et al., 2003, Taylor et al., 2010, Berenson et al., 2013).

Macrophages isolated from BALF are considered the ‘gold standard’ for investigating AM function in COPD. BAL requires bronchoscopy however, which is invasive and carries inherent risks (Du Rand et al., 2013). This is of particular concern for subjects with a poor cardiorespiratory reserve, such as those with significant COPD and/or associated comorbidities (Peacock et al., 1994). Research bronchoscopies are therefore deemed unnecessarily high risk for such patients, which can result in a sampling bias. The potential for side-effects and complications can also lead to difficulties recruiting patients and healthy controls alike, as well as retaining an adequate number of willing volunteers for repeated procedures (Martinsen et al., 2016). Bronchoscopy is also not a viable option for sampling AM during acute exacerbations. As such, the examination of macrophage function during these important events requires alternative sampling techniques or the use of models. Other potential limitations of BAL include a low cell yield from healthy controls (Lofdahl et al., 2005) and a poor recovery of BALF from COPD patients. The latter has been reported to be as low as 10-40% (Klech and Hutter, 1990), with poorer returns being more likely to reflect the larger airways (Martin et al., 1985). Furthermore, the volume of BALF recovered from COPD patients is inversely proportional to the degree of emphysema (Lofdahl et al., 2005), which further compounds the sampling bias in favour of milder disease.

Pulmonary macrophages can also be isolated from induced sputum or lung tissue that has been surgically resected. Both however, do have significant limitations. Although induction with
hypertonic saline is less invasive, not all subjects can produce a sputum sample (approximately 10%) (Peter et al., 2013) and some patients may experience bronchoconstriction (Pizzichini et al., 2002). Moreover, induced sputum does not closely resemble the distal airways, containing a higher proportion of neutrophils and fewer macrophages than BALF (Maestrelli et al., 1995). Sputum samples are also contaminated by upper respiratory tract secretions, saliva and by cells or bacteria from the mouth (Holz et al., 1998). Samples of resected lung tissue are in short supply and almost exclusively donated by patients with lung cancer, which can influence macrophage function (Pouniotis et al., 2006). The tissue is also frequently digested during processing, with samples therefore containing both IM and AM. In short, neither induced sputum nor the use of lung tissue can be considered superior, or even comparable to BAL.

The limitations and risks associated with sampling AM have led to the development of surrogates. The most commonly adopted is the MDM model. In vivo, AM are derived from circulating blood monocytes that have migrated into the lung and differentiated into resident macrophages (Gordon et al., 1992). This process can be reproduced in vitro by isolating monocytes from peripheral blood and differentiating them in the presence of GM-CSF to generate MDM (Burgess and Metcalf, 1980). A significant proportion of these monocytes will differentiate (> 80%) into a highly pure MDM monolayer (Winkler et al., 2008) (Section 1.6.1.1 and Figure 1.8).

MDM have a consistent and stable phenotype and share many similarities with AM in terms of surface molecular expression (SR included), cytokine responses, resistance to oxidative stress, phagocytic capacity and susceptibility to pathogens, such as human immunodeficiency virus and M. tuberculosis (Akagawa et al., 2006, Winkler et al., 2008). Unlike BAL, venepuncture can also be undertaken during exacerbations (Singh et al., 2013) or in patients with severe airflow obstruction. This allows macrophage function to be studied in all patients, thereby avoiding
sampling bias. Furthermore, venepuncture is less invasive, has fewer adverse effects and is favoured by patients. It is also better suited to studies requiring repeated measures. To date however, a direct comparison of phagocytosis by individually-paired AM and MDM has yet to be undertaken, i.e. comparing and correlating the phagocytic responses of AM and MDM, the latter being differentiated from monocytes that were sampled concurrently.

MDM internalise both inert particles and bacteria, as confirmed by confocal and electron microscopy, which has demonstrated engulfment by phagocytic processes that involve pseudopodia and phagosome formation (Figure 1.8) (Taylor et al., 2010). Moreover, the defective nature of phagocytosis is also replicated in MDM from COPD patients. In a detailed analysis, Taylor et al. (2010) found that MDM from COPD patients internalised H. influenzae, S. pneumoniae and E. coli to a lesser extent than MDM derived from non-smokers and smoking controls. In contrast, beads were internalised to the same extent by MDM from all three groups (Taylor et al., 2010). Such findings mirror results with AM and support the continued use of this validated model to further investigate defective phagocytosis in COPD. They also suggest that any defect is likely to be inherent to the circulating monocytes of susceptible individuals, rather than the result of environmental exposure within the alveolus.

This chapter will explore whether the use of AM is suitable for a PhD investigation into defective bacterial phagocytosis in COPD, or whether BAL would hinder such a study. It will also examine for the first time, whether phagocytosis by paired AM and MDM do indeed correlate, in order to lend further support to the argument that MDM are a reliable model for studying phagocytosis.
3.2. Hypothesis

The hypothesis for this chapter is: ‘MDM provide a surrogate measure of phagocytosis by AM for an investigation into defective bacterial phagocytosis in COPD’.

3.3. Aims

The following aims were adopted in order to investigate the above hypothesis:

- Screen a population of subjects, to ascertain their willingness to consent to bronchoscopy and BAL (non-smokers, smokers and non-severe COPD patients).
- Undertake bronchoscopies and BAL in all consenting subjects to determine the rates of procedural complications and number of AM that can be isolated from BALF. Specifically, to determine whether an adequate number of cells can be consistently obtained from all subjects for phagocytosis assays with three prey (beads, *H. influenzae* and *S. pneumoniae*).
- Perform phagocytosis assays (beads, *H. influenzae* and *S. pneumoniae*) with AM and compare results from non-smokers, smokers and COPD patients. If defective bacterial phagocytosis is not replicated in COPD, these data will be used to perform power calculations to determine whether a practical sample size might be able to detect statistically significant differences between COPD patients and controls.
- Obtain paired AM and MDM from non-smokers, smokers and COPD patients in order to correlate phagocytosis by the two macrophage types (beads, *H. influenzae* and *S. pneumoniae*).

3.4. Methods

3.4.1. Subject selection

Healthy non-smokers, smoking controls and COPD patients were recruited as described in Section 2.2.1. Subjects were asked if they would be willing to consent to bronchoscopy and
BAL. Patients with severe COPD (FEV₁ < 50 %) were not included, as they have a higher risk of bronchoscopy complications and are less likely to provide an adequate return of BALF.

3.4.2. Bronchoscopy

Bronchoscopy was performed in order to collect BALF for the isolation of AM. The technique is described in Section 2.2.5.5. Briefly, a total of 200 ml of 0.9% (w/v) saline was instilled via the operating channel of the bronchoscope and any returning fluid was aspirated. The total volume of returning BALF was recorded. Subjects were assessed by a clinician prior to hospital discharge and followed up by telephone at 48 h and 1 week. Any procedural complications were recorded.

3.4.3. Isolation of alveolar macrophages

AM were isolated from BALF, as described in Section 2.2.6.4. Cell viability was determined using 4% (v/v) trypan blue and only samples with > 70% viability were used. The total number of AM were adjusted according to the proportion of live cells. AM were seeded into black 96-well tissue culture plates at 1 x 10⁵ viable macrophages/well and rested overnight, prior to phagocytosis assays the following morning. AM were assessed for any signs of infection, including changes in cell media (e.g. turbidity or colour), malodour or the visualisation of microorganisms under light microscopy. Any infected samples were discarded.

3.4.4. Isolation and culture of MDM

MDM were generated from 60 ml of peripheral blood, as described in Section 2.2.6. Briefly, monocytes were isolated by adherence from the PBMC fractions of Percoll gradients, and seeded into black 96-well tissue culture plates at 1 x 10⁵ cells/well. MDM were then cultured in the presence of GM-CSF (2 ng/ml) for 12 days.
3.4.5. **Paired AM and MDM**

Paired AM and MDM were obtained using the methodology described in Section 3.4.3 and Section 3.4.4, respectively. Subjects were intravenously cannulated immediately before bronchoscopy, allowing 60 ml of whole blood to be drawn. These samples were used to generate MDM and paired with the AM that were isolated from the corresponding BALF. Both monocytes and AM were seeded in black 96-well tissue culture plates at $1 \times 10^5$ cells/well.

3.4.6. **Phagocytosis assays**

Phagocytosis assays were performed with AM and MDM using fluorimetry, as described in Section 2.2.11. Incubation was for 4h and any extracellular fluorescence was quenched with trypan blue 1% ($v/v$). Fluorescence was determined using a plate reader (excitation $\lambda 480\text{nm}$ and emission $\lambda 520\text{nm}$) and the relative fluorescence units (RFU) were used as a measure of phagocytosis. Intra- and inter-assay variability was limited by ensuring that all assays were run in quadruplicate and by using the same batch of labelled *H. influenzae* and *S. pneumoniae* for every experiment, respectively.

3.4.6.1. **Phagocytosis of beads**

Fluorescently-labelled microspheres of 2.0 μm diameter (4.5 x 10⁹ beads/ml) were used (Section 2.2.11.1). They were diluted in RPMI (11μl/ml) before being added to individual wells containing $1 \times 10^5$ of either MDM or AM (final working concentration of $50 \times 10^6$ microspheres/ml). The intra-assay CV, *i.e.* the mean CV between sample replicates was < 10% for phagocytosis assays involving beads. This was true for all three subject groups and for assays involving both AM and MDM.
3.4.6.2. Phagocytosis of bacteria

Aliquots of *H. influenzae* and *S. pneumoniae* were used (Section 2.2.9.4). They were diluted in RPMI (100μl bacteria/ml) before being added to individual wells containing 1 x 10^5 of either MDM or AM (final working concentration of 5x10^6 CFU/ml). The intra-assay CV for bacterial phagocytosis was < 10% for assays with both *H. influenzae* and *S. pneumoniae*. This was true for all three subject groups and for assays involving both AM and MDM.

3.4.7. Viability assays

The viability of AM and MDM was assessed immediately after phagocytosis using MTT assays. This is described in Section 2.2.12.

3.4.8. Data analysis

Normal distributions were not assumed and all analyses were therefore performed using non-parametric tests. Demographic data were compared using a Kruskal-Wallis with Dunn’s post-hoc test. Comparisons between 2 groups were made using either Mann-Whitney U tests (unpaired) or Wilcoxon signed rank tests (paired). Comparisons between ≥ 3 groups were made using a Kruskal-Wallis with Dunn’s post-hoc test. Correlations were performed using a Spearman’s rank correlation coefficient. In all instances, the null hypothesis (i.e. no difference or an absent correlation) was rejected at p < 0.05. Power calculations were performed by comparing two means, as previously described (Rosner, 2015). A statistical power of 80%, significance levels of 5% and a 1:1 study group ratio were applied. To allow for the subsequent use of non-parametric tests, an additional 15% was added to the calculated sample size (Lehmann, 2006). All analyses (except power calculations) were performed using GraphPad Prism software (USA).
3.5. Results

3.5.1. Sampling AM by bronchoalveolar lavage

3.5.1.1. Subject demographics

The characteristics of the non-smoking, smoking and COPD subjects who underwent bronchoscopy are shown in Table 3.1. The groups were matched for age. There were an equal number of male and female non-smokers, but more male subjects than females in the smoking group, and there were more female than male COPD patients. The smokers and COPD patients were matched for smoking history. Most of the COPD patients were ex-smokers. All COPD patients had a FEV$_1 \geq$ 50% predicted. Nevertheless, the COPD group still had a lower FEV$_1$ (L and % predicted) than both the non-smokers and smokers. The FEV$_1$/FVC ratio was also lower for the COPD group, but only when compared to the non-smokers. No differences were observed in FVC (L).

3.5.1.2. Recruitment and bronchoscopy complications

Recruitment data and complications from bronchoscopy are shown in Table 3.2. A total of 23 non-smokers, 19 smokers and 18 COPD patients (all potentially suitable) were asked to participate. However, only 8 (35%), 7 (37%) and 11 (61%) respectively consented and underwent bronchoscopy. Of the 34 subjects who declined, 31 (91%) cited the procedure’s invasiveness and potential complications as reasons to withhold consent, 2 (6%) felt the reimbursement amount to be inadequate and 1 (3%) reported severe symptoms following a previous research bronchoscopy. Actual complications were only reported by 2 of the subjects who underwent bronchoscopy. Both were COPD patients who complained of fever within 24 h of BAL.
<table>
<thead>
<tr>
<th></th>
<th>Non-Smoker (n = 8)</th>
<th>Smoker (n = 7)</th>
<th>COPD (n = 11)</th>
</tr>
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<tbody>
<tr>
<td>Age (years)</td>
<td>61.0 ± 2.8</td>
<td>58.9 ± 3.2</td>
<td>62.9 ± 1.9</td>
</tr>
<tr>
<td>Gender (male : female)</td>
<td>4:4</td>
<td>4:3</td>
<td>3:8</td>
</tr>
<tr>
<td>Smoking history (pack years)</td>
<td>0.0 ± 0.0</td>
<td>31.7 ± 8.1**</td>
<td>32.0 ± 3.8***</td>
</tr>
<tr>
<td>Current smoker (yes : no)</td>
<td>-</td>
<td>7:0</td>
<td>4:7</td>
</tr>
<tr>
<td>FEV₁ (L)</td>
<td>3.12 ± 0.25</td>
<td>2.75 ± 0.31</td>
<td>1.74 ± 0.12***#</td>
</tr>
<tr>
<td>FEV₁ (% predicted)</td>
<td>105.6 ± 6.2</td>
<td>92.9 ± 3.7</td>
<td>66.6 ± 2.5****#</td>
</tr>
<tr>
<td>FVC (L)</td>
<td>3.72 ± 0.29</td>
<td>3.57 ± 0.36</td>
<td>2.99 ± 0.28</td>
</tr>
<tr>
<td>FEV₁/FVC</td>
<td>0.79 ± 0.03</td>
<td>0.77 ± 0.03</td>
<td>0.63 ± 0.04*</td>
</tr>
</tbody>
</table>

**Table 3.1. Subject demographics.** Participants underwent spirometry, with FEV₁ and FVC being recorded. A single pack year is equivalent to 20 cigarettes smoked every day for 1 year. Data presented are mean ± SEM. *** represents p < 0.001, ** represents p < 0.01, * represents p < 0.05 relative to non-smokers. # represents p < 0.05 relative to smoking controls.

<table>
<thead>
<tr>
<th></th>
<th>Non-Smoker</th>
<th>Smoker</th>
<th>COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects asked to participate</td>
<td>23</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>Bronchoscopies</td>
<td>8 (35%)</td>
<td>7 (37%)</td>
<td>11 (61%)</td>
</tr>
<tr>
<td>Procedure complications (a)</td>
<td>0</td>
<td>0</td>
<td>2 (18%)</td>
</tr>
</tbody>
</table>

**Table 3.2. Recruitment data and complications from bronchoscopy.** Non-smokers, smokers and COPD patients were asked to participate. Subjects who provided consent underwent bronchoscopy and BAL. Data relating to recruitment and any complications were recorded. (a) complications within 1 week of follow up. Presented are the raw numbers and percentages.
3.5.1.3. Isolation of AM from BALF

Data pertaining to the isolation of AM from BALF are shown in Table 3.3. Smaller BALF returns (ml and % of instilled saline) were obtained from COPD patients, relative to non-smokers but not smokers. The greatest number of recovered AM were from smokers. Non-smokers and COPD patients yielding a similar but lower number of AM. A minimum of $1.2 \times 10^6$ AM was required for phagocytosis assays with beads, *H. influenzae* and *S. pneumoniae*. However, $< 1.2 \times 10^6$ AM were isolated from the BALF of 1 non-smoker and 3 COPD patients (13% and 27% of bronchoscopies, respectively). A single sample was also infected and was therefore not used (smoker). In summary, from the 26 subjects who underwent BAL (8 non-smokers, 7 smokers and 11 COPD patients), a total of 21 samples were subsequently available for phagocytosis assays (7 non-smokers, 6 smokers and 8 COPD patients).
### Table 3.3. Isolation of AM from BALF

Participants underwent bronchoscopy and BAL was performed. AM were isolated from the BALF and data relating to this isolation were recorded. Cells were rested overnight and inspected immediately before phagocytosis assays. Presented are medians ± IQR, raw numbers and percentages. **(a)** % relative to instilled volume of saline (200 ml). **(b)** $1.2 \times 10^6$ AM are the minimum number of AM required for phagocytosis assays; samples containing fewer cells were considered insufficient for use in this study. **(c)** infection of AM that precluded use in phagocytosis assays. *represents $p < 0.05$ relative to non-smokers. *represents $p < 0.05$ relative to smoking controls.

<table>
<thead>
<tr>
<th></th>
<th>Non-Smoker (n = 8)</th>
<th>Smoker (n = 7)</th>
<th>COPD (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALF return</td>
<td>ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% <em>(a)</em></td>
<td>125 ± 69</td>
<td>100 ± 60</td>
<td>80 ± 60*</td>
</tr>
<tr>
<td>Number of AM isolated $(x 10^6)$</td>
<td>63 ± 34</td>
<td>50 ± 30</td>
<td>40 ± 30*</td>
</tr>
<tr>
<td>Insufficient AM for assay <em>(b)</em></td>
<td>9.5 ± 6.1#</td>
<td>24.8 ± 58.4</td>
<td>5.0 ± 12.0#</td>
</tr>
<tr>
<td>AM infection <em>(c)</em></td>
<td>1 (13%)</td>
<td>0</td>
<td>3 (27%)</td>
</tr>
<tr>
<td>Samples available for phagocytosis</td>
<td>7 (88%)</td>
<td>6 (86%)</td>
<td>8 (73%)</td>
</tr>
</tbody>
</table>

**3.5.1.4. Relationship between the volume of recovered BALF and airflow obstruction**

To determine whether the volume of recovered BALF was related to the degree of airflow obstruction, correlations were performed with FEV$_1$ (% predicted) (**Figure 3.1**). When analysing all subjects together, a moderate positive correlation was observed between the volume of recovered BALF and FEV$_1$ (% predicted). When non-smokers and smokers were analysed independently, no correlations were found. For COPD patients, however, a strong positive correlation was present.
Figure 3.1. Correlation between the volume of recovered BALF and airflow obstruction (FEV₁ % predicted). Non-smokers (▲), smokers (■) and COPD patients (●) underwent bronchoscopy and BAL was performed, n = 8, n = 7 and n = 11 respectively. The volume of recovered BALF was recorded. Spirometry was performed to determine FEV₁ (% predicted). Data presented are Spearman rank correlation coefficients.

3.5.2. Phagocytosis by AM

3.5.2.1. Subject demographics

Demographic data for the subjects who donated AM for phagocytosis experiments are shown in Table 3.4. The non-smokers, smokers and COPD patients were matched for age. Male participants outnumbered females in the non-smoking and smoking groups, while the COPD patients were predominantly female. The smokers and COPD patients were matched for smoking history. Most of the COPD patients were ex-smokers. Non-smokers and smokers were matched for all measures of spirometry. COPD patients however, had a lower FEV₁ (L and % predicted) when compared to the non-smokers, but not the smokers. Patients with severe COPD were not recruited. As such, all COPD patients had a FEV₁ ≥ 50% predicted. No
differences were observed in FVC (L) or FEV₁/FVC between the COPD patients and any of the other two subject groups.

<table>
<thead>
<tr>
<th></th>
<th>Non-Smoker (n = 7)</th>
<th>Smoker (n = 6)</th>
<th>COPD (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>60.7 ± 3.2</td>
<td>57.2 ± 3.2</td>
<td>60.4 ± 1.9</td>
</tr>
<tr>
<td><strong>Gender (male : female)</strong></td>
<td>4:3</td>
<td>4:2</td>
<td>2:6</td>
</tr>
<tr>
<td><strong>Smoking history (pack years)</strong></td>
<td>0.0 ± 0.0</td>
<td>32.0 ± 9.6*</td>
<td>30.5 ± 2.8**</td>
</tr>
<tr>
<td><strong>Current smoker (yes : no)</strong></td>
<td>-</td>
<td>6:0</td>
<td>2:6</td>
</tr>
<tr>
<td><strong>FEV₁ (L)</strong></td>
<td>3.21 ± 0.27</td>
<td>2.82 ± 0.35</td>
<td>1.74 ± 0.15**</td>
</tr>
<tr>
<td><strong>FEV₁ (% predicted)</strong></td>
<td>109.4 ± 5.6</td>
<td>91.7 ± 4.2</td>
<td>68.3 ± 3.1***</td>
</tr>
<tr>
<td><strong>FVC (L)</strong></td>
<td>3.73 ± 0.33</td>
<td>3.69 ± 0.41</td>
<td>2.68 ± 0.28</td>
</tr>
<tr>
<td><strong>FEV₁/FVC</strong></td>
<td>0.81 ± 0.02</td>
<td>0.76 ± 0.03</td>
<td>0.69 ± 0.04</td>
</tr>
</tbody>
</table>

**Table 3.4. Subject demographics.** Participants underwent spirometry, with FEV₁ and FVC being recorded. A single pack year is equivalent to 20 cigarettes smoked every day for 1 year. Data presented are mean ± SEM. *** represents p < 0.001, ** represents p < 0.01, * represents p < 0.05 relative to non-smokers.

3.5.2.2. **Effect of COPD on the phagocytosis of beads by AM**

The effect of COPD on the phagocytosis of beads by AM was examined by comparing the results of phagocytosis assays from COPD patients and control subjects (Figure 3.2). No differences were observed between COPD patients, non-smokers and smokers.
Figure 3.2. Comparison of bead phagocytosis by AM from non-smokers, smokers and COPD patients. AM were isolated from the BALF of non-smokers ( ), smokers ( ) and COPD patients ( ), n = 7, n = 6 and n = 8 respectively. Cells were rested overnight prior to performing phagocytosis assays with fluorescently-labelled beads. Data presented are median RFU ± IQR and range.

3.5.2.3. Effect of COPD on the phagocytosis of bacteria by AM

Any effect of COPD on the phagocytosis of bacteria (H. influenzae and S. pneumoniae) was examined by comparing the results of phagocytosis assays from COPD patients and control subjects (Figure 3.3). No differences were observed between COPD patients, non-smokers and smokers for both H. influenzae and S. pneumoniae. A trend was apparent however, suggesting a reduction in the phagocytosis of H. influenzae by AM from COPD patients, relative to non-smoking controls. The median RFU x 10³ (± IQR) for the phagocytosis of H. influenzae was 2.2 (± 1.6) for COPD patients and 3.8 (± 3.1) for non-smokers (p = 0.08). This trend was less clear for S. pneumoniae, where median RFU x 10³ (± IQR) were 2.0 (± 1.5) for COPD patients and 2.4 (± 2.1) and for non-smokers (p = 0.50).

The data presented in Figure 3.3 were used to perform power calculations in order to determine the minimum sample size required to detect a statistically significant difference in
bacterial phagocytosis between non-smokers and COPD patients. 17 subjects would be required in each group to detect a difference in the phagocytosis of *H. influenzae* by AM from non-smokers and COPD patients. In contrast, 40 non-smokers, smokers and COPD patients would be needed to observe a difference in the phagocytosis of *S. pneumoniae*.

![Figure 3.3](image)

**Figure 3.3. Comparison of bacterial phagocytosis by AM from non-smokers, smokers and COPD patients:** (A) *H. influenzae* (B) *S. pneumoniae*. AM were isolated from the BALF of non-smokers (■), smokers (■) and COPD patients (■), n = 7, n = 6 and n = 8 respectively. Cells were rested overnight prior to performing phagocytosis assays with fluorescently-labelled bacteria. Data presented are median RFU ± IQR and range. p = 0.08 for COPD patients relative to non-smokers (*H. influenzae* only).

### 3.5.2.4. Effect of beads and bacteria on the viability of AM

To ensure that exposure to prey was not cytotoxic, MTT assays were performed with AM after phagocytosis with beads, *H. influenzae* and *S. pneumoniae* (Figure 3.4). Exposure to prey for 4h did not lead to a reduction in the viability of AM from non-smokers, smokers or COPD patients. Conversely, an apparent improvement in the mitochondrial reduction of MTT was observed after incubation with both bacteria. Importantly however, similar data were observed for all three subject groups.
Figure 3.4. Effect of beads and bacteria on the viability of AM: (A) Non-smokers (B) Smokers (C) COPD. AM were isolated from the BALF of non-smokers (□), smokers (□) and COPD patients (□), n = 7, n = 6, n = 8 respectively. They were incubated for 4h with beads, *H. influenzae* (HI) or *S. pneumoniae* (SP). Controls were left untreated (UT). After phagocytosis, AM were incubated with 50 μl MTT solution (45 min at 37°C). Supernatants were then aspirated and AM lysed with 50 μl DMSO. Plates were read at λ570 nm on a spectrophotometer. Data presented are mean cell viability (± SEM) as a percentage of the UT controls (UT = 100% viability, represented by horizontal dotted lines). ** represents p < 0.01, * represents p < 0.05 relative to UT control.
3.5.2.5. Relationship between phagocytosis of bacteria and beads

The ability of AM to phagocytose inert beads was compared to their ability to phagocytose bacteria by correlating the RFU of experiments with beads to results obtained with *H. influenzae* (Figure 3.5; Panel A) and *S. pneumoniae* (Figure 3.5; Panel B). When analysing all subjects together, a moderate positive correlation was observed between the phagocytosis of beads and *H. influenzae*. No correlation was present between the phagocytosis of beads and *S. pneumoniae*. Most importantly however, when the three groups were analysed independently, no correlations were found between the phagocytosis of beads and either of the bacteria.

![Figure 3.5. Correlations between the phagocytosis of beads and bacteria by AM: (A) *H. influenzae* (HI) (B) *S. pneumoniae* (SP). AM were isolated from the BALF of non-smokers (▲), smokers (■) and COPD patients (●), n = 7, n = 6 and n = 8 respectively. Cells were rested overnight prior to performing phagocytosis assays with fluorescently-labelled beads and bacteria. Data presented are Spearman rank correlation coefficients (subject groups analysed together).](image)
3.5.2.6. Relationship between phagocytosis of Gram-negative and -positive bacteria

The ability of AM to phagocytose Gram-negative bacteria was compared to their ability to phagocytose Gram-positive bacteria by correlating the RFU from experiments with *H. influenzae* and *S. pneumoniae* respectively (Figure 3.6). When analysing all subjects together, a moderate positive correlation was observed between the phagocytosis of *H. influenzae* and *S. pneumoniae*. Most importantly however, no correlations were found when the three groups were analysed independently.

**Figure 3.6. Correlations between the phagocytosis of *H. influenzae* and *S. pneumoniae* by AM.** AM were isolated from the BALF of non-smokers (▲), smokers (■) and COPD patients (●), n = 7, n = 6 and n = 8 respectively. Cells were rested overnight prior to performing phagocytosis assays with fluorescently-labelled *H. influenzae* (HI) and *S. pneumoniae* (SP). Data presented are Spearman rank correlation coefficients (subject groups analysed together).
3.5.3. Comparing phagocytosis by MDM and AM

Section 3.5.1 highlighted some of the limitations involved in using AM isolated from BALF to study phagocytosis in COPD. Furthermore, it was determined in Section 3.5.2 that a significant number of bronchoscopies would be required to obtain an adequate number of AM in order to detect a statistically significant difference in bacterial phagocytosis between COPD patients and controls. As such, a more readily available surrogate is required. MDM are a validated and commonly adopted model. This section explores whether MDM provide a reliable surrogate measure of bead and bacterial phagocytosis by AM, by correlating the results from assays involving paired MDM and AM. Importantly, the AM and monocyte precursors had been sampled concurrently from the same patients.

3.5.3.1. Subject demographics

Demographic data for the subjects who donated paired AM and MDM are shown above (Table 3.4) and discussed in Section 3.5.2.1.

3.5.3.2. Comparing the phagocytosis of beads by paired MDM and AM

Phagocytosis assays were undertaken with fluorescently-labelled beads and correlations were performed between the RFU from individually-paired MDM and AM (Figure 3.7). A positive correlation was observed between the phagocytosis of beads by AM and MDM, but only when independently analysing the COPD patients, or with the subject groups as a whole (Table 3.5).
Figure 3.7. Correlation between the phagocytosis of beads by paired AM and MDM. AM were isolated from the BALF of non-smokers (▲; n = 6), smokers (■; n = 6) and COPD patients (●; n = 7). They were rested overnight before incubation with fluorescently-labelled beads for 4h. Phagocytosis was then quantified by fluorimetry. Monocytes were concurrently isolated from the peripheral blood of the same participants (drawn immediately before bronchoscopy). They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. MDM underwent phagocytosis, as described above. Data presented are Spearman rank correlation coefficients.

3.5.3.3. Comparing the phagocytosis of \textit{H. influenzae} by paired MDM and AM

Phagocytosis assays were undertaken with fluorescently-labelled \textit{H. influenzae} and correlations were performed between the RFU from individually-paired MDM and AM (Figure 3.8). A positive correlation was observed between the phagocytosis of \textit{H. influenzae} by AM and MDM, but only when analysing all of the subject groups together (Table 3.5).
Figure 3.8. Correlation between the phagocytosis of *H. influenzae* by paired AM and MDM. AM were isolated from the BALF of non-smokers (▲; n = 7), smokers ( ■; n = 6) and COPD patients ( ●; n = 7). They were rested overnight before incubation with fluorescently-labelled *H. influenzae* for 4h. Phagocytosis was then quantified by fluorimetry. Monocytes were concurrently isolated from the peripheral blood of the same participants (drawn immediately before bronchoscopy). They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. MDM underwent phagocytosis, as described above. Data presented are Spearman rank correlation coefficients.

### 3.5.3.4. Comparing the phagocytosis of *S. pneumoniae* by paired MDM and AM

Phagocytosis assays were undertaken with fluorescently-labelled *S. pneumoniae* and correlations were performed between the RFU from individually-paired MDM and AM (Figure 3.9). A positive correlation was observed between the phagocytosis of *S. pneumoniae* by AM and MDM, but only when independently analysing the smokers, or with the subject groups as a whole (Table 3.5).
Figure 3.9. Correlation between the phagocytosis of *S. pneumoniae* by paired AM and MDM. AM were isolated from the BALF of non-smokers (▲; n = 7), smokers (■; n = 6) and COPD patients (●; n = 7). They were rested overnight before incubation with fluorescently-labelled *S. pneumoniae* for 4h. Phagocytosis was then quantified by fluorimetry. Monocytes were concurrently isolated from the peripheral blood of the same participants (drawn immediately before bronchoscopy). They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. MDM underwent phagocytosis, as described above. Data presented are Spearman rank correlation coefficients.

Table 3.5 summarises the data presented in Figures 3.7, 3.8 and 3.9 (i.e. correlations for phagocytosis by paired AM and MDM). In summary, although positive correlations were observed for all 3 prey, this was only when the subject groups were analysed as a whole and there were an insufficient number of samples to demonstrate significant correlations within each subject group. However, when considering the COPD patients alone, a strong positive correlation was still seen between the phagocytosis of beads by paired AM and MDM. Likewise, a strong positive correlation was observed for the phagocytosis of *S. pneumoniae* by paired AM and MDM from smokers.
Table 3.5. Correlation coefficients for phagocytosis by paired AM and MDM. AM were isolated from the BALF of non-smokers (n = 6-7), smokers (n = 6) and COPD patients (n = 7). They were rested overnight before incubation with fluorescently-labelled prey for 4h. Phagocytosis was then quantified by fluorimetry. Monocytes were concurrently isolated from the peripheral blood of the same participants (drawn immediately before bronchoscopy). They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. MDM underwent phagocytosis, as described above. Data presented are Spearman rank correlation coefficients (and corresponding p values) for phagocytosis by paired AM and MDM (subject groups analysed independently and as a whole). Data in bold is significant (p < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Non-smoker</th>
<th>Smoker</th>
<th>COPD</th>
<th>All Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Beads</strong></td>
<td>Spearman r</td>
<td>0.49</td>
<td>0.37</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.18</td>
<td>0.25</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>H. influenzae</strong></td>
<td>Spearman r</td>
<td>0.54</td>
<td>0.37</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.12</td>
<td>0.25</td>
<td>0.39</td>
</tr>
<tr>
<td><strong>S. pneumoniae</strong></td>
<td>Spearman r</td>
<td>0.29</td>
<td>0.94</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.28</td>
<td><strong>0.008</strong></td>
<td>0.28</td>
</tr>
</tbody>
</table>

3.6. Discussion

The data presented in this chapter highlights many of the limitations associated with BAL and the use of AM for research. However, strong correlations were found between the phagocytic responses of AM and MDM, and this supports the use of MDM as an alternative for studying phagocytosis in COPD.

There was a marked reluctance among volunteers to consent to bronchoscopy. Indeed, less than half of all suitable non-smokers, smokers and COPD patients provided their consent (43% overall). This was most notable among control subjects, with only 36% consenting, but less of
a concern for COPD patients (61% consented). Although no studies have specifically addressed the recruitment of COPD patients for research bronchoscopy, similar data have been reported for patients with cystic fibrosis and HIV (Martinsen et al., 2016). It has also been identified previously that control subjects are less likely to offer consent (Chudleigh et al., 2013). In keeping with these reports, potential volunteers for the current study almost universally cited the procedure’s invasiveness and possible complications as reasons to withhold consent (Lipman et al., 1998, Kye et al., 2009, Mtunthama et al., 2008). In truth, the actual rate of adverse events was low (8%), all were non-severe (fever only), self-limiting and exclusive to COPD patients. Regardless, a failure to recruit an adequate number of volunteers is always likely to hamper studies involving the use of an invasive procedure, such as bronchoscopy.

When analysing the data pertaining to the recovery of BALF, further important limitations were identified. Despite only recruiting COPD patients with mild or moderate airflow obstruction, BALF returns were still notably lower from COPD patients when compared to non-smokers (36% less). Although not statistically significant, there also appeared to be a lower recovery relative to smokers (20% less). In support of these findings, moderate correlations were observed between the volumes of BALF recovered from all subjects and the degree of airflow obstruction (FEV₁ % predicted). A strong correlation was observed for COPD patients alone, despite the limited number of patients and narrow range of non-severe FEV₁ measures (54.1 – 79.5% predicted). This likely represents a failure to aspirate the instilled saline from the narrowed airways and emphysematous lung during lavage. This has been described previously (Lofdahl et al., 2005). When taken together, these data highlight that the recovery of adequate volumes of BALF could be an issue for studies involving COPD patients, especially when recruiting those with advanced disease.
Current smokers without airflow obstruction yielded the greatest number of AM, approximately 5 and 2.5 times more than COPD patients and non-smokers respectively. It is possible that this reflects an increased recruitment of leukocytes to the alveolar space in response to cigarette smoking (Barnes, 2004a). Although the median number of AM isolated from all three groups was greater than the 1.2 \times 10^6 cells required to undertake a complete series of phagocytosis assays, a significant proportion of subjects still failed to yield this minimum number (15% in total). These subjects were exclusively non-smokers and COPD patients. In summary, from the 60 subjects that were invited to participate, only 21 AM samples were ultimately available for phagocytosis (35%). While the major limitation for control subjects appeared to be recruitment due to a lack of their willingness to consent, the recovery of an adequate volume of BALF and a poor AM yield, hindered the acquisition of data for COPD patients. As such, it is clear that BAL and the use of AM is a very labour intensive, inefficient and unrealistic approach for this PhD investigation.

Defective bacterial phagocytosis by AM from COPD patients was not confirmed in this chapter. This was most likely the result of underpowered data and possibly by limiting recruitment to those COPD patients with milder disease (mean FEV_1 \pm SEM was only 66.6 \pm 2.5 % predicted). Indeed, bacterial phagocytosis has been shown to positively correlate with FEV_1 % predicted (Berenson et al., 2013). Previously published work has convincingly shown that AM from COPD patients are defective in their phagocytosis of both H. influenzae and S. pneumoniae (Berenson et al., 2006, Marti-Lliteras et al., 2009, Taylor et al., 2010, Berenson et al., 2013). All of these studies however, had recruited considerably more subjects (n = 14 – 64 per group) and one had included COPD patients with more pronounced airflow obstruction (mean FEV_1 % predicted \pm SEM of 50.6 \pm 4.0) (Taylor et al., 2010).
A clear trend towards a reduction in the phagocytosis of *H. influenzae* by AM was observed for COPD patients. This led to power calculations being performed. Using these data, it was estimated that a total of 17 subjects would be required in each group in order to detect a statistically significant difference in the phagocytosis of *H. influenzae*. These findings were very similar to the number of subjects recruited by Berenson *et al.* (2006), Marti-Lliteras *et al.* (2009) and Taylor *et al.* (2010). Although any reduction in the phagocytosis of *S. pneumoniae* by COPD patients was less clear from the data presented herein, power calculations were still performed. They suggested that 40 subjects would be required in each group for a statistically significant difference in *S. pneumoniae* phagocytosis. This is considerably more than that required for *H. influenzae*, but still in keeping with the number of subjects recruited by Berenson *et al.* (2013). When considering the aforementioned difficulties with subject recruitment, BALF return and AM isolation, these sample sizes equate to a substantial number of bronchoscopies (50 per group) and subject screenings (potentially 115 for each group). This was not possible for the present study.

Potential relationships were demonstrated between the phagocytosis of different prey by AM. When considering all subjects, positive correlations were observed between the phagocytosis of beads and *H. influenzae*, as well as between Gram-negative (*H. influenzae*) and Gram-positive bacteria (*S. pneumoniae*). However, these data were underpowered and therefore unable to identify any correlations when analysing each group independently, or a definitive relationship between the phagocytosis of beads and *S. pneumoniae* (*p = 0.10* for the subject groups analysed as a whole). It might be argued that any correlations simply reflect differences in the phagocytosis of beads, *H. influenzae* and *S. pneumoniae* by non-smokers, smokers and COPD patients. This same dataset however, did not confirm these differences.
The effect of beads, *H. influenzae* and *S. pneumoniae* on the viability of AM was also explored in this chapter. A MTT assay was employed, whereby the metabolic activity of AM was used as a surrogate for viability, specifically the cellular reduction of MTT by mitochondrial oxidoreductase enzymes. Using this assay, beads were not found to significantly impair the viability of AM from non-smokers, smokers or COPD patients. The presence of bacteria however, did lead to an apparent improvement in the enzymatic reduction of MTT, rather than any decrease in AM viability in cells from all groups and may reflect oxidative changes within the cell upon bacterial phagocytosis.

The data presented above have highlighted that the use of AM from BALF is not practical for an investigation into defective phagocytosis in COPD. Other limitations include a sampling bias (bronchoscopy is not suitable in severe COPD) and an inability to sample during acute exacerbations or repeatedly over short time courses. MDM are a validated, commonly-used and accepted surrogate for AM that avoid many of these limitations (Winkler *et al.*, 2008). Given that they are generated from peripheral blood monocytes, sampling is minimally invasive, relatively risk free and can be undertaken in severe COPD, repeatedly over short time courses or during acute exacerbations. Venepuncture is also likely to be favoured by research volunteers. For the first time, the phagocytic responses of individually-paired AM and MDM were correlated; the latter being generated from peripheral blood monocytes sampled immediately before bronchoscopy. When analysing all subjects, significant positive correlations were observed for all the phagocytic prey used in these assays. However, positive correlations were not consistently identified when independently analysing each subject group. This most likely reflects the underpowered nature of the data and confirming such findings in a larger population is therefore a priority. This would lend further support to the use of MDM as a model for exploring defective phagocytosis in COPD.
MDM remain a model however, and any limitations must therefore be acknowledged. For example, MDM do not replicate the corticosteroid-resistant nature of AM isolated from COPD patients (Ito et al., 2006). Moreover, AM are highly heterogenous (Mosser and Edwards, 2008) and plastic (Galli et al., 2011), and this cannot be replicated by culturing monocytes in the presence of a single growth factor (i.e. GM-CSF). MDM can be polarised in vitro by differentially culturing monocytes in either GM-CSF or M-CSF to generate cells of varying morphologies and cell surface receptor expression, that are primed to respond in a more M1-like (pro-inflammatory) or M2-like (inflammatory-resolving) manner, respectively (Akagawa et al., 2006). This includes cytokine (Akagawa et al., 2006) and phagocytic responses, with MDM cultured in M-CSF internalising greater quantities of bacteria (Such et al., 2013). As such, future studies might want to consider incorporating MDM that have been polarised through culture in either GM-CSF or M-CSF. However, little is known about the predominant AM phenotype in health or indeed COPD. As such, AM isolated from BALF must therefore still be considered the gold standard.

The hypothesis for this chapter was: ‘MDM provide a surrogate measure of phagocytosis by AM for an investigation into defective bacterial phagocytosis in COPD’. This hypothesis can be accepted. The data presented herein have confirmed a number of limitations associated with using AM to study phagocytosis in COPD, many of which can be avoided with MDM. These include difficulties with subject recruitment, procedural complications, sampling bias towards a milder spectrum of disease and low cell yields. Power calculations also confirmed that a significant number of subjects and an unrealistic number of bronchoscopies would be required. An even greater number would be needed to explore the underpinning mechanisms of defective phagocytosis and trial any potential treatments. Both of these research goals are important aims of subsequent chapters. Strong correlations were observed between the
phagocytic capability of AM and MDM, lending weight to the argument that MDM should be adopted as a surrogate. With this in mind, this thesis will use MDM to further explore the defective nature of bacterial phagocytosis. MDM are a model however, with potential limitations. Attempts have therefore been made to validate any findings in AM.
CHAPTER 4

Defective Phagocytosis in COPD and its Relationship to Disease Severity, Symptom Burden, Exacerbation Frequency, Pharmacotherapy & Microtubule Polymer Stability
4.1. Introduction

Micro-aspiration from the upper respiratory tract continually supplies the lower airways with bacteria, but competent host defences prevent pathogen colonisation and infection (King et al., 2013). In COPD however, multiple defects have been reported in both adaptive (Bhat et al., 2015) and innate immunity (Shaykhiev and Crystal, 2013) (Section 1.5). These allow PPB to persist, thereby promoting bacterial colonisation and dysbiosis, i.e. a potentially harmful and pro-inflammatory microbiome (Hilty et al., 2010, Erb-Downward et al., 2011). Indeed, PPB are cultured from the lower respiratory tract of approximately 30% of stable COPD patients (Rosell et al., 2005); *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* being the predominant species (Cabello et al., 1997, Rosell et al., 2005). These bacteria play an important role in the pathogenesis of COPD. They amplify inflammation (Hill et al., 2000, Banerjee et al., 2004b, Sethi et al., 2006, Marin et al., 2010, Marin et al., 2012, Singh et al., 2014) and are associated with accelerated declines in lung function (Wilkinson et al., 2003) and a poorer health status (Banerjee et al., 2004b, Marin et al., 2012).

COPD exacerbations are important determinants of disease progression. They can be non-infective or viral in origin (Sapey and Stockley, 2006) but as many as 50% are associated with PPB (Sethi et al., 2007, Papi et al., 2006). Such bacterial exacerbations can result from the acquisition of a new strain (Sethi et al., 2012) but many will in fact arise from pre-existing colonising PPB (Patel et al., 2002, Sethi et al., 2007) with similar bacterial pathogens isolated during clinical stability and exacerbation, namely *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* (Monso et al., 1995, Rosell et al., 2005). Furthermore, airway inflammation is significantly amplified by bacterial exacerbations (Aaron et al., 2001, Fujimoto et al., 2005, Hurst et al., 2006a) and more rapid declines in FEV$_1$ are reported in patients who exacerbate frequently (Seemungal et al., 1998).
Phagocytosis by resident leukocytes, such as AM and neutrophils is the predominant mechanism through which bacteria are cleared from the lower airway (Martin and Frevert, 2005). It is also one of the cellular processes that has been consistently reported as dysfunctional in COPD (Donnelly and Barnes, 2012). As such, it might be postulated that a failure to clear PPB by phagocytosis promotes colonisation of the lower airways with resultant inflammation, recurrent bacterial exacerbations and COPD progression (Dickson et al., 2013). AM account for the vast majority of airway leukocytes (Harris et al., 1970) and have an expanded population in COPD (Barnes, 2004a). They are therefore the primary phagocyte and any defect in their phagocytosis is likely to be a major determinant of PPB colonisation. AM from COPD patients are not only defective in the phagocytosis of potential respiratory pathogens, such as H. influenzae (Berenson et al., 2006, Marti-Lliteras et al., 2009), S. pneumoniae (Taylor et al., 2010), M. catarrhalis (Berenson et al., 2013) and A. fumigatus (Belchamber et al., 2016), but they are also less effective in clearing pathogenically less relevant bacterial and fungal species, such as E. coli (Taylor et al., 2010, Hodge and Reynolds, 2012) and C. albicans (Ferrara et al., 1996, Vecchiarelli et al., 1991).

Despite the importance of AM phagocytosis in clearing PPB from the lower respiratory tract, the wealth of data demonstrating its defective nature in COPD and its potential role in permitting bacterial colonisation and promoting exacerbations, few studies have identified relationships between this defect and markers of disease severity. Berenson et al. (2013) demonstrated strong correlations between FEV$_1$ % predicted and the phagocytosis of H. influenzae and M. catarrhalis, concluding that the ‘severity of COPD correlated with impaired AM phagocytosis’. They incorporated all subjects in their regression analysis however, including those with a FEV$_1$ > 80%, many of whom would arguably have not had COPD. Furthermore, they failed to demonstrate any correlation between the phagocytosis of S.
pneumoniae and FEV₁ % predicted, or exclude/comment on any potential effect from confounders, such as the medication taken by the COPD patients. By the same token, there are currently no published reports on any associations between the magnitude of defective bacterial phagocytosis and airway inflammation, symptom burden or exacerbation frequency. It might be anticipated that those patients with less effective bacterial phagocytosis would have greater airway inflammation, exacerbate most frequently and suffer with more severe symptoms.

The mechanism(s) underpinning defective AM phagocytosis remain unclear. They might arise at any point along its complex pathway (Section 1.6.2), including the recognition of PAMPs by cell surface receptors, the rearrangement of actin to enfold prey, the formation and migration of phagosomes and/or bacterial killing within phagolysosomes (Aderem and Underhill, 1999). Inhaled particles and pathogens are unlikely to be opsonised due to the lack of serum in the lung and phagocytosis will therefore be predominantly mediated by SR (Donnelly and Barnes, 2012). However, although a study by Taylor et al. (2010) did confirm a reduction in the phagocytosis of S. pneumoniae and H. influenzae by MDM from COPD patients, it did not demonstrate a differential expression of MARCO, TLR2, TLR4, CD163, CD36, CD14 or CD206 (MR), when comparing MDM from non-smokers, smokers and COPD patients. This suggests that any defect(s) in the phagocytosis of non-opsonised prey is likely to lie downstream of receptor expression. With this in mind, the role of the actin cytoskeleton has been examined but experiments using MDM have not demonstrated any differential sensitivity to the actin microfilament inhibitor, cytochalasin, in COPD (Taylor et al., 2009, Thomas, 2012). As detailed in Section 1.7, the effective phagocytosis of non-opsonised prey stringently requires an intact microtubule network (Newman et al., 1991, Allen and Aderem, 1996, Sulahian et al., 2008).
Defects in microtubule arrangements might therefore contribute to defective bacterial phagocytosis in COPD.

A detailed description of microtubule structure, polymerisation, depolymerisation, stability and post-translational modification is provided in Section 1.7. Briefly, they are dynamic filamentous polymers, that continually polymerise or depolymerise according to cellular requirements (Joshi, 1998). They are composed of α- and β-tubulin subunits that are bound to GTP and incorporated into the growing end of the microtubule to form a GTP-tubulin cap, which stabilises and prevents unravelling (Walczak, 2000). Tubulin possesses intrinsic GTPase activity (Nogales et al., 1998, Roychowdhury et al., 1999) and once the GTP-cap is hydrolysed, the polymer undergoes ‘catastrophe’, by rapidly dissociating (Weisenberg et al., 1976). Two subpopulations of microtubules exist within any cell, those that are dynamic and easily disassembled (within minutes), and those that are inherently more stable (hours) (Schulze and Kirschner, 1987). While a dynamic state facilitates cell motility and mitosis, stable microtubules are required for effective phagocytosis (Peachman et al., 2004). Although both microtubule subtypes will co-exist within AM from COPD patients, it is possible that skewing towards a predominantly dynamic population could contribute to ineffective phagocytosis.

Stable microtubules associate with various MAPs (Hirokawa, 1994) and remain as polymers for several hours (Infante et al., 2000), enabling post-translational modification, including α-tubulin acetylation (Janke and Bulinski, 2011). Under conditions that promote depolymerisation (e.g. cold or certain pharmacological agents), stable and acetylated microtubules are significantly more resistant to disassembly (Piperno et al., 1987, Matsuyama et al., 2002, Xie et al., 2010). Acetylation can therefore act as a marker of microtubule stability. With this in mind, preliminary data with MDM have shown that lower concentrations of
acetylated tubulin are found in COPD patients, relative to non-smoking controls, suggesting the presence of less stable microtubule arrangements (Thomas, 2012).

Several pharmacological agents are capable of interfering with microtubule dynamics (Section 1.7.2). Nocodazole however, specifically binds tubulin (Jordan and Wilson, 1998) and at micromolar concentrations acts as a ‘microtubule destabiliser’ by increasing GTPase activity to induce ‘catastrophe’ (Lin and Hamel, 1981, Mejillano et al., 1996) and inhibit phagocytosis (De Brabander et al., 1976, Cannon and Swanson, 1992). The efficacy of nocodazole-induced depolymerisation is influenced by polymer-stabilising factors. In particular, α-tubulin acetylation has been shown to offer resistance against the effects of nocodazole (Piperno et al., 1987, Xie et al., 2010). Such characteristics make nocodazole an attractive tool for investigating the relative presence of stability among a diverse microtubule population. Unpublished data from within the research group has already shown that bacterial phagocytosis by MDM can be inhibited by 10 μM nocodazole and that COPD patients display an increased sensitivity (Taylor et al., 2009, Thomas et al., 2011, Thomas, 2012). Moreover, improvements in phagocytosis by COPD MDM and increased tubulin acetylation can be achieved with ‘microtubule stabilisers’, such as epothilone B and trichostatin A (Taylor et al., 2009, Thomas et al., 2011). Taken together, these preliminary data suggest a lack of microtubule stability in COPD MDM. A more comprehensive examination of microtubule arrangements is therefore warranted and could provide a much-needed novel target for COPD therapy.

Studying phagocytosis by AM requires bronchoscopy and the isolation of macrophages from BALF. It was demonstrated in Chapter 3 however, that this can be problematic. Issues with subject recruitment, procedural complications, a sampling bias in favour of milder disease and difficulties in obtaining an adequate number of AM from both non-smokers and COPD patients,
were all identified. Chapter 3 also highlighted many advantages to using MDM as a surrogate. Specifically, they are a validated and commonly-adopted model (Winkler et al., 2008) that avoids many of the aforementioned limitations. Furthermore, data from this thesis has also demonstrated correlations between the phagocytosis of paired AM and MDM, indicating that MDM provide a reliable surrogate measure of AM phagocytosis (Section 3.5.3).

The current chapter will use MDM from non-smokers, smokers and COPD patients to explore defective bacterial phagocytosis in COPD. It will examine for the first time whether this relates to markers of disease severity (e.g. airflow obstruction) or symptom burden and/or is associated with particular disease phenotypes (e.g. frequent exacerbators and GOLD combined assessment groups) or the use of certain medication. Relative microtubule stability will also be explored as a possible mechanism for defective bacterial phagocytosis using two complementary markers of polymer stability, i.e. the sensitivity of phagocytosis to the inhibitory effects of nocodazole and levels of acetylated tubulin within cell lysates.

4.2. Hypothesis

The hypothesis for this chapter is: ‘Bacterial phagocytosis by MDM is defective in COPD, correlates with markers of disease severity, is most attenuated in frequent exacerbators and results from a predominance of unstable microtubule arrangements’.

4.3. Aims

The following aims were adopted in order to investigate the above hypothesis:

- Use MDM as a surrogate for AM and perform phagocytosis assays with beads, H. influenzae and S. pneumoniae, comparing results from non-smokers, smokers and COPD patients. A sample size of 20 subjects per group was chosen. This was based on power calculations performed in Chapter 3.
- Correlate the results of phagocytosis assays from COPD patients with markers of disease severity. Specifically, to determine whether bacterial phagocytosis correlates with airflow obstruction, or is associated with symptom burden (as determined by CAT and mMRC scores) or the combined GOLD assessment.

- Compare the results of phagocytosis assays from COPD patients with frequent and infrequent exacerbations.

- Examine whether defective bacterial phagocytosis may be related to the use of commonly-prescribed COPD medication. In other words, to exclude any potential confounding effects from such medications.

- Examine and compare microtubule arrangements in MDM from COPD patients, non-smokers and smokers by interrogating polymer stability with nocodazole. Specifically, to determine whether phagocytosis by COPD-derived MDM is more sensitive to the effects of nocodazole-induced depolymerisation by constructing dose-response curves across a range of micromolar concentrations and comparing EC$_{50}$ values to experiments undertaken with MDM from control subjects.

- Use Western blotting to compare the levels of acetylated tubulin in MDM from non-smokers, smokers and COPD patients. Specifically, to determine whether MDM from COPD patients contain less acetylated tubulin.

- Validate any findings in AM isolated from BALF.

### 4.4. Methods

#### 4.4.1. Subject selection

Healthy non-smokers, smoking controls and COPD patients were recruited as described in **Section 2.2.1.** All subjects underwent spirometry (**Section 2.2.2.1**). All COPD patients completed a CAT questionnaire (**Appendix B**). They also reported on their degree of
breathlessness according to the mMRC dyspnoea scale (Appendix A) and the number of exacerbations experienced during the preceding year. These data were used to stratify the COPD cohort according to the combined GOLD assessment (Figure 1.1).

4.4.2. Isolation and culture of MDM

All subjects donated 60 ml of peripheral blood, which was used to generate MDM as described in Section 2.2.6. Briefly, PBMC fractions were obtained using Percoll gradients and monocytes subsequently isolated by adherence. These monocytes were seeded into black 96-well tissue culture plates at $1 \times 10^5$ cells/well, and cultured in the presence of GM-CSF (2 ng/ml) for 12 days in order to generate MDM.

4.4.3. Isolation of alveolar macrophages

AM were isolated from BALF obtained during flexible bronchoscopy (Section 2.2.6.4). As with MDM, they were seeded into black 96-well tissue culture plates at $1 \times 10^5$ cells/well. AM were rested overnight prior to assays. Insufficient samples were obtained to perform concurrent Western blotting for acetylated tubulin. As such, only nocodazole experiments were undertaken with AM samples.

4.4.4. Phagocytosis assays

Phagocytosis assays were performed using fluorimetry, as described in Section 2.2.11. MDM were incubated with prey for 4 h, before being washed and any extracellular fluorescence quenched with trypan blue 1% (v/v). Fluorescence was determined using a plate reader (excitation $\lambda_{480}$nm and emission $\lambda_{520}$nm) and the RFU were used as a measure of phagocytosis. Fluorescent microscopy images were also obtained, in order to provide a qualitative assessment of phagocytosis. Data from the MDM phagocytosis assays performed in Chapter 3 (RFU) were also incorporated (all subject groups). Intra- and inter-assay variability
was limited by ensuring that all phagocytosis assays were run in quadruplicate and by using the same batch of labelled *H. influenzae* and *S. pneumoniae* for every experiment, across both Chapter 3 and Chapter 4. The intra-assay CV (*i.e.* the mean CV between sample replicates) was < 10% for all of the phagocytosis assays presented in this chapter. This was true across all of the subject groups, for assays with both AM and MDM, and for all three prey (*i.e.* beads, *H. influenzae* and *S. pneumoniae*).

### 4.4.4.1. Phagocytosis of beads

Fluorescently-labelled microspheres of 2.0 μm diameter (4.5 x 10⁹ beads/ml) were used (Section 2.2.11.1). After sonication for 2 min, they were diluted in RPMI (11μl/ml) before being added to individual wells containing 1 x 10⁵ of MDM (final working concentration of 50 x 10⁶ microspheres/ml).

### 4.4.4.2. Phagocytosis of bacteria

Aliquots of *H. influenzae* and *S. pneumoniae* were used (Sections 2.2.9.4) and diluted in RPMI (100μl bacteria/ml) before being added to individual wells containing 1 x 10⁵ of MDM (final working concentration of 5 x 10⁸ CFU/ml).

### 4.4.5. Nocodazole assays

Prior to performing phagocytosis assays, MDM and AM (1 x 10⁵ cells/well) were pre-treated with 90 μl of either nocodazole, vehicle control (0.1% (*v/v*) DMSO) or RPMI by incubation for 1h at 37°C, 5% (*v/v*) CO₂. Solutions of nocodazole were prepared in RPMI at concentrations ranging from 10⁻⁶ to 10⁻⁴ M. Phagocytosis assays were then performed using fluorescently-labelled beads or bacteria (Sections 4.4.5.1 and 4.4.5.2). These experiments were undertaken in a subset of non-smokers, smokers and COPD patients. MDM from all of these subjects had also undergone untreated phagocytosis assays (Section 4.4.4). Nocodazole assays were run in
quadruplicate and with the same batch of labelled *H. influenzae* and *S. pneumoniae*, in an attempt to limit intra- and inter-assay variability, respectively. The intra-assay CV was < 10% for all of the nocodazole assays. This was true across all of the subject groups, for assays with both AM and MDM, and for all three prey (*i.e.* beads, *H. influenzae* and *S. pneumoniae*).

### 4.4.5.1. Nocodazole and phagocytosis of beads

The same fluorescently-labelled microspheres were used (Section 4.4.4.1) but they were diluted using 11μl/100ml of RPMI. 10 μl of the resulting bead suspension were then added to individual wells, already containing 90 μl RPMI (± 0.1% (v/v) DMSO vehicle control ± nocodazole, at 10⁻⁴ to 10⁻⁶ M). The final working concentration for phagocytosis was therefore 50 x 10⁶ microspheres/ml, the same as Section 4.4.4.1. Fluorometric assays were then completed according to Section 2.2.11.3.

### 4.4.5.2. Nocodazole and phagocytosis of bacteria

Undiluted stock suspensions of bacteria were used (Section 2.2.9.4). 10 μl of *H. influenzae* or *S. pneumoniae* were added to individual wells, already contained 90 μl of RPMI (± 0.1% (v/v) DMSO vehicle control ± nocodazole at 10⁻⁴ to 10⁻⁶ M). The final working concentration for phagocytosis was therefore 5 x 10⁸ CFU/ml, the same as Section 4.4.4.2. Fluorometric assays were then completed according to Section 2.2.11.3.

### 4.4.6. Western blotting

Western blots were performed for acetylated tubulin, as described in Section 2.2.13. Membranes were then stripped and re-probed for actin, the loading control (Section 2.2.13.6). Insufficient numbers of AM were obtained for Western blotting. As such, Western blotting was only performed with MDM. Dilutions of the primary monoclonal antibodies against the protein of interest (either acetylated tubulin or actin) and their corresponding HRP-conjugated
secondary antibodies are shown in Table 4.1. These experiments were undertaken in a subset of non-smokers, smokers and COPD patients, many of whom had not donated MDM for phagocytosis (Section 4.4.4) or nocodazole assays (Section 4.4.5).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-acetylated tubulin primary antibody</td>
<td>1:1000</td>
</tr>
<tr>
<td>(produced in mouse)</td>
<td></td>
</tr>
<tr>
<td>Anti-actin primary antibody</td>
<td>1:10000</td>
</tr>
<tr>
<td>(produced in rabbit)</td>
<td></td>
</tr>
<tr>
<td>Anti-mouse secondary antibody</td>
<td>1:1000</td>
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<tr>
<td>(HRP-conjugated)</td>
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<tr>
<td>Anti-rabbit secondary antibody</td>
<td>1:1000</td>
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<tr>
<td>(HRP-conjugated)</td>
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</tr>
</tbody>
</table>

Table 4.1. Dilutions of the primary monoclonal antibodies and HRP-conjugated secondary antibodies. Western blotting was performed using the above dilutions of antibody. Solutions were prepared in 5% milk (w/v).

4.4.7. Viability assays

MDM and AM viability was assessed immediately after phagocytosis using a MTT assay, as described in Section 2.2.12. Viability data from the MDM used in Chapter 3 were also incorporated (all subject groups).

4.4.8. Data analysis

Normal distributions were not assumed and all analyses were therefore performed using non-parametric tests. Demographic data were compared using a Kruskal-Wallis with Dunn’s post-hoc test. Comparisons between 2 groups were made using either Mann-Whitney U tests
(unpaired) or Wilcoxon signed rank tests (paired). Comparisons between ≥ 3 groups were made using a Kruskal-Wallis with Dunn’s post-hoc test. Correlations were performed using a Spearman’s rank correlation coefficient. Nocodazole dose-response curves were constructed by plotting the log nocodazole concentration (M) against percentage phagocytosis and assigning a three-parameter non-linear regression curve. Percentage phagocytosis was calculated relative to the vehicle control (i.e. RFU of condition ÷ RFU of vehicle control, expressed as a percentage (VC = 100%). Phagocytosis responses at different concentrations of nocodazole were then compared to the response with vehicle using a Kruskal-Wallis with Dunn’s post-hoc test. Values for EC_{50} and maximum percentage inhibition were calculated from the curve of each individual experiment. Mean (± SEM) values for EC_{50} and maximum percentage inhibition were then calculated for non-smokers, smokers and COPD patients. In all instances, the null hypothesis (i.e. no difference or an absent correlation) was rejected at p < 0.05. All analyses were performed using GraphPad Prism software (USA).

4.5. Results

4.5.1. Effect of COPD on phagocytosis by MDM

4.5.1.1. Subject demographics

Demographic data for the subjects who donated MDM for phagocytosis assays are shown in Table 4.2. The COPD patients were older than the smokers, but not the non-smokers. The smokers and non-smokers were matched for age. Male participants marginally outnumbered females in all of the three subject groups. The smokers and COPD patients were matched for smoking history. Most COPD patients were ex-smokers (almost twice as many). Non-smokers and smokers were matched for all measures of spirometry. The COPD patients however, had a lower FEV₁ (L and % predicted), FVC and FEV₁/FVC ratio than both control groups.
Table 4.2. Subject demographics. Participants underwent spirometry, with FEV₁ and FVC being recorded. A single pack year is equivalent to 20 cigarettes smoked every day for 1 year. Data presented are mean ± SEM. *** represents p < 0.001 relative to non-smokers. ## represents p < 0.01, ### p < 0.001 relative to smoking controls.

### 4.5.1.2. Effect of COPD on the phagocytosis of beads

The effect of COPD on the phagocytosis of beads was qualitatively assessed by comparing the fluorescent microscopy images of MDM from non-smokers, smokers and COPD patients (Figure 4.1). No differences were observed between COPD patients and controls. These findings were confirmed when comparing the median RFU from phagocytosis assays involving beads (Figure 4.3). In summary, non-smokers, smokers and COPD patients internalised equivalent quantities of beads.

Results from phagocytosis assays undertaken within this chapter were compared to those reported in Chapter 3 for MDM (Figure 3.7). Similar median RFU x 10³ (± IQR) were observed
in Chapter 3 and 4 for non-smokers (20.13 (± 8.74) vs 20.00 (± 8.26), p = 0.76), smokers (20.49 (± 15.30) vs 16.65 (± 10.28), p = 0.52) and COPD patients (18.08 (± 7.60) vs 17.04 (± 6.36), p = 0.84).

4.5.1.3. Effect of COPD on the phagocytosis of bacteria

The effect of COPD on the phagocytosis of bacteria was qualitatively assessed by comparing the fluorescent microscopy images of MDM from non-smokers, smokers and COPD patients (Figure 4.2). For both *H. influenzae* and *S. pneumoniae*, there was a reduced uptake of bacteria by MDM from COPD patients relative to the non-smokers. Experiments with smoking subjects produced a variable range of images. Some were comparable to non-smokers, while others closely resembled those obtained with COPD patients. Microscopy findings were confirmed when comparing the median RFU from phagocytosis assays involving bacteria (Figure 4.4). MDM from COPD patients internalised less *H. influenzae* and *S. pneumoniae* than MDM from non-smokers (41% and 53% less respectively). Assays involving smokers produced a wide range of RFU, and the median was equivalent to results obtained with both non-smokers and COPD patients.

Results from bacterial phagocytosis assays undertaken within this chapter were compared to those reported in Chapter 3 for MDM (Figures 3.8 and 3.9). Similar median RFU x 10^3 (± IQR) were observed for *S. pneumoniae* in Chapter 3 and 4 for non-smokers (2.25 (± 2.05) vs 1.59 (± 1.43), p = 0.19), smokers (1.25 (± 2.80) vs 1.40 (± 2.42), p = 0.87) and COPD patients (0.97 (± 1.17) vs 0.83 (± 1.06), p = 0.60). However, different median RFU x 10^3 (± IQR) were observed for *H. pneumoniae* with non-smokers (3.60 (± 2.88) vs 1.44 (± 0.85), p = 0.003) and COPD patients (1.68 (± 1.23) vs 0.77 (± 0.42), p = 0.002), but not smokers (1.41 (± 2.25) vs 1.35 (± 1.54), p = 0.87), when comparing results from Chapters 3 and 4 respectively.
Figure 4.1. Fluorescent microscopy images demonstrating bead phagocytosis by MDM: (A) Non-smokers (B) Smokers (C) COPD patients. Monocytes were isolated from the peripheral blood of non-smokers ( ), smokers ( ) and COPD patients ( ). They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. MDM were then incubated with fluorescently-labelled beads for 4h, before being washed and viewed on an inverted fluorescent microscope (x 40 objective lens). Scale bar = 50 μm. Images are representative of n = 5.
Figure 4.2. Fluorescent microscopy images demonstrating bacterial phagocytosis by MDM: (A-B) Non-smokers (C-D) Smokers (E-F) COPD patients. Monocytes were isolated from the peripheral blood of non-smokers (☐), smokers (☐) and COPD patients (☐). They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. MDM were then incubated for 4h with fluorescently-labelled *H. influenzae* (A, C, E) or *S. pneumoniae* (B, D, F), before being washed and viewed on an inverted fluorescent microscope (×40 objective lens). Scale bar = 50 μm. Images are representative of n = 5.
Figure 4.3. **Comparison of bead phagocytosis by MDM from non-smokers, smokers and COPD patients.** Monocytes were isolated from the peripheral blood of non-smokers (■), smokers (▲) and COPD patients (□), n = 23, n = 20 and n = 23 respectively. They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. MDM were then incubated with fluorescently-labelled beads for 4h and phagocytosis quantified by fluorimetry. Data presented are median RFU ± IQR and range.

Figure 4.4. **Comparison of bacterial phagocytosis by MDM from non-smokers, smokers and COPD patients:** (A) *H. influenzae* (B) *S. pneumoniae*. Monocytes were isolated from the peripheral blood of non-smokers (■), smokers (▲) and COPD patients (□), n = 22-23, n = 20 and n = 23 respectively. They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. MDM were then incubated with fluorescently-labelled bacteria for 4h and phagocytosis quantified by fluorimetry. Data presented are median RFU ± IQR and range.
4.5.1.4. **Effect of beads and bacteria on the viability of MDM**

To ensure that exposure to prey was not cytotoxic, MTT assays were performed with MDM after phagocytosis with beads, *H. influenzae* and *S. pneumoniae* (Figure 4.5). Exposure to prey for 4h did not result in a reduction in the viability of MDM from any of the three subject groups. For the COPD patients however, a small improvement in the mitochondrial reduction of MTT was observed after incubation with beads (16% greater), but not with bacteria. This was not seen with non-smokers or COPD patients.
Figure 4.5. Effect of beads and bacteria on the viability of MDM: (A) Non-smokers (B) Smokers (C) COPD. MDM were generated from peripheral blood monocytes for non-smokers ( ), smokers ( ) and COPD patients ( ), n = 23, n = 20, n = 23 respectively. MDM were incubated for 4h with beads, *H. influenzae* (HI) or *S. pneumoniae* (SP). Control cells were left untreated (UT). After phagocytosis, MDM were treated with 50 μl of MTT (45 min at 37°C) before being lysed with 50 μl of DMSO. Plates were read at λ570 nm on a spectrophotometer. Data presented are mean viability (± SEM) as a percentage of UT controls (UT = 100% viability, represented by horizontal dotted lines). *** represents p < 0.001, relative to UT control.
4.5.2. Relationships between the phagocytosis of different prey by MDM

4.5.2.1. Relationship between the phagocytosis of bacteria and beads

The ability of MDM to phagocytose inert beads was compared to their ability to phagocytose either *H. influenzae* or *S. pneumoniae* (Figure 4.6; Panels A and B, respectively). When analysing the subject groups as a whole, no correlation was present between the phagocytosis of beads and *H. influenzae*. In contrast, a weak positive correlation was observed between the phagocytosis of beads and *S. pneumoniae*. Most importantly however, when the three groups were analysed independently, no correlations were found between the phagocytosis of beads and either of the two bacteria.

Figure 4.6. Correlations between the phagocytosis of beads and bacteria by MDM: (A) *H. influenzae* (HI) (B) *S. pneumoniae* (SP). Monocytes were isolated from the peripheral blood of non-smokers (▲), smokers (■) and COPD patients (●), n = 22, n = 20 and n = 23 respectively. They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled beads and bacteria. Data presented are Spearman rank correlation coefficients (subject groups analysed together).
4.5.2.2. Relationship between the phagocytosis of Gram-negative and -positive bacteria

The ability of MDM to phagocytose Gram-negative bacteria was compared to their ability to phagocytose Gram-positive bacteria by correlating the RFU from experiments with *H. influenzae* and *S. pneumoniae*, respectively (Figure 4.7). When analysing the subject groups as a whole, a strong positive correlation was observed between the phagocytosis of *H. influenzae* and *S. pneumoniae*. When the three groups were analysed independently, positive correlations were also observed for non-smokers, smokers and COPD patients.

Figure 4.7. Correlation between the phagocytosis of *H. influenzae* and *S. pneumoniae* by MDM. Monocytes were isolated from the peripheral blood of non-smokers (▲), smokers (■) and COPD patients (●), n = 22, n = 20 and n = 23 respectively. They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled *H. influenzae* (HI) and *S. pneumoniae* (SP). Data presented are Spearman rank correlation coefficients.
4.5.3. Effect of demographic variables on phagocytosis by MDM

The data were analysed to determine whether the phagocytic ability of MDM was related to any of the demographic variables, such as age, gender, lung function, pack years and smoking status.

4.5.3.1. Effect of age on the phagocytosis of beads and bacteria

The COPD patients were older than the smokers (Table 4.2). Therefore, any relationship between age and the phagocytosis of beads and bacteria was examined by performing correlations (Figure 4.8). No correlations were observed between age and the phagocytosis of beads, *H. influenzae* or *S. pneumoniae*, when the subject groups were analysed as a whole or independently.

4.5.3.2. Effect of gender on the phagocytosis of beads and bacteria

All of the subject groups contained slightly more males than females (Table 4.2). Therefore, any effect of gender on the phagocytosis of beads and bacteria was examined (Figure 4.9). There were no differences in the phagocytosis of *H. influenzae* and *S. pneumoniae* by the male and female participants (subject groups analysed as a whole or independently). MDM from males however, internalised more beads than MDM from females (29% more). During the independent group analysis, the phagocytosis of beads by smokers was greater for males (57% more) but no differences were observed between the male and female non-smokers or COPD patients.
Figure 4.8. Correlations between MDM phagocytosis and subject age: (A) Beads (B) *H. influenzae* (C) *S. pneumoniae*. Monocytes were isolated from the peripheral blood of non-smokers (▲), smokers (■) and COPD patients (●), n = 22-23, n = 20 and n = 23 respectively. They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled beads and bacteria. Data presented are Spearman rank correlation coefficients (subject groups analysed together).
Figure 4.9. Phagocytosis by MDM from male and female subjects: (A) Beads (B) *H. influenzae* (C) *S. pneumoniae*. Monocytes were isolated from the peripheral blood of non-smokers (▲), smokers (■) and COPD patients (●), n = 22-23, n = 20 and n = 23 respectively. They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled beads and bacteria. Data presented are medians ± IQR. ** represents p < 0.01.
4.5.3.3. **Effect of lung function on the phagocytosis of beads**

To determine whether the phagocytosis of beads was related to lung function, correlations were performed with \( FEV_1 \) (L and % predicted), FVC and \( FEV_1/FVC \) (**Figure 4.10**). No correlations were observed for \( FEV_1 \) (% predicted) or \( FEV_1/FVC \). Similar data were obtained when each subject group was analysed independently or when all the subjects were analysed as a whole. \( FEV_1 \) (L) did however weakly and positively correlate with the phagocytosis of beads, but only when all the groups were combined. A moderate correlation was also observed with FVC. This was present when analysing all of the subjects and during the independent analysis of the smokers \((r = 0.49, p = 0.03)\) and COPD patients \((r = 0.44, p = 0.03)\), but not the non-smokers \((r = 0.28, p = 0.2)\).

4.5.3.4. **Effect of lung function on the phagocytosis of *H. influenzae***

To determine whether the phagocytosis of *H. influenzae* was related to lung function, correlations were performed with \( FEV_1 \) (L and % predicted), FVC and \( FEV_1/FVC \) (**Figure 4.11**). When analysing the subject groups as a whole, the phagocytosis of *H. influenzae* weakly and positively correlated with \( FEV_1 \) (L), \( FEV_1 \) (% predicted), FVC and \( FEV_1/FVC \) ratio. No correlations were observed when each of the subject groups were analysed independently.

4.5.3.5. **Effect of lung function on the phagocytosis of *S. pneumoniae***

To determine whether the phagocytosis of *S. pneumoniae* was related to lung function, correlations were performed with \( FEV_1 \) (L and % predicted), FVC and \( FEV_1/FVC \) (**Figure 4.12**). When analysing the subject groups as a whole, the phagocytosis of *S. pneumoniae* weakly and positively correlated with \( FEV_1 \) (L), \( FEV_1 \) (% predicted) and FVC, but not \( FEV_1/FVC \). No correlations were observed when each of the subject groups were analysed independently.
Figure 4.10. Correlations between the phagocytosis of beads by MDM and (A) $FEV_1$ (L) (B) $FEV_1$ (% predicted) (C) FVC (D) $FEV_1$/FVC ratio. Monocytes were isolated from the peripheral blood of non-smokers (▲), smokers (■) and COPD patients (●), n = 23, n = 20 and n = 23 respectively. They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled beads. Spirometry was undertaken to determine $FEV_1$ and FVC. Data presented are Spearman rank correlation coefficients (subject groups analysed together).
Figure 4.11. Correlations between the phagocytosis of *H. influenzae* by MDM and (A) FEV\(_1\) (L) (B) FEV\(_1\) (% predicted) (C) FVC (D) FEV\(_1\)/FVC ratio. Monocytes were isolated from the peripheral blood of non-smokers (▲), smokers (■) and COPD patients (●), n = 23, n = 20 and n = 23 respectively. They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled *H. influenzae*. Spirometry was undertaken to determine FEV\(_1\) and FVC. Data presented are Spearman rank correlation coefficients (subject groups analysed together).
Figure 4.12. Correlations between the phagocytosis of *S. pneumoniae* by MDM and (A) FEV₁ (L) (B) FEV₁ (% predicted) (C) FVC (D) FEV₁/FVC ratio. Monocytes were isolated from the peripheral blood of non-smokers (▲), smokers (■) and COPD patients (●), n = 22, n = 20 and n = 23 respectively. They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled *S. pneumoniae*. Spirometry was undertaken to determine FEV₁ and FVC. Data presented are Spearman rank correlation coefficients (subject groups analysed together).
4.5.3.6. Effect of smoking on the phagocytosis of beads and bacteria

To determine whether the phagocytosis of beads and bacteria were related to smoking, correlations were performed with the number of pack years smoked by the smoking controls and COPD patients (Figure 4.13). When analysed together, no correlations were observed between the number of pack years and the phagocytosis of beads and *H. influenzae* (Figure 4.13; Panels A & B). Similar data were also obtained when analysing the subject groups independently. A weak negative correlation was present however, for the phagocytosis of *S. pneumoniae* (Figure 4.13; Panel C). When the groups were analysed independently, this negative correlation was only apparent for COPD patients (*r* = -0.46, *p* = 0.03) and may have been driven in part by an outlier (196 pack years and very low RFU = 0.44 x 10³).

The phagocytosis of beads and bacteria were also compared between current and ex-smoking participants (Figure 4.14). When analysing the smokers and COPD patients together, no differences were observed for the phagocytosis of beads, *H. influenzae* or *S. pneumoniae*. Similar data were also seen when independently analysing the COPD patients (Figure 4.15).
Figure 4.13. Correlations between phagocytosis by MDM and number of pack years: (A) Beads (B) *H. influenzae* (C) *S. pneumoniae*. Monocytes were isolated from the peripheral blood of smokers (■) and COPD patients (●), *n* = 20 and *n* = 23 respectively. They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled beads and bacteria. Data presented are Spearman rank correlation coefficients (subject groups analysed together).
Figure 4.14. Phagocytosis by MDM from current and ex-smoking subjects: (A) Beads (B) *H. influenzae* (C) *S. pneumoniae*. Monocytes were isolated from the peripheral blood of smokers (■) and COPD patients (●), n = 20 and n = 23 respectively. They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled beads and bacteria. Data presented are medians ± IQR.
Figure 4.15. Phagocytosis by MDM from current and ex-smoking COPD patients: (A) Beads (B) *H. influenzae* (C) *S. pneumoniae*. Monocytes were isolated from the peripheral blood of COPD patients (*n* = 23). They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled beads and bacteria. Data presented are medians ± IQR.
4.5.4. Relationship between phagocytosis and COPD severity or phenotype

The data from COPD patients were analysed to determine whether the phagocytic ability of MDM was related to markers of disease severity (airflow obstruction), symptoms and specific disease phenotypes (frequent exacerbators or GOLD combined assessment groups).

4.5.4.1. Clinical data for COPD cohort

Clinical data for the COPD cohort are presented in Table 4.3. All patients had moderate (57%) or severe (43%) airflow obstruction according to the GOLD spirometry classification. No patient had mild or very severe airflow obstruction. The mean CAT score was 17 (± 2), and most patients scored ≥ 10 points (70%), suggesting that the cohort was moderately impacted by symptoms. They were also notably breathless. The mean mMRC dyspnoea score was 2.0 (± 0.2) and most patients scored ≥ 2 points (65%). There was an approximately equal number of frequent and infrequent exacerbators (48% and 52% respectively). However, most patients (53%) were classified as Group D according to the combined GOLD assessment (i.e. more symptoms and high risk of exacerbation).
Table 4.3. Clinical data for the COPD cohort. All COPD patients underwent spirometry and FEV₁ was recorded (L and % predicted). Patients completed a CAT questionnaire and their degree of dyspnoea was scored using the mMRC scale. The number of COPD exacerbations during the preceding year were self-reported. All patients were categorised according to GOLD spirometry classification, symptom-burden (CAT and mMRC score), exacerbation frequency and combined GOLD assessment. Patients with more symptoms and those who were most breathless, were categorised as having a CAT score ≥ 10 or mMRC ≥ 2 respectively. Data presented are numbers, percentages and mean ± SEM.
4.5.4.2. Relationship between airflow obstruction and the phagocytosis of beads

Any association between airflow obstruction and the phagocytosis of beads was examined by correlating FEV₁ (% predicted) with RFU (Figure 4.16). No correlation was observed. The phagocytosis of beads was also compared between COPD patients of varying airflow obstruction, according to the GOLD spirometry classification (Figure 4.17). Only patients with moderate or severe airflow obstruction were available for comparison. No differences in the phagocytosis of beads were found between these subjects.

![Figure 4.16. Correlations between the phagocytosis of beads by MDM from COPD patients and FEV₁ (% predicted). Monocytes were isolated from the peripheral blood of COPD patients (n = 23). They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled beads. Spirometry was undertaken to determine FEV₁ (% predicted). Data presented are Spearman rank correlation coefficients.](image-url)
Figure 4.17. Phagocytosis of beads by MDM from COPD patients according to the severity of airflow obstruction. Monocytes were isolated from the peripheral blood of COPD patients (n = 23). They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled beads. The severity of airflow obstruction was classified according to GOLD criteria. Only patients with moderate (FEV₁ = 50-79%) or severe (FEV₁ = 30-49%) airflow obstruction were identified (n = 13 and n = 10 respectively). Data presented are medians ± IQR.

4.5.4.3. Relationship between airflow obstruction and the phagocytosis of bacteria

Any association between airflow obstruction and the phagocytosis of bacteria was examined by correlating FEV₁ (% predicted) with the RFU from experiments with both *H. influenzae* and *S. pneumoniae* (Figure 4.18). No statistically significant correlations were observed.

The phagocytosis of bacteria was also compared between COPD patients of varying airflow obstruction (Figure 4.19). Only patients with moderate or severe airflow obstruction were available for comparison. No statistical differences in the phagocytosis of either *H. influenzae* or *S. pneumoniae* were identified. There were possible trends however, suggesting MDM from patients with severe airflow obstruction internalised less bacteria.
Figure 4.18. Correlations between the phagocytosis of bacteria by MDM from COPD patients and FEV\(_1\) (% predicted): (A) *H. influenzae* (HI) (B) *S. pneumoniae* (SP). Monocytes were isolated from the peripheral blood of COPD patients (n = 23) and cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled bacteria. Spirometry was undertaken to determine FEV\(_1\) (% predicted). Data presented are Spearman rank correlation coefficients.

![Figure 4.18](image)

Figure 4.19. Phagocytosis of bacteria by MDM from COPD patients according to the severity of airflow obstruction: (A) *H. influenzae* (B) *S. pneumoniae*. Monocytes were isolated from the peripheral blood of COPD patients (n = 23) and cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled bacteria. The severity of airflow obstruction was classified according to GOLD criteria. Only patients with moderate (FEV\(_1\) = 50-79%) or severe (FEV\(_1\) = 30-49%) airflow obstruction were identified (n = 13 and n = 10 respectively). Data presented are medians ± IQR.

![Figure 4.19](image)
4.5.4.4. Relationship between symptom burden and phagocytosis

COPD symptoms can be graded according to the mMRC dyspnoea scale or CAT. According to GOLD (2015), patients are more breathless with mMRC ≥ 2 and more symptomatic with CAT ≥ 10. To determine whether symptom severity was related to phagocytosis, the COPD cohort was divided according to these suggested mMRC and CAT score cut-offs and comparisons were made for the phagocytosis of beads (Figure 4.20), H. influenzae (Figure 4.21) and S. pneumoniae (Figure 4.22).

No differences were observed between the phagocytosis of beads by COPD patients with mMRC < 2 and those with mMRC ≥ 2. Likewise, bead phagocytosis was unaffected by CAT score. Similar data were also obtained for the phagocytosis of both H. influenzae and S. pneumoniae. There was a trend however, suggesting that MDM from patients with more symptoms (mMRC ≥ 2 or CAT ≥ 10) internalised less S. pneumoniae (p = 0.17 and p = 0.09 for mMRC and CAT respectively).
Figure 4.20. Phagocytosis of beads by MDM from COPD patients according to: (A) mMRC dyspnoea score (B) CAT score. Monocytes were isolated from the peripheral blood of COPD patients \textit{(n} = 23)\textit{) and cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled beads. Dyspnoea was scored with the mMRC scale. Patients completed a CAT questionnaire. Patients who were most breathless were categorised as those with a mMRC score $\geq 2$. Patients with more symptoms were categorised as those with a CAT score $\geq 10$. Data presented are medians $\pm$ IQR.

Figure 4.21. Phagocytosis of \textit{H. influenzae} by MDM from COPD patients according to: (A) MRC dyspnoea score (B) CAT score. Monocytes were isolated from the peripheral blood of COPD patients \textit{(n} = 23)\textit{) and cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled \textit{H. influenzae}. Dyspnoea was scored with the mMRC scale. Patients completed a CAT questionnaire. Patients who were most breathless were categorised as those with a mMRC score $\geq 2$. Patients with more symptoms were categorised as those with a CAT score $\geq 10$. Data presented are medians $\pm$ IQR.
Figure 4.22. Phagocytosis of \textit{S. pneumoniae} by MDM from COPD patients according to: (A) MRC dyspnoea score (B) CAT score. Monocytes were isolated from the peripheral blood of COPD patients (\(n = 23\)) and cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled \textit{S. pneumoniae}. Dyspnoea was scored with the mMRC scale. Patients completed a CAT questionnaire. Patients who were most breathless were categorised as those with a mMRC score ≥ 2. Patients with more symptoms were categorised as those with a CAT score ≥ 10. Data presented are medians ± IQR.

4.5.4.5. Relationship between COPD exacerbation frequency and phagocytosis

Frequent exacerbators are defined as COPD patients who experience ≥ 2 exacerbations per year (Wedzicha \textit{et al.}, 2013). To determine whether there was a relationship between phagocytosis and exacerbation frequency, the COPD cohort was first divided into frequent (≥ 2 / year) and infrequent exacerbators (< 2 / year) on the basis of the number of exacerbations during the preceding year. Demographic data for frequent and infrequent exacerbators are shown in Table 4.4. The mean number of exacerbations were 3.5 ± 0.2 and 0.7 ± 0.1 respectively (\(p < 0.001\)). Both groups were matched for age, smoking history, FEV\(_1\) (L and % predicted), FVC and FEV\(_1\)/FVC. Infrequent exacerbators were predominantly female. Males outnumbered females in the frequent exacerbator group. Section 4.5.3.2 does confirm however, that gender has no effect on phagocytosis by MDM from COPD patients.
Table 4.4. Subject demographics for infrequent and frequent exacerbators. COPD patients were categorised as infrequent (< 2 / year) or frequent (≥ 2 / year) exacerbators. All patients underwent spirometry, with FEV₁ and FVC being recorded. A single pack year is equivalent to 20 cigarettes smoked every day for 1 year. Data presented are mean ± SEM. *** represents p < 0.001 relative to infrequent COPD exacerbators.

After dividing the COPD cohort into frequent and infrequent exacerbators, the phagocytosis of beads (Figure 4.23) and bacteria (Figure 4.24) were then compared between these two phenotypes. Exacerbation frequency did not affect the phagocytosis of beads, H. influenzae or S. pneumoniae. However, there was a possible trend suggesting that MDM from frequent exacerbators internalised less H. influenzae (p = 0.15) than those who exacerbated less frequently; a single outlier appeared to prevent statistical significance (frequent exacerbator with RFU = 3.1 x 10³).
Figure 4.23. Phagocytosis of beads by MDM from COPD patients with infrequent (< 2 / year) and frequent (≥ 2 / year) exacerbations. Monocytes were isolated from the peripheral blood of COPD patients (n = 23). They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled beads. Data presented are medians ± IQR.

Figure 4.24. Phagocytosis of bacteria by MDM from COPD patients with infrequent (< 2 / year) and frequent (≥ 2 / year) exacerbations: (A) H. influenzae (B) S. pneumoniae. Monocytes were isolated from the peripheral blood of COPD patients (n = 23). They were cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled bacteria. Data presented are medians ± IQR.
4.5.4.6. Relationship between phagocytosis and the combined GOLD assessment

COPD patients can be categorised according to the combined GOLD assessment as Groups A – D (Figure 1.1). To determine whether there was a relationship between phagocytosis and GOLD group, the COPD cohort was first divided into groups A, B, C or D, before comparisons were made for the phagocytosis of beads (Figure 4.25) and bacteria (Figure 4.26). Too few patients were categorised as A, B or C (Table 4.3) and no differences in the phagocytosis of beads, *H. influenzae* or *S. pneumoniae* were observed.

![Graph showing phagocytosis of beads by MDM from COPD patients according to the combined GOLD assessment](image)

**Figure 4.25. Phagocytosis of beads by MDM from COPD patients according to the combined GOLD assessment.** Monocytes were isolated from the peripheral blood of COPD patients (n = 23) and cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled beads. COPD patients were classified according GOLD: A (n = 3), B (n = 4), C (n = 4) and D (n = 12). Data are medians ± IQR.
Figure 4.26. Phagocytosis of bacteria by MDM from COPD patients according to the combined GOLD assessment: (A) *H. influenzae* (B) *S. pneumoniae*. Monocytes were isolated from the peripheral blood of COPD patients (n = 23) and cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled bacteria. COPD patients were classified according GOLD: A (n = 3), B (n = 4), C (n = 4) and D (n = 12). Data are medians ± IQR.

4.5.5. Relationship between bacterial phagocytosis and pharmacotherapy

Data from COPD patients were analysed to determine whether bacterial phagocytosis was related to the regular use of any class of medication (inhaled or oral). This was performed in order to exclude the possibility that a commonly-prescribed treatment was a confounder and potentially accounted for the defective bacterial phagocytosis observed in COPD. No COPD patient was prescribed regular oral prednisolone, *N*-acetylcysteine, roflumilast or LTOT, and no patients used domiciliary NIV. As such, these treatments were not considered as potential confounders.

4.5.5.1. Pharmacotherapy in the COPD cohort

Drug histories were available for 19 out of the 23 COPD patients (Tables 4.5 and 4.6). A significant proportion of the cohort was taking a regular oral medication (79%). These included
carbocisteine (42%), azithromycin (37%) and theophylline (21%). The theophyllines were exclusively slow/sustained-release preparations. No patient was taking regular prednisolone. Nearly all of these patients (90%) were prescribed one of the three major classes of inhaled treatment. Significant proportions were taking LABAs or LAMAs, either as monotherapy or in combination with other inhaled treatments (74% and 63% respectively). Most of these prescriptions were combined with other medication. Over half of the patients (63%) were taking an ICS, almost exclusively in combination with a LAMA or LABA.

<table>
<thead>
<tr>
<th></th>
<th>COPD (n = 19)</th>
</tr>
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<tbody>
<tr>
<td>Carbocisteine</td>
<td>8 (42%)</td>
</tr>
<tr>
<td>Azithromycin (a)</td>
<td>7 (37%)</td>
</tr>
<tr>
<td>Theophylline (b)</td>
<td>4 (21%)</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>0 (%)</td>
</tr>
<tr>
<td><strong>Use of regular oral medication</strong></td>
<td><strong>15 (79%)</strong></td>
</tr>
</tbody>
</table>

Table 4.5. Use of oral medication by the COPD cohort. COPD patients were asked to provide drug histories, including the use of regular oral medication. Data presented are numbers and percentages. (a) 250 mg taken on Monday, Wednesday and Friday. (b) exclusively slow/sustained-release aminophylline.
Table 4.6. Use of inhaled medication by the COPD cohort. COPD patients were asked to provide drug histories, including the use of regular inhaled medication. Data presented are numbers and percentages. LABA = any long-acting β₂-agonist, LAMA = any long-acting muscarinic antagonist, ICS = any inhaled corticosteroid.

<table>
<thead>
<tr>
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<th>COPD (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LABA monotherapy</td>
<td>2 (11%)</td>
</tr>
<tr>
<td>LABA in combination</td>
<td>12 (63%)</td>
</tr>
<tr>
<td>LABA (monotherapy or in combination)</td>
<td>14 (74%)</td>
</tr>
<tr>
<td>LAMA monotherapy</td>
<td>2 (11%)</td>
</tr>
<tr>
<td>LAMA in combination</td>
<td>10 (53%)</td>
</tr>
<tr>
<td>LAMA (monotherapy or in combination)</td>
<td>12 (63%)</td>
</tr>
<tr>
<td>ICS monotherapy</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>ICS in combination</td>
<td>11 (58%)</td>
</tr>
<tr>
<td>ICS (monotherapy or in combination)</td>
<td>12 (63%)</td>
</tr>
<tr>
<td>Use of regular inhaled medication</td>
<td>17 (90%)</td>
</tr>
</tbody>
</table>

4.5.5.2. Relationships between bacterial phagocytosis and inhaled medication

Any potential relationship between bacterial phagocytosis and the use of inhaled medication was explored by comparing the RFU from phagocytosis experiments with patients taking the medication to the RFU from experiments with patients who were not (Figure 4.27). Patients were classed as taking the inhaler, if it had been prescribed for the preceding month, either as monotherapy or in combination with other inhaled treatments. No differences were observed in the phagocytosis of *H. influenzae* or *S. pneumoniae* for LABAs, LAMAs or ICS.
Figure 4.27. Bacterial phagocytosis by MDM from COPD patients according to the use of inhaled medication: (A-B) LABA (C-D) LAMA (E-F) ICS. Monocytes were isolated from the peripheral blood of COPD patients (n = 19) and cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled *H. influenzae* (A, C or E) and *S. pneumoniae* (B, D or F). Drug histories were taken to determine the use of inhaled medication. Data presented are medians ± IQR. LABA = long-acting β₂-agonist, LAMA = long-acting muscarinic antagonist, ICS = inhaled corticosteroid.
4.5.5.3. **Relationships between bacterial phagocytosis and oral medication**

Any potential relationship between bacterial phagocytosis and the use of oral medication was explored by comparing the RFU from phagocytosis experiments with patients taking the medication to the RFU from experiments with patients who were not (Figure 4.28). Patients were classed as taking the drug, if it had been prescribed for the preceding month, either alone or in combination with other oral treatments and inhalers. No differences were observed in the phagocytosis of *H. influenzae* or *S. pneumoniae* for carbocisteine and theophylline. The MDM from COPD patients who were taking azithromycin internalised significantly more *S. pneumoniae* than the MDM from patients who were not prescribed this macrolide (Figure 4.28; Panel D). There also appeared to be a similar trend in the phagocytosis of *H. influenzae*, for those patients taking azithromycin (*p* = 0.23).
**Figure 4.28. Bacterial phagocytosis by MDM from COPD patients according to the use of oral medication:** (A-B) Carbocisteine (C-D) Azithromycin (E-F) Theophylline. Monocytes were isolated from the peripheral blood of COPD patients (n = 19) and cultured for 12 days in the presence of GM-CSF (2 ng/ml) to generate MDM. Phagocytosis assays were performed with fluorescently-labelled *H. influenzae* (A, C or E) and *S. pneumoniae* (B, D or F). Drug histories were taken to determine the use of oral medication. Data presented are medians ± IQR. ** represents p < 0.01.
Having confirmed that the phagocytosis of *H. influenzae* and *S. pneumoniae* by MDM is defective in COPD (Section 4.5.1.3), the comparative stability of microtubule polymers was then explored in a subset of non-smokers, smokers and COPD patients. The relative sensitivity of phagocytosis to nocodazole-induced depolymerisation and the levels of acetylated tubulin within MDM were investigated as two markers of microtubule stability (Sections 4.5.6 and 4.5.7 respectively).

### 4.5.6. Effect of nocodazole on phagocytosis by MDM

#### 4.5.6.1. Subject Demographics

Demographic data for the subset of subjects who donated MDM for nocodazole experiments are shown in Table 4.7. The mean age was greater in the COPD group when compared to smokers, but not non-smokers. There were equal numbers of male and female smokers, however male subjects outnumbered females in the non-smoking group, and there were more female than male COPD patients. The smokers and COPD patients were matched for smoking history. Almost all of the COPD patients were ex-smokers. The COPD patients had a lower FEV₁ (L) and FEV₁/FVC ratio than both the non-smokers and smokers. The FEV₁ (% predicted) was also lower in the COPD group, but only when compared to the non-smokers. No differences were observed in FVC (L) between the three subject groups.
### Table 4.7. Subject demographics.

Participants underwent spirometry and the FEV\textsubscript{1} and FVC were recorded. A single pack year is equivalent to 20 cigarettes smoked every day for 1 year. Data presented are mean ± SEM. ** represents p < 0.01, * represents p < 0.05 relative to non-smokers. # represents p < 0.05 relative to smoking controls.

<table>
<thead>
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<th>Non-Smoker (n = 6)</th>
<th>Smoker (n = 6)</th>
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<tr>
<td><strong>Age (years)</strong></td>
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<td>54.6 ± 2.4</td>
<td>61.4 ± 1.8*#</td>
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<tr>
<td><strong>Gender (male : female)</strong></td>
<td>4:2</td>
<td>3:3</td>
<td>2:4</td>
</tr>
<tr>
<td><strong>Smoking history (pack years)</strong></td>
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<td>37.7 ± 5.5**</td>
<td>60.8 ± 27.4*</td>
</tr>
<tr>
<td><strong>Current Smoker (yes : no)</strong></td>
<td>-</td>
<td>6:0</td>
<td>1:5</td>
</tr>
<tr>
<td><strong>FEV\textsubscript{1} (L)</strong></td>
<td>2.99 ± 0.39</td>
<td>2.64 ± 0.33</td>
<td>1.22 ± 0.19**#</td>
</tr>
<tr>
<td><strong>FEV\textsubscript{1} (% predicted)</strong></td>
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<td>87.4 ± 6.7</td>
<td>51.1 ± 6.6**</td>
</tr>
<tr>
<td><strong>FVC (L)</strong></td>
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<td>3.54 ± 0.52</td>
<td>2.42 ± 0.31</td>
</tr>
<tr>
<td><strong>FEV\textsubscript{1}/FVC</strong></td>
<td>0.79 ± 0.02</td>
<td>0.76 ± 0.03</td>
<td>0.53 ± 0.03**#</td>
</tr>
</tbody>
</table>

4.5.6.2. **Effect of nocodazole on the phagocytosis of beads (MDM)**

Nocodazole inhibited the phagocytosis of beads by MDM from non-smokers and COPD patients, but not smokers (Figure 4.29). The maximum percentage inhibition of bead phagocytosis was no different for the non-smoking controls and COPD patients (mean (± SEM) of 42.6 % (± 6.6) and 41.6 % (± 5.4), respectively). Within this concentration range, the response was not concentration-dependent. As such, EC\textsubscript{50} values could not be calculated.
Figure 4.29. Effect of nocodazole on the phagocytosis of beads by MDM. Monocytes from non-smokers (▲), smokers (■) and COPD patients (●) were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM (n = 6 for all 3 groups). Cells were then pre-treated for 1h with different concentrations of nocodazole or vehicle control (VC; DMSO 0.1%), prior to performing phagocytosis assays with beads. Data presented are mean percentage phagocytosis ± SEM (calculated relative to VC). * represents p < 0.05 relative to own VC.

4.5.6.3. Effect of nocodazole on the phagocytosis of bacteria (MDM)

Nocodazole inhibited the phagocytosis of *H. influenzae* by MDM from non-smokers, smokers and COPD patients (Figure 4.30; Panel A). The phagocytosis of *S. pneumoniae* was also inhibited by nocodazole, and for all of the three subject groups (Figure 4.30; Panel B). The responses for both bacteria were concentration-dependent and the curves for COPD MDM appeared to be shifted leftward, relative to the control groups. As such, values for maximum percentage inhibition and EC50 were calculated and compared (Tables 4.8 and 4.9).
Figure 4.30. Effect of nocodazole on the phagocytosis of bacteria by MDM: (A) *H. influenzae* (B) *S. pneumoniae*. Monocytes from non-smokers (▲), smokers (■) and COPD patients (●) were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM (n = 6 for all 3 groups). Cells were then pre-treated for 1h with different concentrations of nocodazole or vehicle control (VC; DMSO 0.1%), prior to performing phagocytosis assays with bacteria. Data presented are mean percentage phagocytosis ± SEM (calculated relative to VC). *** represents p < 0.001, ** represents p < 0.01, * represents p < 0.05 relative to own VC.

The maximum percentage inhibition for the phagocytosis of *H. influenzae* and *S. pneumoniae* was no different across the three groups (Table 4.8). EC50 values for nocodazole-induced inhibition of bacterial phagocytosis were lower for MDM from COPD patients, relative to non-smokers but not smokers (Table 4.9). This was true of both *H. influenzae* and *S. pneumoniae* phagocytosis. These findings confirm the apparent leftward shift of the concentration-response curves for COPD patients, as displayed in Figure 4.30. This suggests that bacterial phagocytosis by MDM from COPD patients was more sensitive to the effects of nocodazole than MDM from non-smokers, but not smokers.
Table 4.8. Maximum percentage inhibition of bacterial phagocytosis by MDM in response to nocodazole. Monocytes from non-smokers (n = 6), smokers (n = 6) and COPD patients (n = 6) were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM. Cells were then pre-treated for 1h with different concentrations of nocodazole or vehicle control (VC; DMSO 0.1%), prior to performing phagocytosis assays with *H. influenzae* (HI) or *S. pneumoniae* (SP). The maximum percentage inhibition was calculated relative to the VC. Data presented are mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Non-Smoker (n = 6)</th>
<th>Smoker (n = 6)</th>
<th>COPD (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phagocytosis of HI</td>
<td>44.1 ± 14.0</td>
<td>58.3 ± 7.5</td>
<td>53.2 ± 6.7</td>
</tr>
<tr>
<td>Phagocytosis of SP</td>
<td>45.6 ± 10.6</td>
<td>55.2 ± 9.1</td>
<td>69.5 ± 8.2</td>
</tr>
</tbody>
</table>

Table 4.9. EC₅₀ for the inhibition of bacterial phagocytosis by nocodazole (MDM). Monocytes from non-smokers (n = 6), smokers (n = 6) and COPD patients (n = 6) were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM. Cells were then pre-treated for 1h with different concentrations of nocodazole or vehicle control (DMSO 0.1%), prior to performing phagocytosis assays with *H. influenzae* (HI) or *S. pneumoniae* (SP). Individual EC₅₀ values were calculated. Data presented are mean ± SEM. * represents p < 0.05 relative to non-smoking controls.

<table>
<thead>
<tr>
<th></th>
<th>Non-Smoker (n = 6)</th>
<th>Smoker (n = 6)</th>
<th>COPD (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phagocytosis of HI</td>
<td>10.0 ± 2.2</td>
<td>7.7 ± 1.8</td>
<td>2.0 ± 0.4*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phagocytosis of SP</td>
<td>27.1 ± 5.3</td>
<td>18.0 ± 6.3</td>
<td>4.5 ± 1.6*</td>
</tr>
</tbody>
</table>

4.5.6.4. Effect of nocodazole on MDM viability

Viability assays were performed with MDM, in order to confirm that any effect of nocodazole on phagocytosis was not the result of a reduction in cell viability with higher concentrations. When compared to untreated cells, the viability of MDM were unaffected by the vehicle or nocodazole, up to concentrations of 10⁻⁴ M (*Figure 4.31*).
Figure 4.31. Effect of nocodazole on MDM cell viability. Monocytes from non-smokers (▲), smokers (■) and COPD patients (●) were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM (n = 6 for all 3 groups). Cells were pre-treated for 1h with different concentrations of nocodazole, vehicle control (VC; DMSO 0.1%) or left untreated (UT), before being used in phagocytosis assays. They were then incubated with 50 μl of MTT solution for 45 min at 37°C. Supernatants were aspirated and 50 μl of DMSO were added. Plates were read at λ570 nm on a spectrophotometer. Data presented are percentage cell viability, relative to the UT cells (i.e. UT cells = 100% viability, represented by the horizontal dotted line).

4.5.7. Levels of acetylated tubulin in MDM

Having shown that the phagocytosis of bacteria by MDM from COPD patients is more sensitive to the inhibitory effects of nocodazole when compared to non-smokers (but not smokers), the next step was to investigate whether these findings were reflected in the levels of acetylated tubulin contained within these cells. Unstimulated MDM from non-smokers, smokers and COPD patients were therefore lysed and the levels of acetylated tubulin analysed by Western blotting (relative to actin, the loading control).
4.5.7.1. Subject Demographics

Demographic data for the subjects who donated venous blood for MDM culture, cell lysis and the quantification of acetylated-tubulin are shown in Table 4.10. The mean age was greater in the COPD group when compared to the smokers, but not the non-smokers. Male participants outnumbered females in the non-smoking and smoking groups. There were however, an equal number of male and female COPD patients. The smokers and COPD patients were matched for smoking history. Almost all of the COPD patients were ex-smokers. COPD patients had a lower FEV$_1$ (L), FEV$_1$ (% predicted), FVC (L) and FEV$_1$/FVC ratio, when compared to both the non-smokers and smokers. Non-smokers and smokers were matched for all measures of spirometry.
<table>
<thead>
<tr>
<th></th>
<th>Non-Smoker (n = 9)</th>
<th>Smoker (n = 8)</th>
<th>COPD (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>60.1 ± 3.3</td>
<td>53.5 ± 1.5</td>
<td>71.4 ± 2.3##</td>
</tr>
<tr>
<td><strong>Gender</strong> (male : female)</td>
<td>6:3</td>
<td>7:1</td>
<td>4:4</td>
</tr>
<tr>
<td><strong>Smoking history</strong> (pack years)</td>
<td>0.0 ± 0.0</td>
<td>30.8 ± 5.0*</td>
<td>51.4 ± 5.5***</td>
</tr>
<tr>
<td><strong>Current smoker</strong> (yes : no)</td>
<td>-</td>
<td>8:0</td>
<td>2:6</td>
</tr>
<tr>
<td><strong>FEV₁ (L)</strong></td>
<td>3.10 ± 0.33</td>
<td>3.16 ± 0.22</td>
<td>1.26 ± 0.10***##</td>
</tr>
<tr>
<td><strong>FEV₁ (% predicted)</strong></td>
<td>102.4 ± 3.4</td>
<td>88.9 ± 4.1</td>
<td>49.2 ± 5.2****#</td>
</tr>
<tr>
<td><strong>FVC (L)</strong></td>
<td>3.96 ± 0.40</td>
<td>4.38 ± 0.37</td>
<td>2.58 ± 0.18***##</td>
</tr>
<tr>
<td><strong>FEV₁/FVC</strong></td>
<td>0.79 ± 0.03</td>
<td>0.76 ± 0.04</td>
<td>0.49 ± 0.04****##</td>
</tr>
</tbody>
</table>

Table 4.10. Subject demographics. Participants underwent spirometry and FEV₁ and FVC were recorded. A single pack year is equivalent to 20 cigarettes smoked every day for 1 year. Data presented are mean ± SEM. *** represents p < 0.001, ** represents p < 0.01, * represents p < 0.05 relative to non-smokers. ## represents p < 0.01, # represents p < 0.05 relative to smoking controls.

4.5.7.2. Levels of acetylated tubulin in MDM

Levels of acetylated tubulin within MDM were compared between non-smokers, smokers and COPD patients. MDM from COPD patients contained less acetylated tubulin than non-smokers, but not smokers (Figure 4.32).
Figure 4.32. Comparison of the levels of acetylated tubulin (Ac-T) in MDM from non-smokers, smokers and COPD patients. Monocytes from non-smokers (NS; n = 9), smokers (Sm; n = 8) and COPD patients (n = 8) were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM. Cells were then lysed in RIPA buffer and proteins separated by electrophoresis. Western blotting was then performed using an anti-acetylated tubulin antibody. Blots were stripped and re-probed with a primary anti-actin antibody, which was used as a loading control. Presented blots are representative examples. Data are median ± IQR, and range. * represents p < 0.05 relative to non-smoking controls.

4.5.7.3. Effect of demographic variables on the levels of acetylated tubulin in MDM

The subject groups were not matched for age and did not contain an equal number of male and female participants (Table 4.10). Therefore, any potential confounding effects on the levels of acetylated tubulin were examined. No correlations were observed between age and the levels of acetylated tubulin, across all of the three subject groups (Spearman r = -0.16, p = 0.44) or when analysing each group independently. Moreover, there was no difference in the levels of acetylated tubulin contained within MDM from the male and female participants.
(subject groups analysed together). The median acetylated tubulin/actin ratios (± IQR) were 0.57 (± 0.22) and 0.50 (± 0.15) for males and females respectively (p = 0.12).

4.5.7.4. Effect of lung function on the levels of acetylated tubulin in MDM

To determine whether levels of acetylated tubulin were related to lung function, correlations were performed with FEV₁ (L and % predicted), FVC and FEV₁/FVC (Figure 4.33). No correlations were observed for FVC or FEV₁/FVC, when the subject groups were analysed as a whole or independently. FEV₁ (L) and FEV₁ (% predicted) did, however, moderately correlate with the levels of acetylated tubulin contained within MDM, but this was only apparent when the three groups were analysed together. When the groups were analysed independently, no correlations were observed for FEV₁ (L) or FEV₁ (% predicted).
Figure 4.33. Correlation between lung function and the levels of acetylated tubulin (Ac-T) in MDM:
(A) FEV₁ (L) (B) FEV₁ (% predicted) (C) FVC (D) FEV₁/FVC ratio. Monocytes from non-smokers (▲; n = 9), smokers (■; n = 8) and COPD patients (●; n = 8) were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM. Cells were then lysed in RIPA buffer and proteins separated by electrophoresis. Western blotting was then performed using an anti-acetylated tubulin antibody. Blots were stripped and re-probed with a primary anti-actin antibody as a loading control. Data shown are Spearman rank correlation coefficients (subject groups analysed together).
4.5.7.5. Effect of smoking on the levels of acetylated tubulin in MDM

To determine whether levels of acetylated tubulin were related to smoking, correlations were performed with the number of pack years smoked (Figure 4.34). No correlation was observed between the levels of acetylated tubulin contained within MDM and the number of pack years (subject groups analysed together and independently). Comparisons were also made between current and ex-smokers, to determine whether levels of acetylated tubulin were related to smoking status (Figure 4.35). There were no differences in the levels of acetylated tubulin between current and ex-smokers.

Figure 4.34. Correlation between pack years and the level of acetylated tubulin (Ac-T) in MDM from smokers and COPD patients. Monocytes from smokers (■; n = 8) and COPD patients (●; n = 8) were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM. Cells were then lysed in RIPA buffer and proteins separated by electrophoresis. Western blotting was then performed using an anti-acetylated tubulin antibody. Blots were stripped and re-probed with a primary anti-actin antibody as a loading control. Data shown are Spearman rank correlation coefficients (subject groups analysed together).
Figure 4.35. Effect of smoking status on the levels of acetylated tubulin (Ac-T) in MDM from smokers and COPD patients. Monocytes from smokers (■; n = 8) and COPD patients (●; n = 8) were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM. Cells were then lysed in RIPA buffer and proteins separated by electrophoresis. Western blotting was then performed using an anti-acetylated tubulin antibody. Blots were stripped and re-probed with a primary anti-actin antibody as a loading control. Data shown are Spearman rank correlation coefficients (subject groups analysed together).

4.5.8. Effect of nocodazole on phagocytosis by AM

Bacterial phagocytosis by MDM from COPD patients was more susceptible to the effects of nocodazole (Section 4.5.6). These findings were therefore investigated in AM.

4.5.8.1. Subject Demographics

Demographic data for the subjects who underwent bronchoscopy and donated AM for nocodazole experiments are shown in Table 4.11. The three groups were matched for age. There were an unequal number of male and female participants in each group. In the non-smoking and smoking groups, males outnumbered females. In the COPD group, there were more female than male patients. The smokers and COPD patients were matched for smoking
Chapter 4

history. The COPD patients comprised both current and ex-smokers. They had a lower mean FEV₁ (L) than both the non-smokers and smokers. The mean FEV₁ (% predicted) and FEV₁/FVC ratio were also lower in the COPD group, but only when compared to the non-smokers. There were no differences in FVC (L).

<table>
<thead>
<tr>
<th></th>
<th>Non-Smoker (n = 7)</th>
<th>Smoker (n = 5)</th>
<th>COPD (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>60.7 ± 3.2</td>
<td>54.6 ± 2.4</td>
<td>61.4 ± 1.8</td>
</tr>
<tr>
<td>Gender (male : female)</td>
<td>4:3</td>
<td>4:1</td>
<td>1:6</td>
</tr>
<tr>
<td>Smoking history (pack years)</td>
<td>0.0 ± 0.0</td>
<td>24.2 ± 6.9*</td>
<td>30.6 ± 3.2**</td>
</tr>
<tr>
<td>Current Smoker (yes : no)</td>
<td>-</td>
<td>5:0</td>
<td>4:3</td>
</tr>
<tr>
<td>FEV₁ (L)</td>
<td>3.21 ± 0.27</td>
<td>3.05 ± 0.33</td>
<td>1.63 ± 0.11***#</td>
</tr>
<tr>
<td>FEV₁ (% predicted)</td>
<td>109.4 ± 5.6</td>
<td>91.7 ± 5.2</td>
<td>68.0 ± 3.6***</td>
</tr>
<tr>
<td>FVC (L)</td>
<td>3.73 ± 0.33</td>
<td>3.92 ± 0.41</td>
<td>2.52 ± 0.26</td>
</tr>
<tr>
<td>FEV₁/FVC</td>
<td>0.81 ± 0.02</td>
<td>0.78 ± 0.03</td>
<td>0.67 ± 0.03*</td>
</tr>
</tbody>
</table>

Table 4.11. Subject demographics. Participants underwent spirometry and the FEV₁ and FVC were recorded. A single pack year is equivalent to 20 cigarettes smoked every day for 1 year. Data presented are mean ± SEM. *** represents p < 0.001, ** represents p < 0.01, * represents p < 0.05 relative to non-smokers. # represents p < 0.05 relative to smoking controls.

4.5.8.2. Effect of nocodazole on the phagocytosis of beads (AM)

Nocodazole inhibited the phagocytosis of beads by AM from non-smokers, smokers and COPD patients (Figure 4.36). No differences were observed between the maximum percentage inhibition of bead phagocytosis for non-smokers, smokers and COPD patients (mean (± SEM).
of 58.7 % (± 8.7), 69.6 (± 7.4) and 50.4 % (± 11.8) respectively). Within this concentration range, the response was not concentration-dependent. As such, EC50 values could not be calculated.

![Graph](image)

**Figure 4.36. Effect of nocodazole on the phagocytosis of beads by AM.** AM were isolated from the BALF of non-smokers (▲), smokers (■) and COPD patients (●), n = 7, n = 5 and n = 7 respectively. Cells were rested overnight before pre-treatment with different concentrations of nocodazole or vehicle control (VC; DMSO 0.1%) for 1h prior to performing phagocytosis assays with beads. Data presented are mean percentage phagocytosis ± SEM (calculated relative to VC). *** represents p < 0.001, ** represents p < 0.01, * represents p < 0.05 relative to own VC.

### 4.5.8.3. Effect of nocodazole on the phagocytosis of bacteria (AM)

Nocodazole inhibited the phagocytosis of both *H. influenzae* and *S. pneumoniae* by AM derived from non-smokers, smokers and COPD patients (**Figure 4.37; Panels A and B**). The response to nocodazole was concentration-dependent for both bacteria. The curves for COPD patients also appeared to be shifted leftward, relative to the control subjects (non-smokers but not smokers).
Figure 4.37. Effect of nocodazole on the phagocytosis of bacteria by AM. (A) *H. influenzae* (B) *S. pneumoniae*. AM were isolated from the BALF of non-smokers (▲), smokers (■) and COPD patients (●), n = 7, n = 5 and n = 7 respectively. Cells were rested overnight before pre-treatment with different concentrations of nocodazole or vehicle control (VC; DMSO 0.1%) for 1h prior to performing phagocytosis assays with bacteria. Data presented are mean percentage phagocytosis ± SEM (calculated relative to VC). *** represents p < 0.001, ** represents p < 0.01, * represents p < 0.05 relative to own VC.

As with MDM, values for maximum percentage inhibition (Table 4.12) and EC₅₀ (Table 4.13) were calculated and compared between the three groups. The maximum percentage inhibition of bacterial phagocytosis by AM was no different across the three groups for both *H. influenzae* and *S. pneumoniae*. EC₅₀ values for the inhibition of bacterial phagocytosis by nocodazole were lower for AM from COPD patients, when compared to non-smokers, but not smokers. This was true of both *H. influenzae* and *S. pneumoniae*. This confirms the apparent leftward shift of the curves for COPD patients relative to non-smokers, as demonstrated in Figure 4.37 (Panels A & B). In other words, bacterial phagocytosis by AM from COPD patients was more sensitive to the effects of nocodazole than AM from non-smokers, but not smokers.
Table 4.12. Maximum percentage inhibition of bacterial phagocytosis by AM in response to nocodazole. BAL was used to sample AM from non-smokers (n = 7), smokers (n = 5) and COPD patients (n = 7). Cells were pre-treated for 1h with different concentrations of nocodazole or vehicle control (VC; DMSO 0.1%), prior to performing phagocytosis assays with *H. influenzae* (HI) or *S. pneumoniae* (SP). The maximum percentage inhibition was calculated relative to the VC. Data presented are mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Non-Smoker (n = 7)</th>
<th>Smoker (n = 5)</th>
<th>COPD (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phagocytosis of HI (% inhibition)</strong></td>
<td>62.2 ± 6.1</td>
<td>63.8 ± 8.6</td>
<td>80.1 ± 8.6</td>
</tr>
<tr>
<td><strong>Phagocytosis of SP (% inhibition)</strong></td>
<td>61.1 ± 9.7</td>
<td>84.6 ± 7.3</td>
<td>88.6 ± 5.3</td>
</tr>
</tbody>
</table>

Table 4.13. EC$_{50}$ for the inhibition of bacterial phagocytosis by nocodazole (AM). BAL was used to sample AM from non-smokers (n = 7), smokers (n = 5) and COPD patients (n = 7). Cells were pre-treated for 1h with different concentrations of nocodazole or vehicle control (VC; DMSO 0.1%), prior to performing phagocytosis assays with *H. influenzae* (HI) or *S. pneumoniae* (SP). Individual EC$_{50}$ values were calculated. Data presented are mean ± SEM. * represents p < 0.05 relative to non-smoking controls.

<table>
<thead>
<tr>
<th></th>
<th>Non-Smoker (n = 7)</th>
<th>Smoker (n = 5)</th>
<th>COPD (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phagocytosis of HI EC$_{50}$ (µM)</strong></td>
<td>24.3 ± 4.1</td>
<td>25.4 ± 7.7</td>
<td>6.9 ± 2.1*</td>
</tr>
<tr>
<td><strong>Phagocytosis of SP EC$_{50}$ (µM)</strong></td>
<td>8.8 ± 2.5</td>
<td>4.8 ± 2.3</td>
<td>2.6 ± 0.6*</td>
</tr>
</tbody>
</table>

The results presented in Figure 4.37 and Table 4.13 mirror those obtained with MDM. In both sets of experiments, cells from COPD patients were more sensitive to the inhibitory effects of nocodazole than those from non-smokers but not smokers.
4.5.8.4. Effect of nocodazole on AM viability

Viability assays were performed with AM, in order to confirm that the effect of nocodazole on phagocytosis was not the result of a reduction in cell viability with higher concentrations. When compared to untreated cells, the viability of AM from all groups were unaffected by the vehicle or nocodazole, up to concentrations of $10^{-4}$ M (Figure 4.38).

![Figure 4.38. Effect of nocodazole on AM viability.](image)

**Figure 4.38. Effect of nocodazole on AM viability.** AM were isolated from the BALF of non-smokers (▲), smokers (■) and COPD patients (●), n = 7, n = 5 and n = 7 respectively. Cells were rested overnight before pre-treatment (1 h) with different concentrations of nocodazole or vehicle control (VC; DMSO 0.1%). Some cells were left untreated (UT). After performing phagocytosis assays, AM were then incubated with 50 μl of MTT solution for 45 min at 37°C. Supernatants were aspirated and 50 μl of DMSO were added. Plates were read at $\lambda_{570}$ nm on a spectrophotometer. Data presented are percentage cell viability, relative to the UT cells (i.e. UT cells = 100% viability, represented by the horizontal dotted line).
4.6. Discussion

MDM from COPD patients were defective in bacterial phagocytosis, relative to non-smokers, but not smokers. There were sizeable 41% and 53% reductions in the internalisation of *H. influenzae* and *S. pneumoniae*, respectively. Similar results have been previously reported for both MDM (Taylor et al., 2010, Hodge and Reynolds, 2012) and AM (Berenson et al., 2006, Marti-Lliteras et al., 2009, Taylor et al., 2010, Hodge and Reynolds, 2012, Berenson et al., 2013), using either adherent cells and fluorometry (Taylor et al., 2010, Berenson et al., 2013) or non-adherent macrophages and flow cytometry (Hodge and Reynolds, 2012). This strongly implies that the results presented in this chapter are reproducible and representative of macrophage function/dysfunction *in vitro*. *H. influenzae* and *S. pneumoniae* were selected for assay, as they are important respiratory pathogens (Monso et al., 1995) that commonly reside in the lungs of COPD patients (Cabello et al., 1997, Rosell et al., 2005). Their ineffective clearance by defective phagocytosis is therefore likely to be clinically relevant and might be a key mechanism through which they colonise COPD airways and perpetuate inflammation.

No differences in the phagocytosis of bacteria were observed between smokers and COPD patients, or between the two control groups. As such, it still remains unclear if bacterial phagocytosis is defective in smokers without COPD. One explanation might be the wide variability in results from smoking controls. Despite a similar number of participants, the IQR for the phagocytosis of *S. pneumoniae* was almost twice as wide for smokers (± 2.32) than for non-smokers (± 1.28) or COPD patients (± 1.01). This might reflect the highly heterogenous nature of the smoking group. Although a FEV₁/FVC ratio < 0.7 and FEV₁ < 80% were used to define COPD, the smoking group did contain some patients with spirometry results that were close to these cut-offs. These subjects may have had as yet undiagnosed ‘mild GOLD stage 1’ disease (Aaron et al., 2017) and their phagocytosis results might have been more reflective of
COPD. The smoking group also contained subjects with normal spirometry, who had a minimal smoking history (barely > 10 pack years). Results from these participants may have more closely resembled those from non-smoking controls.

In contrast to bacterial phagocytosis, there were no differences in the uptake of inert beads by MDM from non-smokers, smokers and COPD patients. This dichotomy in results has been observed before with both MDM and AM (Taylor et al., 2010). It suggests that inert particles are recognised by different cell surface receptors and/or internalised via alternative phagocytosis pathways, which are not defective in COPD. Importantly however, it also highlights that macrophages from all three groups are capable of equivalent phagocytosis, or that macrophages from COPD patients are not completely dysfunctional as phagocytes. This raises the possibility that defective bacterial phagocytosis might be correctable pharmacologically, which could reduce airway bacterial load and associated inflammation.

Defective bacterial phagocytosis was a feature of MDM from COPD patients. These cells were cultured from circulating monocytes, had never entered the lung parenchyma or alveoli, and were therefore not directly exposed to any of the inflammatory- or environmental-stimuli associated with COPD. This strongly implies that the defect is inherent to the monocyte-macrophage lineage, rather than being acquired by AM as they reside in the lungs of COPD patients. Alternatively, the monocytes may have been influenced by systemic inflammation that had ‘spilled over’ from the lungs into the circulation (Agusti et al., 2012). Taylor et al. (2010) examined the phagocytosis of *E. coli* by monocytes, MDM and AM, sampled from non-smokers, smokers and COPD patients. Although defects in bacterial phagocytosis were demonstrated by both MDM and AM from COPD patients, monocytes from all of the three groups internalised equivalent quantities of *E. coli*. These findings not only support the data
presented in this chapter but also suggest that monocyte differentiation is required for defective bacterial phagocytosis.

Heat-killed bacteria were used for phagocytosis assays. Although this avoided the added complexity of live bacteria, heat denatures bacterial proteins and this is not representative of phagocytosis in vitro. Although this could be considered a limitation, studies utilising live bacteria have demonstrated that phagocytosis by MDM and AM is still defective in COPD (Berenson et al., 2006, Taylor et al., 2010, Thomas, 2012). Furthermore, the fluorometric assay employed in this thesis only examined the overall internalisation of prey. It was unable to isolate any differences in specific phagocytic processes between the three groups, such as the committal of prey to the phagolysosome or bacterial killing. Bacteria can be labelled with the pH sensitive dye pHrodo™ (Invitrogen, UK), which will only fluoresce at the low pH encountered in the phagolysosome (Prosser et al., 2013). Using pHrodo™ and fluorometry, any differences in phagolysosome formation could also have been assessed. ‘Killing assays’ with live bacteria would have allowed bacterial killing to have been quantified and compared between the subject groups. Such assays have been previously described and shown that the intracellular killing of bacteria by macrophages is unaffected by COPD (Berenson et al., 2006, Thomas, 2012).

Another potential limitation is a lack of data regarding the repeatability of fluorometric phagocytosis assays. It was anticipated that fluorescent-labelling might vary between batches of H. influenzae and S. pneumoniae, with subsequent effects upon RFU. As such, single batches of H. influenzae and S. pneumoniae were used for all experiments. However, detailed data regarding repeatability could not be collected during this study due to the limited cell availability and an inability to reuse MDM/AM for repeated assays. Moreover, it is unknown whether similar results would be obtained if MDM/AM were sampled from the same subject,
but at different timepoints. That said, median RFU from phagocytosis assays with MDM were comparable across Chapters 3 and 4, i.e. results with beads and *S. pneumoniae* were similar when comparing the three subject groups. Although this was not consistently demonstrated for *H. influenzae* (for smokers only), it nevertheless provides some evidence of assay repeatability.

The subject groups were not matched for age and all contained marginally more males than females. As such, any potential confounding effects were examined. Age did not correlate with the uptake of beads, *H. influenzae* or *S. pneumoniae*, suggesting that any differences in phagocytosis were not simply a phenomenon of ageing. Likewise, gender was not associated with any differences in the phagocytosis of bacteria. For unclear reasons however, males internalised beads more avidly than females. This was only the case for male smokers, when the groups were analysed independently. Weak to moderate correlations were observed between most of the lung function parameters and the phagocytosis of both *H. influenzae* and *S. pneumoniae*. This included $\text{FEV}_1$ (L and % predicted), FVC and $\text{FEV}_1/\text{FVC}$ (for *H. influenzae* but not *S. pneumoniae*). These were only present when the subjects were analysed as a whole and no associations were found during independent group analyses. Given that the COPD patients had a significantly lower $\text{FEV}_1$ (L and % predicted), FVC and $\text{FEV}_1/\text{FVC}$ ratio, these weak correlations are most likely representative of the underlying differences in bacterial phagocytosis between the subject groups, and do not serve as evidence for an association with lung function.

A significant proportion of the COPD cohort was prescribed a regular inhaled treatment (*i.e.* LABA, LAMA or ICS) and/or oral medication (*i.e.* carbocisteine, azithromycin or slow/sustained-release theophylline). Many of these drugs target the actions of innate immune cells, macrophages included. Given that the control subjects were not exposed to any of these
pharmacotherapies, it is possible that they might have acted as confounders and potentially accounted for defective phagocytosis in COPD. However, when analysing the COPD data, no reductions in bacterial phagocytosis were found for those patients who were taking any of these medications. This further strengthens the argument that defective bacterial phagocytosis results from COPD itself and is not a by-product of using immunomodulatory treatments. In contrast, the regular use of azithromycin was associated with an increased (rather than decreased) uptake of *S. pneumoniae* by MDM from COPD patients. This suggests that this macrolide may be capable of augmenting bacterial phagocytosis by AM. This is further explored *in vitro*, within Chapter 5.

The data presented in this chapter does not indicate that cigarette smoking is an important aetiological factor for defective phagocytosis. No correlations were seen between pack years and the internalisation of either beads or *H. influenzae*. Although, there was a correlation between the phagocytosis of *S. pneumoniae* and pack years, this was weak, only present for COPD patients during an individual group analysis, and appeared to be driven to a large extent by a single outlier. Furthermore, no differences were found between the phagocytosis of *H. influenzae* and *S. pneumoniae* by current and ex-smokers. This was apparent when analysing the smoking controls and COPD patients together, or when individually assessing the COPD group.

In Chapter 3, correlations were demonstrated between the phagocytosis of Gram-negative and Gram-positive bacteria by AM (*Figure 3.6*). A similar, albeit stronger correlation, was also observed for MDM in this chapter. Unlike AM, independent correlations were also found for non-smokers, smokers and COPD patients. This might simply reflect the increased subject recruitment. However, when taken together, these complementary data suggest that the phagocytic capacity of macrophages is not specific to bacterial strain. They also support the
hypothesis first introduced in Chapter 3, that MDM are a reliable surrogate for studying AM phagocytosis.

If, as suggested, defective phagocytosis by AM has a pivotal role in bacterial colonisation and airway inflammation, then one would expect a close association between the capacity of AM for bacterial phagocytosis and markers of disease severity. However, no correlations were found between FEV$_1$ (% predicted) and the phagocytosis of *H. influenzae* or *S. pneumoniae* by MDM from COPD patients. Furthermore, the MDM from patients with severe airflow obstruction did not internalise less bacteria than those from patients with moderate airflow obstruction. These data were clearly limited by a relatively narrow range of FEV$_1$ values (34 – 79%), which did not encompass all severities of airflow obstruction. Moreover, as detailed in Section 1.5, other innate and adaptive immune responses are important in eliminating bacteria and many of these are also defective in COPD (Shaykhiev and Crystal, 2013). Nonetheless, this should be evaluated further in a larger population with a wider array of FEV$_1$. Attempts could also be made to correlate bacterial phagocytosis with more sensitive measures of small airway dysfunction, such as R$_{5-20}$ (using IOS).

By the same token, relationships between bacterial phagocytosis and COPD-specific symptoms might also be expected. However, no statistically-significant associations for dyspnoea (mMRC score) or symptom burden (CAT score) were identified. There was a trend towards a reduction in the phagocytosis of *S. pneumoniae* by MDM from those COPD patients who were more breathless (mMRC ≥ 2) or had the greatest symptom burden (CAT ≥ 10). However, the COPD cohort was significantly skewed towards patients with greater CAT and mMRC scores.

It was hypothesised that patients who exacerbate most frequently, do so as their AM are less capable of clearing bacteria from the airways. However, when comparing frequent (≥ 2 / year)
and infrequent (< 2 / year) exacerbators, no differences were found in the phagocytosis of beads, *H. influenzae* or *S. pneumoniae*. This was despite the two COPD phenotypes being matched for all demographic variables and there being a clear separation in the mean number of exacerbations per year (5 times as many for frequent exacerbators). Fifty percent of exacerbations are not associated with bacteria (Sapey and Stockley, 2006) and it therefore seems highly unlikely in retrospect that differential bacterial phagocytic responses could solely account for exacerbation frequency. A more detailed analysis, that pairs results from phagocytosis assays with prospective data on the presence or absence of bacteria in airway samples, would be better placed to determine whether defective bacterial phagocytosis was an important aetiological factor in bacterial colonisation and COPD exacerbations. Such studies could also correlate the presence of specific bacterial species to their internalisation by MDM or AM.

The combined GOLD assessment encompasses airflow obstruction, symptoms and exacerbation frequency to provide a more complete assessment of COPD severity (*Figure 1.1*). As such, the COPD cohort was categorised into Groups A, B, C and D, to identify any differences in bacterial phagocytosis between these groups. However, nearly all of the patients were categorised as Group D, which prevented any meaningful comparisons.

Possible mechanism(s) underpinning defective bacterial phagocytosis were also explored in this chapter and for reasons detailed in *Section 4.1*, the relative stability of microtubules was chosen as the focus for this investigation. It was hypothesised that defective bacterial phagocytosis arises from a predominance of unstable microtubule polymers in COPD. As such, two markers of stability were compared in a subset of MDM from non-smokers, smokers and COPD patients. These were the relative sensitivity of phagocytosis to micromolar concentrations of nocodazole and levels of acetylated tubulin from unstimulated cells. Any
leftward shift in nocodazole dose-response curves (i.e. lower EC$_{50}$ values) and/or reductions in acetylated-tubulin were interpreted as evidence of less stable microtubule arrangements. Conscious that MDM are a model, attempts were also made to validate any findings in AM.

Pre-incubation with nocodazole for 1 hour inhibited the phagocytosis of beads, *H. influenzae* and *S. pneumoniae* by both MDM and AM. High micromolar concentrations were required, but they were not associated with any reduction in cell viability. While bacterial phagocytosis was inhibited in a concentration-dependent manner, the responses for experiments with beads were concentration-independent. Nocodazole did not completely inhibit the phagocytosis of either beads or bacteria by macrophages from non-smokers, smokers or COPD patients. The maximum percentage inhibition for bead phagocytosis was 30-70% for MDM and AM, while bacterial phagocytosis was inhibited by 40-90% (approximate ranges based on all three subject groups, MDM and AM). These data either suggest that a significant proportion of microtubules are stable (in all subject groups) and therefore resistant to depolymerisation, or that phagocytosis during these assays was not completely dependent on an intact network of microtubule polymers, and that some internalisation occurred via microtubule-independent pathways. Although both suggestions are plausible, higher concentrations of nocodazole and longer pre-incubation times were not examined in this thesis, and these may have led to a complete inhibition of phagocytosis. Nonetheless, future investigations should still examine the relative contribution of microtubule-independent pathways, such as antibody-dependent FcR-mediated phagocytosis (Newman et al., 1991) and the possibility of any defects in COPD.

Bacterial phagocytosis by COPD macrophages was found to be more sensitive to the inhibitory effects of nocodazole than macrophages from non-smokers, but not smokers (both MDM and AM). This was evidenced by a leftward shift in the inhibitory concentration-response curves and lower corresponding EC$_{50}$ values. Concurrently, MDM from COPD patients were found to
contain less acetylated tubulin than MDM from non-smokers, but not smokers. These results were complementary and were not otherwise explained by confounding variables. Indeed, the subject groups were either matched and/or the levels of acetylated tubulin failed to correlate with age, gender, smoking history/status and lung function. Moderate correlations were observed for FEV₁ (both L and % predicted) but these might be explained by the differences in acetylated tubulin that were found between the subject groups.

An inadequate number of AM were harvested from BALF to enable the quantification of acetylated tubulin and potentially validate the work with MDM. This is a limitation and these experiments should be undertaken once additional samples are available. Confirmation of microtubule depolymerisation with nocodazole, could also have been directly visualised using confocal microscopy and Tubulin Tracker™. This green dye preferentially stains polymerised microtubules and a loss of signal may have been seen with the addition of nocodazole (depolymerisation). Nevertheless, the data presented above can be taken together to provide evidence that microtubule polymers are skewed towards an unstable population in COPD macrophages. It is possible that while polymers in AM from healthy non-smokers are stable, acetylated, associated with MAPs and resistant to disassembly, those contained within AM from COPD patients are less stable, rapidly undergo ‘catastrophe’, remain unacetylated/less acetylated and are less likely to be associated with MAPs (Figure 4.39). They would therefore be less effective for SR-mediated phagocytosis.
Figure 4.39. Contrasting microtubule arrangements in AM from healthy non-smokers and COPD patients. This figure summarises the predominant polymer arrangements in AM from non-smokers and COPD patients. AM from healthy non-smokers contain stable and acetylated polymers, which are associated with MAPs, are resistant to disassembly and less likely to depolymerise in the presence of nocodazole. In contrast, those contained within AM from COPD patients are less stable, rapidly undergo ‘catastrophe’, are less likely to be associated with MAPs and remain unacetylated. They are also more susceptible to depolymerisation by nocodazole and less effective for SR-mediated phagocytosis. MAP = microtubule-associated protein.

Data from the microtubule experiments (i.e. nocodazole sensitivity and levels of acetylated tubulin) closely reflect results from the bacterial phagocytosis assays (Figure 4.4). Specifically, both data sets revealed differences between COPD patients and non-smokers, but results with smokers could not be distinguished from either of the other two subject groups. As discussed earlier, this might reflect the heterogeneity of the smoking control group. As such, larger sample sizes are therefore also required to definitively compare smokers to both non-smokers and COPD patients, with respect to microtubule stability.
Statistical differences in EC$_{50}$ values and contrasting levels of acetylated tubulin were observed despite only using a subset of subjects and a relatively small sample size (n < 10 in each group). In Chapter 3, power calculations demonstrated that upwards of 20 subjects were required in each group for defective bacterial phagocytosis to be evident statistically. Moreover, defective bacterial phagocytosis by MDM was detected in this chapter when 20-23 subjects were recruited into each group. It is therefore unclear why the microtubule populations of much smaller subgroups would behave so differently. One explanation might be that macrophages from COPD patients are able to partially compensate for the unstable microtubule polymers by upregulating alternative and less ‘microtubule- or SR-dependent’ pathways. Defective bacterial phagocytosis may therefore be less evident and require a larger sample size for statistical significance. Nonetheless, it remains a limitation of these data and any future work should also compare raw phagocytosis results from the non-smokers, smokers and COPD patients who donate macrophages for nocodazole concentration-responses and Western blotting.

Similar results with nocodazole and acetylated tubulin have been observed by the research group previously. Data with MDM have already shown that bacterial phagocytosis by COPD patients displays an increased sensitivity to nocodazole, but only when considering a single concentration of this inhibitor (10 $\mu$M) (Taylor et al., 2009, Thomas, 2012). The same PhD students were also able to demonstrate that lower concentrations of acetylated tubulin were contained within MDM from COPD patients, relative to non-smokers (Taylor, 2009, Thomas, 2012). The present thesis however, is the first to use a range of nocodazole concentrations and corresponding EC$_{50}$ values, to comprehensively demonstrate a difference in nocodazole sensitivity. It is also the first to validate these findings in AM.
The relative effects of nocodazole on the phagocytosis of beads was also explored within this chapter. Although nocodazole was shown to inhibit their internalisation by MDM and AM, this response was not concentration-dependent. As such, it was not possible to compare non-smokers, smokers and COPD patients. A concentration-response curve, similar to those obtained with bacteria, may have been elicited by using lower concentrations of nocodazole. This could have been explored further, as any differential responses by the subject groups may then have become apparent. It remains possible however, that the inhibition of bead phagocytosis is truly concentration-independent, even at lower concentrations.

This chapter interrogated microtubule stability as a possible explanation for defective phagocytosis in COPD. There is some evidence however, that the expression of SR by AM is also reduced in COPD. Hodge et al. (2008) harvested AM from the BALF of 10 COPD patients and 10 healthy non-smoking controls and used flow cytometry to demonstrate that the expression of CD206 (MR) was significantly lower in both current and ex-smoking COPD patients. It is important to note however, that MARCO, rather than CD206, is the dominantly-expressed SR on human AM and is the primary means by which non-opsonised prey are internalised (Arredouani et al., 2004). Moreover, MARCO is not differentially expressed by AM in COPD (Taylor et al., 2010). Regardless, any differences in the expression and/or function of SR could still be explained by an underlying instability of microtubules, as these polymers are vital for membrane trafficking, receptor mobility, clustering and conformational changes (Swanson et al., 1987, Harrison and Grinstein, 2002, Niedergang and Chavrier, 2004).

The hypothesis for this chapter was: ‘Bacterial phagocytosis by MDM is defective in COPD, correlates with markers of disease severity, is most attenuated in frequent exacerbators and results from a predominance of unstable microtubule arrangements’. This hypothesis can only be partially accepted. Although defective bacterial phagocytosis was confirmed relative to
non-smokers, no convincing relationships were observed between the magnitude of this defect and any of the chosen markers of disease severity. Furthermore, the frequent exacerbator phenotype was not associated with a further reduction in bacterial phagocytosis. Experiments with nocodazole confirmed that microtubules in COPD MDM and AM were more sensitive to depolymerisation and MDM from COPD patients also contained less acetylated tubulin. Although these data are suggestive of less stable microtubule arrangements, depolymerisation with nocodazole should still be confirmed with Tubulin Tracker™ and levels of acetylated tubulin should be validated in AM.

Targeting microtubules by improving their stability in COPD may yet prove to be novel pharmacological approach, capable of improving PPB clearance by AM and eliminating this important inflammatory stimulus. Chapter 5 investigates whether defective bacterial phagocytosis can be augmented in COPD by macrolide antibiotics.
CHAPTER 5

The Immunomodulatory Effects of Solithromycin and Associated Analogues
5.1. Introduction

There is a pressing need to identify new treatments for COPD, as currently available pharmacotherapies are ineffective at suppressing the underlying inflammation and unable to slow disease progression or improve survival (Barnes, 2015). Bacterial phagocytosis by AM is defective in COPD (Berenson et al., 2006, Taylor et al., 2010, Berenson et al., 2013) and could potentially act as a novel drug target. It is one of the mechanisms through which PPB such as *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* colonise the airways (Rosell et al., 2005) and along with their products (*e.g.* LPS) stimulate resident leukocytes and perpetuate inflammation (Dickson et al., 2013). Indeed, correlations have been observed between bacterial loads and concentrations of airway inflammatory mediators in COPD (Hill et al., 2000, Banerjee et al., 2004b, Sethi et al., 2006, Marin et al., 2012, Singh et al., 2014). Augmenting defective bacterial phagocytosis could therefore potentially eliminate this important inflammatory-stimulus.

Improvements in phagocytosis might be achieved with an already-existing drug or drug class. In Chapter 4, an association was observed between the long-term use of azithromycin and an improved uptake of *S. pneumoniae* by MDM from COPD patients (Figure 4.28; Panel D). Stronger evidence for an effect of azithromycin on bacterial phagocytosis is found in a study by Hodge and Reynolds (2012). The administration of low doses of oral azithromycin to COPD patients led to significant improvements in the phagocytosis of *E. coli* by both AM and MDM. The investigators concluded that this might have resulted from an upregulation in phagocytic receptors. Indeed, in one of their earlier studies, azithromycin upregulated the expression of CD206 (MR) on AM in vitro, after incubation for 48h at concentrations of approximately $10^{-5}$ and $10^{-6}$ M (Hodge et al., 2008). This was subsequently confirmed ex vivo with oral azithromycin and was associated with improvements in efferocytosis by AM (uptake of apoptotic bronchial epithelial cells) (Hodge et al., 2008). It is important to note however, that
any concurrent effects on bacterial phagocytosis were not examined. Given that defective bacterial phagocytosis occurs in the absence of any downregulation of CD206 (MR) in COPD (Taylor et al., 2010), it seems likely that azithromycin would improve phagocytosis through other mechanisms, perhaps by targeting microtubule polymers, which are unstable in COPD (Chapter 4). Indeed, a number of related macrolide compounds have already been shown to stabilise microtubules (Mitsui-Saito et al., 2002, Field et al., 2009).

Azithromycin belongs to the macrolide class of drugs, which includes a number of compounds with antibacterial properties, such as erythromycin, clarithromycin and telithromycin (Section 1.3.1.4 and 1.3.1.5). These antibiotics bind to a single site on the 50s subunit of the bacterial ribosome, where they inhibit bacterial protein synthesis (Zuckerman, 2004). They are also capable of exerting numerous immunomodulatory effects on leukocytes and structural cells, which are independent of their antimicrobial actions (Kanoh and Rubin, 2010). Macrolide antibiotics have consistently demonstrated clinical benefits in stable COPD (Donath et al., 2013). In a randomised placebo-controlled trial by Albert et al. (2011), the addition of azithromycin to the normal treatment regimen of a large cohort of COPD patients (250 mg once daily for 1 year) was associated with reductions in exacerbation frequency and improvements in quality of life. This has led to the use of macrolide antibiotics as a supplementary therapy for stable patients. In particular, azithromycin is now frequently prescribed by clinicians in the UK for those with regular exacerbations or an ‘inflammatory clinical phenotype’ (bronchitis) that is unresponsive to standard treatment. Mechanistically, it is still debated whether azithromycin works by direct immunomodulation or whether any effects are simply by-products from antimicrobial activity (i.e. airway sterilisation) (Murphy et al., 2010). Regardless, macrolides significantly accumulate within AM (Rodvold et al., 1997)
and it therefore seems likely that they would be capable of modulating inflammation in COPD via effects on this pivotal leukocyte.

Few studies have specifically examined the effects of macrolides on the function of primary AM in COPD. However, there is indirect evidence from other conditions and in vitro, that macrolides would be capable of supressing the release of a number of macrophage-derived pro-inflammatory mediators that are involved in the pathogenesis of COPD. Using macrophages differentiated from the U937 cell line, Li et al. (2012) demonstrated that incubation for 24h with approximately 10^{-6} M of erythromycin suppressed the release of CXCL8 and TNF-α after stimulation with cigarette smoke extract. Cai et al. (2013) used primary AM from patients with cryptogenic organising pneumonia, and showed that incubation for 24h with both azithromycin and clarithromycin (at approximately 10^{-7} to 10^{-5} M) inhibited baseline and LPS-stimulated levels of several inflammatory mediators in a concentration-dependent manner (CXCL-8, TNF-α, IL-6 and IL-1β). Macrolides also suppress the release of pro-inflammatory mediators in vitro. Following 2 weeks of clarithromycin treatment (500 mg twice daily), Basyigit et al. (2004) found reductions in CXCL8 and TNF-α within both the sputum and sera of 30 male COPD patients. These effects are likely to be exerted through the inhibition of transcription factors, such as NF-κB and AP-1 (Desaki et al., 2000, Kikuchi et al., 2002, Desaki et al., 2004, Li et al., 2012), which are activated in COPD (Di Stefano et al., 2002, Barnes, 2006).

Major concerns exist over the use of macrolide antibiotics for immunomodulation in COPD. In particular, there is a worrying association between longer-term prescriptions and high rates of microbial resistance (Serisier, 2013). Indeed, bacteria can generate resistance to macrolides with relative ease, through methylation or mutation of the solitary ribosomal binding site (Leclercq and Courvalin, 2002). In one meta-analysis, long-term azithromycin use was associated with a 2.7-fold increase in the incidence of colonisation with a macrolide-resistant
organism (Li et al., 2014). Ketolides however, are capable of binding to at least 2 ribosomal sites (Hansen et al., 1999). They are semi-synthetic erythromycin derivatives and include telithromycin, cethromycin and the novel fluoroketolide solithromycin. Solithromycin is manufactured by Cempra pharmaceuticals and is currently in clinical development. It binds to at least three sites on the bacterial ribosome (Llano-Sotelo et al., 2010), thereby further reducing the potential for microbial resistance (Rodgers et al., 2013). It is active against Gram-positive, Gram-negative, atypical and non-tuberculous mycobacteria (Fernandes et al., 2011). It is also effective against macrolide-resistant bacterial strains (McGhee et al., 2010).

Solithromycin possesses immunomodulatory properties in vitro, which are similar to those of other macrolides. In a study by Kobayashi et al. (2013b), a 5h incubation with solithromycin at $10^{-4}$ M significantly inhibited TNF-α and CXCL8 release from LPS-stimulated macrophages that had been differentiated from the U937 cell line. Of the comparators erythromycin, clarithromycin, azithromycin and telithromycin, only clarithromycin was able to inhibit mediator release, but at ten-times the concentration ($10^{-3}$ M). The same authors also demonstrated that overnight incubation with solithromycin ($10^{-5}$ M) suppressed the release of TNF-α and production of MMP-9 by unstimulated PBMC from COPD patients. This effect was 10-times more potent than that of the next most potent macrolide, clarithromycin. In their paper, Kobayashi et al. (2013b) also demonstrated that solithromycin inhibited NF-κB activity and used these data as evidence that solithromycin mediated effects on macrophage secretory-function via the regulation of gene transcription. To date however, no studies have examined the effects of solithromycin on primary AM (or MDM) function in COPD.

The longer-term or wide-spread use of solithromycin may still promote bacterial resistance to earlier generations of macrolide, especially those that share ribosomal binding sites with solithromycin. With this in mind, Cempra Inc. have developed solithromycin analogues without
antimicrobial activity. This concept is not new. Indeed, it has been demonstrated previously that the antimicrobial and immunomodulatory activities of macrolide antibiotics are independent and can be separated (Bosnar et al., 2012, Jakopovic et al., 2012). With particular reference to COPD, Sugawara et al. (2015) found that derivatives of EM900 (an erythromycin analogue without antibacterial effects) were capable of improving lung function and histological changes in a smoke-exposed guinea pig model of emphysema. In another study, EM900 downregulated the expression of IL-1β, TNF-α and CXCL-8 from stimulated A549 cells via the inhibition of NF-kB activity (Otsu et al., 2011).

This chapter will, for the first time, examine the potential immunomodulatory effects of solithromycin and its associated analogues on AM function in COPD. It will use MDM as a surrogate and specifically look at whether solithromycin can augment defective bacterial phagocytosis in COPD and inhibit TNF-α from stimulated MDM. Azithromycin (the most commonly prescribed macrolide in stable COPD) and clarithromycin (a potent immunomodulator) have also been adopted as comparators. Chosen concentrations and experimental conditions have been based on the methods reported by other researchers, as detailed earlier in this introduction.

5.2. **Hypothesis**

The hypothesis for this chapter is: ‘solithromycin (and associated analogues) possess immunomodulatory properties, including the augmentation of defective bacterial phagocytosis in COPD and the inhibition of TNF-α release from stimulated MDM’.
5.3. Aims

The following aims were adopted in order to investigate the above hypothesis:

- Examine and compare the effects of solithromycin and azithromycin (comparator) on the phagocytosis of beads, *H. influenzae* and *S. pneumoniae* by MDM from non-smokers, smokers and COPD patients.
- Examine and compare the effects of solithromycin and azithromycin on the release of TNF-α from MDM that have been stimulated by bacteria (non-smokers, smokers and COPD patients).
- Examine and compare the effects of solithromycin, azithromycin and clarithromycin (second comparator) on the release of TNF-α from MDM that have been stimulated by LPS. This will be screened in non-smokers only.
- If solithromycin is indeed shown to be immunomodulatory (*i.e.* augments defective bacterial phagocytosis or inhibits the release of TNF-α from stimulated MDM), then the effects of its analogues will be similarly examined and compared.
- Examine whether any of the solithromycin analogues are antibiotics. Specifically, to investigate whether any of the analogues with immunomodulatory properties are active against the growth of respiratory pathogens (*H. influenzae, S. pneumoniae* and *M. catarrhalis*).

5.4. Methods

5.4.1. Subject selection

Healthy non-smokers, smoking controls and COPD patients were recruited as described in Section 2.2.1.
5.4.2. **Macrolide compounds**

Azithromycin and clarithromycin were purchased from Sigma-Aldrich (UK). Solithromycin and its analogues (CEM-153, CEM-154, CEM-214, CEM-218, CEM-232, CEM-237, CEM-262, CEM-267 and OP-1055) were obtained from Cempra Pharmaceuticals (USA). The structures and antimicrobial effects of these analogues were not provided by Cempra. All compounds were available as powders. The same vehicle (0.1% (v/v) DMSO) was used to prepare stock solutions of all compounds (10^{-4} M in 100% (v/v) DMSO). These stock solutions were subsequently diluted for experiments. Inter-assay variability was limited by using the same dilutions for each experiment (i.e. all MDM cell treatments with every subject, or each bacterial lawn growth).

5.4.3. **Isolation and culture of MDM**

MDM were generated from peripheral blood monocytes, as described in Section 2.2.6. Briefly, subjects donated 60 ml of venous blood and PBMC fractions were obtained using Percoll gradients. Monocytes were subsequently isolated by adherence. They were seeded in black 96-well tissue culture plates at 1 x 10^5 cells/well and cultured in the presence of GM-CSF (2 ng/ml) for 12 days, in order to generate MDM.

5.4.4. **Phagocytosis assays**

Phagocytosis assays were performed using fluorimetry, as described in Section 2.2.11. MDM were incubated with fluorescently-labelled beads or bacteria for 4 h, before being washed and any extracellular fluorescence quenched with trypan blue 1% (v/v). Fluorescence was determined using a plate reader (excitation λ480nm and emission λ520nm) and RFU were used as a measure of phagocytosis. Prior to performing the assay, MDM were pre-treated for 24 h (at 37°C, 5% (v/v) CO2) with 90 μl of azithromycin, solithromycin or vehicle (0.1% (v/v) DMSO). Control cells were left untreated (RPMI only). Solutions of azithromycin and solithromycin
were prepared in RPMI at concentrations ranging from $10^{-6.5}$ to $10^{-4}$ M. All assays were run in quadruplicate and with the same batch of labelled *H. influenzae* and *S. pneumoniae*, in an attempt to limit intra- and inter-assay variability, respectively. The intra-assay CV for all phagocytosis assays was < 10%. This was true of each subject group and for all three prey (beads, *H. influenzae* and *S. pneumoniae*).

5.4.4.1. Phagocytosis of beads

Fluorescently-labelled microspheres of 2.0 μm diameter (4.5 x 10⁹ beads/ml) were used (Section 2.2.11.1). After sonication for 2 min, they were diluted using 11μl/100ml of RPMI. 10 μl of the resulting bead suspension were then added to individual wells, already containing 90 μl of RPMI (± 0.1% (v/v) DMSO vehicle control ± macrolide). The final working concentration for phagocytosis was therefore 50 x 10⁶ microspheres/ml, the same as described in Section 2.2.11.1. Assays were then completed according to Section 2.2.11.3.

5.4.4.2. Phagocytosis of bacteria

Undiluted stock suspensions of bacteria were used (Section 2.2.9.4). 10 μl of *H. influenzae* or *S. pneumoniae* were added to individual wells, already contained 90 μl of RPMI (± 0.1% (v/v) DMSO vehicle control ± macrolide). The final working concentration for phagocytosis was therefore 5 x 10⁸ CFU/ml, the same as described in Section 2.2.11.2. Assays were then completed according to Section 2.2.11.3. Supernatants were harvested following phagocytosis and stored at -80°C, prior to ELISA for TNF-α (Section 5.4.5).

5.4.5. Measurement of TNF-α

ELISA was used to measure the concentrations of TNF-α in MDM supernatants, following stimulation with either 10 ng/ml LPS (for 24h) or 5 x 10⁸ CFU/ml bacteria (i.e. after 4h phagocytosis experiments with *H. influenzae* and *S. pneumoniae*). The effect of macrolides on
TNF-α release was also assessed by pre-treating MDM for 24h with various concentrations of compounds, prior to stimulation. Antibodies were purchased from Invitrogen (UK) and ELISA performed according to the manufacturer’s instructions. For a detailed description of the methodology refer to Sections 2.2.14. Briefly, supernatants were diluted 1:4 with 0.5% (w/v) BSA in D-PBS. TNF-α mouse anti-human capture (2 μg/ml) and detection (0.1 μg/ml) antibodies were used. Concentrations of TNF-α were interpolated from a standard curve. The lower limit of detection (sensitivity) was 31.25 pg/ml. Experiments were undertaken in different subsets of non-smokers, smokers and COPD patients; an exception being the use of the same five non-smokers to evaluate the effects of all the macrolide compounds (i.e. solithromycin, analogues and comparators) on LPS-stimulated TNF-α release (Sections 5.5.4 and 5.5.5).

5.4.6. Viability assays

The effect of all compounds on MDM viability was assessed using a MTT assay, as described in Section 2.2.12.

5.4.7. Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed with the macrolide compounds. Bacterial lawn growths of 3 common respiratory pathogens (H. influenzae, S. pneumoniae and M. catarrhalis) and the Kirby-Bauer disc diffusion method were used (Bauer et al., 1966). Clinical isolates of bacteria were obtained from the microbiology laboratory at RBHT (3 separate COPD patients during exacerbation). A detailed description of the methodology can be found in Section 2.2.10. Briefly, discs of blotting paper (6 mm in diameter) were soaked in varying concentrations of antibiotic (or vehicle) before being placed onto agar plates, which had been freshly inoculated with H. influenzae, S. pneumoniae or M. catarrhalis. These plates were then incubated for 16h at 37°C (5% (v/v) CO₂) before the zones of inhibition were measured for each
condition (i.e. the diameter of inhibited bacterial growth). Photographs were also taken for qualitative assessment.

5.4.8. Data analysis

Normal distributions were not assumed and all analyses were therefore performed using non-parametric tests. Demographic data were compared using a Kruskal-Wallis with Dunn’s post-hoc test. Dose-response curves for the various macrolide compounds were constructed by plotting the log concentration against the response (either percentage phagocytosis, TNF-α concentration (percentage or raw) or zone of inhibition). Where possible, three-parameter non-linear regression curves were assigned. Percentage phagocytosis was calculated relative to the vehicle control (i.e. RFU of condition ÷ RFU of vehicle control, expressed as a percentage (VC = 100%)). Percentage TNF-α release was similarly calculated relative to the response to LPS stimulation. Responses at different concentrations of compound were compared to the response with controls, using a Kruskal-Wallis with Dunn’s post-hoc test. Values for EC_{50} and maximum percentage inhibition were calculated from the curve of each individual experiment. Mean (± SEM) values for EC_{50} and maximum percentage inhibition were then calculated for each compound and comparisons made using a Kruskal-Wallis with Dunn’s post-hoc test. In all instances, the null hypothesis (i.e. no difference) was rejected at p < 0.05. All analyses were performed using GraphPad Prism software (USA).

5.5. Results

5.5.1. Effect of macrolides on phagocytosis by MDM

5.5.1.1. Subject demographics

Demographic data for the subjects who donated MDM for phagocytosis assays are shown in Table 5.1. The COPD patients were older than the smokers, but not non-smokers. The smokers
and non-smokers were matched for age. Male participants marginally outnumbered females (3 to 2) in all of the three subject groups. The smokers and COPD patients were matched for smoking history but nearly all of the COPD patients were ex-smokers. Non-smokers and smokers were matched for all measures of spirometry. The COPD patients however, had a lower FEV₁ (L and % predicted) than the non-smokers but not the smokers. No differences were observed in either FVC (L) or FEV₁/FVC ratio between any of the three subject groups.

<table>
<thead>
<tr>
<th></th>
<th>Non-Smoker (n = 5)</th>
<th>Smoker (n = 5)</th>
<th>COPD (n = 5)</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>57.6 ± 3.3</td>
<td>55.3 ± 3.0</td>
<td>70.6 ± 1.9#</td>
</tr>
<tr>
<td>Gender (male : female)</td>
<td>3:2</td>
<td>3:2</td>
<td>3:2</td>
</tr>
<tr>
<td>Smoking history (pack years)</td>
<td>0.0 ± 0.0</td>
<td>29.8 ± 7.6*</td>
<td>23.9 ± 5.7*</td>
</tr>
<tr>
<td>Current Smoker (yes : no)</td>
<td>-</td>
<td>5:0</td>
<td>1:4</td>
</tr>
<tr>
<td>FEV₁ (L)</td>
<td>3.59 ± 0.16</td>
<td>2.43 ± 0.35</td>
<td>1.82 ± 0.13**</td>
</tr>
<tr>
<td>FEV₁ (% predicted)</td>
<td>115.0 ± 6.2</td>
<td>76.2 ± 4.6</td>
<td>61.5 ± 2.2**</td>
</tr>
<tr>
<td>FVC (L)</td>
<td>4.07 ± 0.24</td>
<td>3.48 ± 0.57</td>
<td>3.87 ± 0.55</td>
</tr>
<tr>
<td>FEV₁/FVC</td>
<td>0.80 ± 0.02</td>
<td>0.71 ± 0.02</td>
<td>0.53 ± 0.07</td>
</tr>
</tbody>
</table>

**Table 5.1. Subject demographics.** Participants underwent spirometry, with FEV₁ and FVC being recorded. A single pack year is equivalent to 20 cigarettes smoked every day for 1 year. Data presented are mean ± SEM. ** represents p < 0.01, * represents p < 0.05 relative to non-smokers. # represents p < 0.05 relative to smoking controls.
5.5.1.2. Effect of azithromycin and solithromycin on the viability of MDM

The effects of azithromycin and solithromycin on MDM viability were explored at concentrations between $10^{-6.5}$ and $10^{-4}$ M (Figure 5.1). Cells were pre-treated with macrolide for 24h prior to 4h phagocytosis assays. Both azithromycin and solithromycin had no effect on the viability of MDM from non-smokers, smokers and COPD patients at concentrations $\leq 10^{-4.5}$ M. Solithromycin but not azithromycin, did however significantly reduce the viability of MDM from COPD patients at concentrations $> 10^{-4.5}$ M by approximately 80% (not examined in control subjects) (Figure 5.1; Panels B and C). Maximum concentrations of $10^{-4.5}$ M were therefore used to determine the effects on phagocytosis by MDM from all subject groups (Section 5.5.1.3 and 5.5.1.4).
Figure 5.1. Effect of azithromycin and solithromycin on MDM viability: (A) Azithromycin (B) Solithromycin (C) Solithromycin (10^{-4.5} to 10^{-4} M in COPD patients only). Monocytes from non-smokers (▲), smokers (■) and COPD patients (●) were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM (n = 3-5). Cells were then pre-treated for 24h with different concentrations of macrolide, vehicle control (VC; 0.1% DMSO) or were left untreated (UT), before being used in 4h phagocytosis assays. MDM were then incubated with 50 μl of MTT solution for 45 min at 37°C. Supernatants were aspirated and 50 μl of DMSO were added. Plates were read at λ570 nm on a spectrophotometer. Data presented are percentage cell viability, relative to the UT cells (i.e. UT cells = 100% viability, represented by the horizontal dotted line). *** represent p < 0.001, ** p < 0.01 relative to UT cells.
5.5.1.3. Effect of azithromycin and solithromycin on the phagocytosis of beads

The effect of azithromycin and solithromycin on the phagocytosis of beads was explored in MDM from non-smokers, smokers and COPD patients (Figure 5.2). At concentrations between $10^{-6.5}$ and $10^{-4.5}$ M, neither azithromycin or solithromycin influenced the phagocytosis of beads by MDM from control subjects or COPD patients.

Figure 5.2. Effect of azithromycin and solithromycin on the phagocytosis of beads by MDM: (A) Azithromycin (B) Solithromycin. Monocytes from non-smokers (▲), smokers (■) and COPD patients (■) were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM (n = 5). Cells were then pre-treated for 24h with different concentrations of macrolide or vehicle control (VC; 0.1% DMSO), prior to performing phagocytosis assays with fluorescently-labelled beads (4h). Data presented are mean percentage phagocytosis ± SEM (relative to VC).
5.5.1.4. Effect of azithromycin and solithromycin on the phagocytosis of bacteria

The effect of azithromycin and solithromycin on the phagocytosis of bacteria was explored in MDM from non-smokers, smokers and COPD patients (Figure 5.3). At concentrations between $10^{-6.5}$ and $10^{-4.5}$ M, azithromycin and solithromycin had no effect on the phagocytosis of *H. influenzae* by any of the subject groups (Figure 5.3; Panel A and B). Similarly, azithromycin (at the same concentrations) did not influence the phagocytosis of *S. pneumoniae* by MDM derived from non-smokers, smokers or COPD patients (Figure 5.3; Panel C). Solithromycin did however inhibit, rather than augment, the phagocytosis of *S. pneumoniae* by approximately 50% (Figure 5.3; Panel D). This was only observed for smokers at $10^{-4.5}$ M. Non-smokers and COPD patients did however exhibit similar trends.
Figure 5.3. Effect of azithromycin and solithromycin on the phagocytosis of *H. influenzae* and *S. pneumoniae* by MDM: (A) Azithromycin (*H. influenzae*) (B) Solithromycin (*H. influenzae*) (C) Azithromycin (*S. pneumoniae*) (D) Solithromycin (*S. pneumoniae*). Monocytes from non-smokers (▲), smokers (■) and COPD patients (●) were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM (n = 5). Cells were then pre-treated for 24h with different concentrations of macrolide or vehicle control (VC; 0.1% DMSO), prior to performing phagocytosis assays with fluorescently-labelled *H. influenzae* or *S. pneumoniae* (4h). Data presented are mean percentage phagocytosis ± SEM (relative to VC). * represent p < 0.05 relative to VC.
5.5.2. **Antimicrobial effects of macrolides**

At concentrations ranging between $10^{-6.5}$ and $10^{-4.5}$ M, azithromycin and solithromycin had no effect, or relatively little effect, on the phagocytosis of beads or bacteria by MDM from non-smokers, smokers and COPD patients. One explanation might have been that the compounds were inactivated in solution. To exclude this possibility, their antimicrobial effects were examined, using the same compounds, concentrations and vehicle.

5.5.2.1. **Effect of azithromycin and solithromycin on the growth of *H. influenzae***

The effects of $10^{-6.5}$ to $10^{-4.5}$ M azithromycin and solithromycin on lawn growths of *H. influenzae* were examined ([*Figures 5.4 and 5.5*]). The growth of *H. influenzae* was only modestly inhibited by both compounds, and only at $10^{-4.5}$ M. Retrospectively, it was determined that this clinical isolate of *H. influenzae* was obtained from a COPD patient who had been taking long term azithromycin. This could have led to a degree of macrolide resistance.

5.5.2.2. **Effect of azithromycin and solithromycin on the growth of *S. pneumoniae***

The effects of $10^{-6.5}$ to $10^{-4.5}$ M azithromycin and solithromycin on lawn growths of *S. pneumoniae* were also examined ([*Figures 5.4 and 5.6*]). The growth of *S. pneumoniae* was inhibited in a concentration-dependent manner by both macrolides.

5.5.2.3. **Effect of azithromycin and solithromycin on the growth of *M. catarrhalis***

The effects of $10^{-6.5}$ to $10^{-4.5}$ M azithromycin and solithromycin on lawn growths of *M. catarrhalis* were then examined ([*Figures 5.4 and 5.7*]). The growth of *M. catarrhalis* was also inhibited in a concentration-dependent manner by both macrolides.
Figure 5.4. Effect of azithromycin and solithromycin on bacterial growth: (A) *H. influenzae* (B) *S. pneumoniae* (C) *M. catarrhalis*. Bacterial lawns were prepared on agar. Discs of blotting paper (6 mm in diameter) were impregnated with varying concentrations of azithromycin (●), solithromycin (●) or vehicle control (VC; 0.1% (v/v) DMSO) before being placed directly onto the media. The plates were incubated for 16h and the zones of inhibition measured. Data presented are mean ± SEM (n = 3). The impregnated disc diameter is represented by the horizontal dotted line. *** represent p < 0.001, ** p < 0.01, * p < 0.05 relative to VC.
Figure 5.5. Zones of inhibition for azithromycin and solithromycin (*H. influenzae*): (A) Azithromycin (B) Solithromycin. Lawns of *H. influenzae* were prepared on agar plates. Discs of blotting paper (6 mm in diameter) were then impregnated with varying concentrations of azithromycin, solithromycin or vehicle control (VC; 0.1% (v/v) DMSO) before being placed directly onto the media. The plates were incubated for 16h at 37°C (5% (v/v) CO₂) and photographs taken. Scale bar = 10 mm. Images representative of n = 3.

Figure 5.6. Zones of inhibition for azithromycin and solithromycin (*S. pneumoniae*): (A) Azithromycin (B) Solithromycin. Lawns of *S. pneumoniae* were prepared on agar plates. Discs of blotting paper (6 mm in diameter) were then impregnated with varying concentrations of azithromycin, solithromycin or vehicle control (VC; 0.1% (v/v) DMSO) before being placed directly onto the media. The plates were incubated for 16h at 37°C (5% (v/v) CO₂) and photographs taken. Scale bar = 10 mm. Images representative of n = 3.
Figure 5.7. Zones of inhibition for azithromycin and solithromycin (*M. catarrhalis*): (A) Azithromycin (B) Solithromycin. Lawns of *M. catarrhalis* were prepared on agar plates. Discs of blotting paper (6 mm in diameter) were then impregnated with varying concentrations of azithromycin, solithromycin or vehicle control (VC; 0.1% (v/v) DMSO) before being placed directly onto the media. The plates were incubated for 16h at 37°C (5% (v/v) CO₂) and photographs taken. Scale bar = 10 mm. Images representative of n = 3.

5.5.3. Effect of macrolides on bacteria-stimulated TNF-α release

The data presented in Sections 5.5.1 and 5.5.2 demonstrated that although azithromycin and solithromycin inhibited bacterial growth *in vitro*, neither compound could upregulate the phagocytosis of bacteria by MDM when using the same concentrations (*i.e.* $10^{-6.5} - 10^{-4.5}$ M).

Macrolides do however possess other immunomodulatory properties, including the inhibition of TNF-α release from stimulated cells of the monocyte-macrophage lineage (Li *et al.*, 2012, Cai *et al.*, 2013, Kobayashi *et al.*, 2013b). As such, it was examined whether azithromycin and/or solithromycin could inhibit the release of TNF-α from MDM that had been stimulated by bacteria.

5.5.3.1. Subject demographics

Demographic data for the subjects who donated MDM are shown above (*Table 5.1*) and discussed in Section 5.5.1.
5.5.3.2. Bacteria-stimulated TNF-α release from MDM

Before examining the relative effects of azithromycin and solithromycin on the release of TNF-α, it was determined whether a 4h incubation with either *H. influenzae* or *S. pneumoniae* stimulated the release of TNF-α from MDM (Figure 5.8). *H. influenzae* and *S. pneumoniae* did indeed stimulate the release of TNF-α from MDM (all subject groups). Similar amounts of TNF-α were detected for non-smokers, smokers and COPD patients, following the same bacterial stimulus. Greater quantities of TNF-α were released from MDM stimulated by *H. influenzae*, relative to *S. pneumoniae* (significant for non-smokers and COPD patients; trend for smokers).

**Figure 5.8. Bacteria-stimulated TNF-α release from MDM.** Monocytes from non-smokers (□), smokers (□) and COPD patients (□) were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM (n = 5 all groups). Cells were then stimulated by incubation for 4h (37°C) with either *H. influenzae* (HI) or *S. pneumoniae* (SP). The supernatants were harvested after 4h and TNF-α was measured by ELISA. Data presented are mean ± SEM. ** represent p < 0.01, * p < 0.05 relative to untreated cells (UT). # represents p < 0.05 relative to HI.
5.5.3.3. Effect of azithromycin and solithromycin on bacteria-stimulated TNF-α

Having demonstrated that a 4h incubation with both *H. influenzae* and *S. pneumoniae* stimulates the release of TNF-α from MDM, the effects of azithromycin and solithromycin on this response were then explored (Figure 5.9). Concentrations ranging between $10^{-6.5}$ and $10^{-4.5}$ M were used, as they were previously shown not to affect MDM viability following a 28h treatment (Section 5.5.1.2). Pre-incubation for 24h with either azithromycin or solithromycin did not affect the release of TNF-α from MDM stimulated with *H. influenzae* (Figure 5.9; Panels A and B) or *S. pneumoniae* (Figure 5.9; Panels C and D). Similar results were observed for non-smokers, smokers and COPD patients.
Figure 5.9. Effect of azithromycin and solithromycin on bacterial-stimulated TNF-α release from MDM (H. influenzae and S. pneumoniae): (A) Azithromycin (H. influenzae) (B) Solithromycin (H. influenzae) (C) Azithromycin (S. pneumoniae) (D) Solithromycin (S. pneumoniae). Monocytes from non-smokers (▲), smokers (■) and COPD patients (●) were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM (n = 3 all groups). Cells were then pre-treated for 24h with varying concentrations of azithromycin, solithromycin, vehicle control (VC; 0.1% DMSO) or left untreated (UT) prior to stimulation with either H. influenzae or S. pneumoniae (for 4h at 37°C). The supernatants were harvested and TNF-α was measured by ELISA. Data presented are mean ± SEM.
5.5.4. Effect of macrolides on LPS-stimulated TNF-α release

The results presented in Section 5.5.3 demonstrated that pre-incubation for 24h with either azithromycin or solithromycin did not inhibit the release of TNF-α from MDM that had been stimulated by bacteria. Studies have however shown that macrolides can inhibit the release of TNF-α from macrophages stimulated by LPS alone (Cai et al., 2013, Kobayashi et al., 2013b). Therefore, the effects of azithromycin and solithromycin on LPS-stimulated TNF-α release were examined in MDM. This was screened in non-smokers only. Clarithromycin was also introduced as a second comparator, as previous work had demonstrated that this macrolide was the most potent immunomodulator of all the commercially-available compounds (Kobayashi et al., 2013b).

5.5.4.1. Subject demographics

The effect of macrolides on LPS-stimulated TNF-α release was only studied using MDM derived from non-smokers. As such, no demographic variables were compared.

5.5.4.2. LPS-stimulated TNF-α release from MDM (non-smokers)

Before examining the relative effects of macrolides on TNF-α release, it was determined whether incubation with 10 ng/ml LPS for 24 h was able to stimulate the release of TNF-α from MDM (Figure 5.10). LPS did indeed stimulate TNF-α release from MDM.
Figure 5.10. LPS-stimulated TNF-α release from MDM donated by non-smokers. Monocytes from non-smokers (□) were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM (n = 6). Cells were then stimulated with 10 ng/ml LPS for 24 h (37°C) and TNF-α was measured in the supernatants using ELISA. Data presented are mean ± SEM. *** represent p < 0.001 relative to untreated cells (UT).

5.5.4.3. Effect of macrolides on MDM viability (non-smokers)

Before determining whether azithromycin, clarithromycin and/or solithromycin were capable of inhibiting the release of TNF-α from LPS-stimulated MDM, the effects of these compounds on MDM viability were examined (Figure 5.11). Cells were pre-treated with macrolide for 24 h prior to LPS stimulation for a further 24 h (total incubation time with macrolide was 48 h). At concentrations ≤ 10^{-5} M, none of the macrolides had any effect on the viability of MDM from non-smokers. Solithromycin, but not azithromycin or clarithromycin, did however significantly reduce the viability of MDM at 10^{-4.5} M (Figure 5.11; Panel C). As such, maximum concentrations of 10^{-5} M were used to test the effects of azithromycin, clarithromycin and solithromycin on the release of TNF-α from LPS stimulated MDM (Section 5.5.4.4).
Figure 5.11. Effect of macrolides on MDM viability: (A) Azithromycin (B) Clarithromycin (C) Solithromycin. Monocytes from non-smokers (▲) were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM (n = 5). MDM were treated for 24h with varying concentrations of macrolide, vehicle control (VC; 0.1% DMSO) or left untreated (UT), prior to stimulation with 10 ng/ml LPS (further 24h). They were then incubated with 50 μl MTT for 45 min (at 37°C), before cell lysis with 50 μl DMSO. Plates were read at λ570 nm on a spectrophotometer. Data presented are percentage cell viability, relative to UT cells (i.e. UT cells = 100% viability, represented by the horizontal dotted line). *** represent p < 0.001 relative to untreated cells (UT).
5.5.4.4. Effect of macrolides on LPS-stimulated TNF-α (non-smokers)

Having confirmed that LPS stimulated the release of TNF-α from MDM, and that azithromycin, clarithromycin and/or solithromycin ≤ 10⁻⁵ M do not influence MDM viability, the effects of these compounds on LPS-stimulated TNF-α release were investigated (Figure 5.12). Azithromycin had no effect on the release of TNF-α (Figure 5.12; Panel A). In contrast, both clarithromycin and solithromycin inhibited the release of TNF-α (Figure 5.12; Panels B & C). The maximal effects of both compounds (i.e. efficacy) were similar; an approximate 40% inhibition. However, only clarithromycin exhibited a concentration-dependent inhibition. This might have been due to a leftward shift in the linear portion of the dose-response curve for solithromycin (i.e. the linear portion may have occurred at concentrations < 10⁻⁶.⁵ M). This suggests that the EC₅₀ for solithromycin might have been lower (i.e. solithromycin was more potent than clarithromycin).
Figure 5.12. Effect of macrolides on LPS-stimulated TNF-α release from MDM: (A) Azithromycin (B) Clarithromycin (C) Solithromycin (D) All macrolides. Monocytes from non-smokers were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM (n = 5). Cells were then pre-treated for 24h with varying concentrations of azithromycin (△), clarithromycin (△) or solithromycin (▲), prior to stimulation with 10 ng/ml LPS for a further 24h. The supernatants were harvested and TNF-α was measured by ELISA. Data presented are mean ± SEM. ** represent p < 0.01, * p < 0.05 relative to LPS-stimulated cells that had not been pre-treated with macrolide.
Mean maximum percentage inhibition and EC$_{50}$ values were calculated for clarithromycin and solithromycin, in order to directly compare their efficacy and potency respectively (Table 5.2). The two compounds were equally efficacious. Mean EC$_{50}$ values could not be calculated for solithromycin, as the linear portion of the concentration-response curve was not present between $10^{-6.5}$ and $10^{-5}$ M. As discussed above, it is possible that this might have been due to a significant leftward shift in the curve for solithromycin, i.e. solithromycin was more potent than clarithromycin.

<table>
<thead>
<tr>
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<th>Clarithromycin (n = 5)</th>
<th>Solithromycin (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum inhibition (%)</td>
<td>NC</td>
<td>41.9 ± 11.2</td>
<td>46.8 ± 7.5</td>
</tr>
<tr>
<td>EC$_{50}$ (μM)</td>
<td>NC</td>
<td>0.4 ± 0.2</td>
<td>NC</td>
</tr>
</tbody>
</table>

Table 5.2. Maximum percentage inhibition and EC$_{50}$ values for the inhibition of TNF-α release by macrolides. Monocytes from non-smokers were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM (n = 5). Cells were then pre-treated for 24h with varying concentrations of azithromycin, clarithromycin or solithromycin, prior to stimulation with 10 ng/ml LPS for a further 24h. The supernatants were harvested and TNF-α was measured by ELISA. The maximum percentage inhibition was calculated relative to LPS-stimulated cells (not pre-treated with macrolide). Individual values were calculated for each subject. Data presented are mean ± SEM. NC = not calculable.
5.5.5. Effect of solithromycin analogues on LPS-stimulated TNF-α release

Having determined that solithromycin is capable of inhibiting the release of TNF-α from LPS-stimulated MDM, the relative effects of its analogues were then examined using the same experimental conditions. The following 9 analogues were screened in MDM from non-smokers only: CEM-153, CEM-154, CEM-214, CEM-218, CEM-232, CEM-237, CEM-262, CEM-267 and OP-1055.

5.5.5.1. Subject demographics

The effect of solithromycin analogues on LPS-stimulated TNF-α release was only studied using MDM from non-smokers. As such, no demographic variables were compared.

5.5.5.2. Effect of solithromycin analogues on MDM viability

Before determining whether any of the solithromycin analogues were capable of inhibiting TNF-α release from LPS-stimulated MDM, the effects of these compounds on the viability of MDM were examined (Figures 5.13 and 5.14). Cells were pre-treated with analogue for 24h prior to undergoing LPS stimulation for 24h (total incubation time with analogue was 48h). The chosen concentration range was based on prior solithromycin experiments, and under these conditions, none of the compounds were found to significantly impair MDM viability.
Figure 5.13. Effect of the solithromycin analogues CEM-214, CEM-237, CEM-262, CEM-267 and OP-1055 on MDM viability: (A) CEM-214 (B) CEM-237 (C) CEM-262 (D) CEM-267 (E) OP-1055. Monocytes from non-smokers (▲) were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM (n = 5). MDM were treated for 24h with varying concentrations of compound, vehicle control (VC; 0.1% DMSO) or left untreated (UT), prior to stimulation with 10 ng/ml LPS (further 24h). They were then incubated with 50 μl MTT solution for 45 min (at 37°C), before cell lysis with 50 μl DMSO. Plates were read at λ570 nm on a spectrophotometer. Data presented are percentage cell viability, relative to UT cells (i.e. UT cells = 100% viability, represented by the horizontal dotted line).
Figure 5.14. Effect of the solithromycin analogues CEM-153, CEM-154, CEM-218 and CEM-232 on MDM viability: (A) CEM-153 (B) CEM-154 (C) CEM-218 (D) CEM-232. Monocytes from non-smokers (▲) were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM (n = 5). MDM were treated for 24h with varying concentrations of compound, vehicle control (VC; 0.1% DMSO) or left untreated (UT), prior to stimulation with 10 ng/ml LPS (further 24h). They were then incubated with 50 μl MTT solution for 45 min (at 37°C), before cell lysis with 50 μl DMSO. Plates were read at λ570 nm on a spectrophotometer. Data presented are percentage cell viability, relative to UT cells (i.e. UT cells = 100% viability, represented by the horizontal dotted line).
5.5.5.3. Effect of solithromycin analogues on TNF-α release (non-smokers)

Having determined that the solithromycin analogues do not influence MDM viability at concentrations ≤ 10⁻⁵ M, the effects of these compounds on LPS-stimulated TNF-α release were then investigated (Figures 5.15 and 5.16). The graphs have been grouped into those compounds without any effect (Figure 5.15) and those which inhibited the release of TNF-α (Figure 5.16). Five compounds (CEM-214, CEM-237, CEM-262, CEM-267 and OP-1055) did not inhibit the release of TNF-α. Four analogues (CEM-153, CEM-154, CEM-218 and CEM-232) inhibited the release of TNF-α from LPS-stimulated MDM.

Figure 5.16 (Panel E) compares the effects of CEM-153, CEM-154, CEM-218 and CEM-232, with that of solithromycin. The maximal effects of all five compounds were similar; a 30-40% inhibition of TNF-α release (i.e. similar efficacy). Concentration-dependent responses were observed for CEM-153, CEM-154 and CEM-218. Concentration-independent responses were seen for CEM-232 and solithromycin. These were very similar, and may have been due to a leftward shift in the dose-response curves for both compounds, suggesting an increased potency relative to CEM-153, CEM-154 and CEM-218.
Figure 5.15. Effect of the solithromycin analogues CEM-214, CEM-237, CEM-262, CEM-267 and OP-1055 on LPS-stimulated TNF-α release from MDM: (A) CEM-214 (B) CEM-237 (C) CEM-262 (D) CEM-267 (E) OP-1055. Monocytes from non-smokers (▲) were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM (n = 5). Cells were then pre-treated for 24h with varying concentrations of solithromycin analogue, prior to stimulation with 10 ng/ml LPS for a further 24h. The supernatants were subsequently harvested and TNF-α was measured by ELISA. Data presented are mean ± SEM.
Figure 5.16. Effect of the solithromycin analogues CEM-153, CEM-154, CEM-218 and CEM-232 on LPS-stimulated TNF-α release from MDM: (A) CEM-153 (B) CEM-154 (C) CEM-218 (D) CEM-232 (E) effective analogues and solithromycin. Monocytes from non-smokers were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM (n = 5). Cells were then pre-treated for 24h with varying concentrations of CEM-153 (△), CEM-154 (▽), CEM-218 (▲), CEM-232 (△) or solithromycin (▲), prior to stimulation with 10 ng/ml LPS for a further 24h. The supernatants were subsequently harvested and TNF-α was measured by ELISA. Data presented are mean ± SEM.
Mean maximum percentage inhibition and EC$_{50}$ values were calculated for CEM-153, CEM-154, CEM-218 and CEM-232, in order to directly compare their efficacy and potency respectively (Table 5.3). Results with solithromycin and clarithromycin were also compared (as calculated in Section 5.5.4.4). All of the 6 compounds were equally efficacious. Mean EC$_{50}$ values were similar for CEM-153, CEM-154, CEM-218 and clarithromycin. Mean EC$_{50}$ values could not be calculated for CEM-232 or solithromycin.

<table>
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<tr>
<td></td>
<td>CEM-153</td>
<td>CEM-154</td>
<td>CEM-218</td>
</tr>
<tr>
<td>MPI (%)</td>
<td>52.5 (± 0.7)</td>
<td>47.8 (± 6.4)</td>
<td>37.0 (± 3.6)</td>
</tr>
<tr>
<td>EC$_{50}$ (μM)</td>
<td>1.1 (± 0.8)</td>
<td>1.2 (± 0.3)</td>
<td>0.3 (± 0.2)</td>
</tr>
</tbody>
</table>

Table 5.3. Maximum percentage inhibition (MPI) and EC$_{50}$ values for the inhibition of TNF-α release by solithromycin analogues. Monocytes from non-smokers were cultured in the presence of GM-CSF (2 ng/ml) for 12 days to generate MDM (n = 5). Cells were then pre-treated for 24h with varying concentrations of CEM-153, CEM-154, CEM-218 and CEM-232, prior to stimulation with 10 ng/ml LPS for a further 24h. The supernatants were harvested and TNF-α was measured by ELISA. The MPI was calculated relative to LPS-stimulated cells (not pre-treated with macrolide). Individual values were calculated for each subject. For comparison, values for clarithromycin and solithromycin are also shown. Data presented are mean (± SEM). NC = not calculable.

5.5.6. Antimicrobial effects of solithromycin analogues

Section 5.5.5 demonstrated that some of the solithromycin analogues (namely CEM-153, CEM-154, CEM-218 and CEM-232) were capable of inhibiting the release of TNF-α from MDM stimulated by LPS. It is possible that one or more of these compounds are purely
immunomodulatory, *i.e.* capable of immunomodulation without antibacterial effects. To test this hypothesis, the antimicrobial effects of all solithromycin analogues were quantitatively screened using lawn growths of *S. pneumoniae* and *M. catarrhalis*. *H. influenzae* was not used, due to the relative lack of inhibition in bacterial growth that was observed with the clinical isolate in Section 5.5.2.1. The macrolides azithromycin, clarithromycin and solithromycin, as well as the non-effective compounds (CEM-214, CEM-237, CEM-262, CEM-267 and OP-1055) were also screened. A single concentration of \(10^{-5} \text{ M}\) was used for all compounds. At this concentration, all of the effective compounds were shown to inhibit the release of TNF-\(\alpha\) from MDM without influencing cell viability.

### 5.5.6.1. Effect of solithromycin analogues on the growth of *S. pneumoniae*

The effects of all the available compounds on lawn growths of *S. pneumoniae* were examined, using a concentration of \(10^{-5} \text{ M}\) (Figure 5.17). As previously observed, solithromycin and azithromycin inhibited the growth of *S. pneumoniae* (Figure 5.4). Clarithromycin also similarly inhibited the growth of *S. pneumoniae*. Of the solithromycin analogues that did not affect the release of TNF-\(\alpha\), all but CEM-237 inhibited the growth of *S. pneumoniae*. Of the solithromycin analogues that did inhibit the release of TNF-\(\alpha\) from MDM, only CEM-218 was found to be active against the growth of *S. pneumoniae*. In other words, CEM-153, CEM-154 and CEM-232 did not inhibit the growth of *S. pneumoniae*. 
Figure 5.17. Effect of macrolides and solithromycin analogues on the growth of S. pneumoniae. Lawns of S. pneumoniae were prepared on agar plates. Discs of blotting paper (6 mm in diameter) were then impregnated with $10^{-5}$ M of macrolide (□), $10^{-5}$ M of solithromycin analogue, or vehicle control (VC; 0.1% DMSO; □), before being placed directly onto the media. Solithromycin analogues with and without effects on LPS-stimulated TNF-α release from MDM are represented by □ and □ respectively. The plates were incubated for 16h at 37°C (5% (V/V) CO₂) and the zones of inhibition were measured. Data presented are mean diameter of zone of inhibition ± SEM (n = 3 for each bacteria). Diameter of impregnated disc is represented by the horizontal dotted line. *** represent p < 0.001, * p < 0.05 relative to VC.

5.5.6.2. Effect of solithromycin analogues on the growth of M. catarrhalis

The effects of all available compounds on lawn growths of M. catarrhalis were also examined, using a concentration of $10^{-5}$ M (Figure 5.18). As previously observed in Figure 5.4, solithromycin and azithromycin inhibited the growth of M. catarrhalis. Clarithromycin also similarly inhibited the growth of M. catarrhalis. Of the solithromycin analogues that did not affect the release of TNF-α, CEM-214, CEM-267 and OP-1055, inhibited the growth of S. pneumoniae. CEM-237 and CEM-262 displayed some activity against this isolate of M. catarrhalis. However, this was not statistically significant. None of the solithromycin analogues
that inhibited the release of TNF-α from MDM, were active against the growth of *M. catarrhalis* (*i.e.* CEM-153, CEM-154, CEM-218 and CEM-232).

**Figure 5.18.** Effect of macrolides and solithromycin analogues on the growth of *M. catarrhalis*. Lawns of *M. catarrhalis* were prepared on agar plates. Discs of blotting paper (6 mm in diameter) were then impregnated with $10^{-5}$ M of macrolide (■), $10^{-5}$ M of solithromycin analogue, or vehicle control (VC; 0.1% DMSO; □), before being placed directly onto the media. Solithromycin analogues with and without effects on LPS-stimulated TNF-α release from MDM are represented by ■ and □ respectively. The plates were incubated for 16h at 37°C (5% (V/V) CO₂) and the zones of inhibition were measured. Data presented are mean diameter of zone of inhibition ± SEM (n = 3 for each bacteria). Diameter of impregnated disc is represented by the horizontal dotted line. *** represent p < 0.001, ** represent p < 0.01, * p < 0.05 relative to VC.

In summary, CEM-232 at a concentration of $10^{-5}$ M was not active against *S. pneumoniae* or *M. catarrhalis*, but did inhibit the release of TNF-α from LPS-stimulated MDM, with a similar efficacy to solithromycin and potentially greater potency than the other effective solithromycin analogues and clarithromycin (**Section 5.5.5.3**).
5.6. Discussion

Solithromycin and azithromycin did not augment bacterial phagocytosis in COPD even at a concentration of $10^{-4.5}$ M and when using a range of concentrations that had previously produced similar immunomodulatory effects in other cell systems (i.e. $10^{-6.5}$ to $10^{-4.5}$ M) (Hodge et al., 2006b, Hodge et al., 2008). Furthermore, neither macrolide influenced the phagocytosis of bacteria by non-smokers or smokers, nor the internalisation of beads by any of the three subject groups. A possible exception being the inhibition, rather than augmentation, of *S. pneumoniae* phagocytosis by MDM exposed to $10^{-4.5}$ M of solithromycin (smokers only). This lack of any effect was unlikely to be due to compound inactivation in solution or the choice of concentration. Indeed, the same concentrations of azithromycin and solithromycin, prepared in the same manner, inhibited the growth of *H. influenzae, S. pneumoniae* and *M. catarrhalis*, while solithromycin was subsequently shown to suppress TNF-α release from LPS-stimulated MDM at $10^{-6}$ and $10^{-5}$ M.

It remains possible that the chosen experimental conditions contributed to the lack of effect on bacterial phagocytosis. Hodge *et al.* (2008) found that similar concentrations of azithromycin ($10^{-6}$ and $10^{-5}$ M) upregulated the expression of CD206 (MR) in vitro and could therefore potentially enhance phagocytosis. This was achieved following 48h of incubation however, rather than the 24h used in this thesis. CD206 recognises glycans with terminal mannose residues on the surface of pathogens (i.e. PAMP) and this could be important in the phagocytosis of bacteria. Yet, bacterial phagocytosis was not investigated in these experiments and defective phagocytosis in COPD is not associated with reductions in CD206 expression (Taylor *et al.*, 2010). Nevertheless, incubation time is considered crucial when determining cellular responses to macrolides. As ‘immunomodulators’, they do not exhibit a simple time-dependent, unidirectional suppression or stimulation of leukocyte function. Instead,
multiphase responses are frequently observed when cells are continually exposed to these compounds in vitro (Culic et al., 2002, Shinkai et al., 2006) or during prolonged administration in vivo (Konno et al., 1992, Parnham et al., 2005) and these likely result from interactions between signalling pathways or feedback mechanisms (Kanoh and Rubin, 2010). Cell stimulation might occur initially, before the cellular response returns to baseline and is subsequently suppressed upon continued exposure. As such, before concluding with certainty that macrolides do not augment bacterial phagocytosis in COPD, assays should be undertaken at both earlier and later time points.

Evidence for an effect of macrolides on bacterial phagocytosis comes from an in vivo trial conducted by Hodge and Reynolds (2012), in which the oral administration of azithromycin to COPD patients was found to improve the phagocytosis of \textit{E. coli} by both AM and MDM. However, this was only undertaken by a single centre, in a small cohort of 10 COPD patients, has not been replicated in the literature or with any other bacteria, and did not involve the concurrent investigation of any potential underlying mechanisms. Therefore, it is possible that the effect on AM arose indirectly through airway sterilisation or via effects on other leukocytes, which could have secondarily influenced macrophage function. The latter could also explain the earlier observation in \textbf{Chapter 4}, that the use of azithromycin was associated with an increased uptake of \textit{S. pneumoniae} by MDM from COPD patients (\textbf{Figure 4.28; Panel D}).

The in vitro data presented in this chapter, suggest that macrolides do not directly upregulate bacterial phagocytosis by MDM. As such, the solithromycin analogues were not screened. However, assays were only undertaken in 5 COPD patients and many more subjects were required to demonstrate defective bacterial phagocytosis in earlier chapters (≥ 20). Furthermore, although some macrolides may be capable of stabilising microtubule polymers (Mitsui-Saito et al., 2002, Field et al., 2009), which could theoretically address their defective
nature in COPD by subsequently enhancing phagocytosis, these compounds contain 20- and 37- members in their monocyclic ring and no study to date has specifically shown that macrolide antibiotics with 14- or 15- members can target microtubules. Finally, there could have been limitations associated with using the MDM model, and any effects on primary AM should also be explored.

Gram-negative bacteria, such as *H. influenzae*, contain LPS in their cell wall (Alexander and Rietschel, 2001). This is recognised by TLR4 receptors on the surface of AM, leading to the activation of NF-κB and subsequent production of inflammatory mediators (Pålsson-McDermott and O'Neil, 2004). This effect was demonstrated in MDM. When the supernatants from phagocytosis assays were analysed for TNF-α, *H. influenzae* was shown to stimulate the release of this important pro-inflammatory cytokine from the MDM of non-smokers, smokers and COPD patients. However, despite evidence that macrolides inhibit NF-κB (Desaki *et al.*, 2000, Kikuchi *et al.*, 2002, Li *et al.*, 2012) and suppress TNF-α release from macrophages (Cai *et al.*, 2013, Kobayashi *et al.*, 2013b) and other leukocytes (Kanoh and Rubin, 2010), neither azithromycin or solithromycin was able to immunomodulate the TNF-α response of MDM to *H. influenzae*. The Gram-positive bacteria *S. pneumoniae* was also found to stimulate the release of TNF-α from MDM. Although LPS is not a component of Gram-positive bacteria, other cell wall components still activate AM (Mogensen *et al.*, 2008, Tomlinson *et al.*, 2014). This response was only approximately half of that demonstrated by *H. influenzae*. Regardless, neither azithromycin or solithromycin suppressed the release of TNF-α from MDM stimulated by *S. pneumoniae*.

The effect of solithromycin on the release of TNF-α was also screened in MDM from non-smokers, using LPS as a stimulus. Following 24h of incubation with LPS, MDM upregulated their release of TNF-α. The addition of solithromycin (pre-incubation for 24h) was subsequently
shown to inhibit this response without any confounding effect from cytotoxicity. Two macrolide comparators were also similarly investigated at the same concentration, namely azithromycin and clarithromycin. However, only the latter inhibited the release of TNF-α, without any effects on cell viability. Both solithromycin and clarithromycin were equally efficacious, with an approximate 40-50% reduction in the release of TNF-α. Although seemingly modest, the effects on MDM from COPD patients and longer incubation times, remain unknown.

These data mirror the work by Kobayashi et al. (2013b), in which solithromycin and clarithromycin (but not azithromycin, erythromycin or telithromycin), inhibited the release of TNF-α from LPS-stimulated macrophages (PMA-differentiated U937 cells). In their study, solithromycin was found to be ten-times more potent than clarithromycin. Indeed, solithromycin may also have been more potent in this cell system, with a possible leftward shift in the TNF-α response relative to clarithromycin, such that the linear portion was not apparent between $10^{-6.5}$ and $10^{-5}$ M. This prevented the calculation and comparison of EC$_{50}$ values but could be confirmed by conducting similar experiments at lower concentrations, identifying any concentration-dependent response and subsequently comparing EC$_{50}$ values with clarithromycin.

A criticism of the experiments conducted in this chapter, and supporting publications, might be the requirement for high concentrations of macrolide to induce effects in leukocytes. However, $10^{-6.5}$ - $10^{-4}$ M is likely to be physiologically relevant. Macrolides concentrate significantly in the ELF of the lung. At typical oral doses of 250 mg once daily, 500 mg twice daily and 400 mg once daily, azithromycin, clarithromycin and solithromycin achieve steady-state within the pulmonary ELF of healthy subjects after 5 days, and at concentrations of approximately $10^{-4}$ M, $10^{-5}$ M and $10^{-5}$ M respectively (Rodvold et al., 1997, Rodvold et al.,...
Resident AM will therefore be bathed in these concentrations, much in the same way that MDM were exposed to similar concentrations in our series of *in vitro* experiments. They are also similar to the MICs for these compounds (Farrell *et al.*, 2010).

Solithromycin may be capable of AM immunomodulation to initiate inflammatory resolution *in vivo*. However, possible inhibitory effects on the phagocytosis of *S. pneumoniae* have not gone unnoticed and could have negative implications. This should be explored in a larger COPD cohort. Furthermore, the concerns that exist over the long-term use of antimicrobials in COPD, are also shared, including the potential to promote macrolide-resistance (Serisier, 2013) and the growth of potentially-harmful microorganisms, such as nontuberculous mycobacteria (Levy *et al.*, 2008, Renna *et al.*, 2011) which may accelerate disease progression (Huang *et al.*, 2012). Although resistance to solithromycin itself would be theoretically difficult to achieve (Rodgers *et al.*, 2013), its introduction as a long term immunomodulator would likely generate bacterial resistance against other macrolides, especially those that share a ribosomal binding site. With this in mind, the effects of several solithromycin analogues on LPS-stimulated TNF-α release were also screened in MDM from non-smokers. Although Cempra did not share the chemical structures or specific characteristics of these compounds, some were expected to be non-antibiotic.

Four out of the nine solithromycin analogues inhibited the release of TNF-α from MDM stimulated by LPS (CEM-153, CEM-154, CEM-218 and CEM-232). All of these compounds were similarly efficacious, when compared to one another, or solithromycin and clarithromycin (approximate 40 – 50% reduction in TNF-α). However, like solithromycin, the linear portion of the dose-response curve for CEM-232 was possibly shifted leftward. Although EC$_{50}$ values could not be calculated, this suggested that CEM-232 was most potent and this should be confirmed using lower concentrations in order to calculate and compare its potency.
Furthermore, CEM-232 was not active against either *S. pneumoniae* or *M. catarrhalis* at $10^5$ M, a concentration at which many of the other compounds inhibited the growth of these bacteria. At this concentration, CEM-232 also inhibited TNF-α release without adversely affecting MDM viability. When taken together, these data suggest that CEM-232 might be capable of immunomodulation without any antibiotic effect. This should be explored more extensively *in vitro* by examining its actions on other AM functions (*e.g.* CXCL8 release), identifying any underlying mechanisms (*e.g.* suppression of NF-kB activity), using MDM from COPD patients, ensuring that these effects are also observed in primary AM and by comprehensively excluding any antibiotic activity against a broader spectrum of bacteria.

The hypothesis for this chapter was: ‘*solithromycin (and associated analogues) possess immunomodulatory properties, including the augmentation of defective bacterial phagocytosis in COPD and the inhibition of TNF-α release from stimulated MDM*.’ This hypothesis can only be partially accepted and further work is required. Solithromycin did not augment bacterial phagocytosis in COPD but was able to inhibit the release of TNF-α from LPS-stimulated MDM (non-smokers only). Four of the solithromycin analogues shared this ability, namely CEM-153, CEM-154, CEM-218 and CEM-232. However, CEM-232 was most promising. It was possibly the most potent and potentially not an antibiotic. The immunomodulatory effects of solithromycin (and CEM-232) could translate into clinical benefits for stable COPD patients. Given the lack of available treatments that are capable of disease modification, this should be investigated urgently in a clinical trial. This is the focus of Chapter 7.
CHAPTER 6

Using Synthetic Absorptive Matrix to Evaluate and Compare Nasal and Bronchial Inflammation in COPD
6.1. Introduction

A complex network of inflammatory mediators are responsible for recruiting, activating and promoting the survival of inflammatory cells within the lungs of COPD patients (Barnes, 2009). They include CXCL8 (a neutrophil chemoattractant), TNF-α (a potent activator of transcription factors, e.g. NF-κB) and IL-6 (a pleiotropic cytokine). As discussed in Chapter 1 (Section 1.2.2), these mediators have widespread and well-researched roles in the pathophysiology of both pulmonary and systemic inflammation in COPD. They are also biomarkers (Barnes et al., 2006), with potentially invaluable roles in clinical assessment and research, including diagnosis, disease progression, phenotyping, clinical outcome prediction and/or evaluating inflammatory kinetics, such as during the assessment of much-needed novel treatments. Valuable information regarding CXCL8, TNF-α and IL-6 is already obtained from induced sputum, bronchial biopsy and BAL. However, the use of such sampling techniques in COPD patients can be problematic and alternatives are urgently required. Some of the limitations associated with bronchoscopy and BAL have already been identified in Chapter 3.

Induced sputum does not closely resemble distal airway inflammation as it selectively samples proximal airways and is contaminated by upper respiratory tract secretions and saliva (Holz et al., 1998). Not all COPD patients can produce an induced sputum sample and some experience bronchoconstriction with hypertonic saline (Pizzichini et al., 2002). Solubilisation of sputum with DTT and the action of intrinsic proteases, may also alter or degrade certain protein mediators prior to assay (Kelly et al., 2002, Woolhouse et al., 2002).

Bronchial biopsy and BAL require bronchoscopy. This is invasive and not without risk, especially for those with significant airflow obstruction and/or comorbidities such as ischaemic heart disease (Krause et al., 1997, Du Rand et al., 2013). BAL also provides a highly variable yield in
COPD, with some series reporting BALF recovery to be as low as 10-40% (Klech and Hutter, 1990). Such poor returns may predominantly reflect the larger airways (Martin et al., 1985) and are most likely with more pronounced emphysema (Lofdahl et al., 2005). As a result, many patients with severe COPD and/or significant comorbidities are either unsuitable or unable to provide adequate samples. This can promote a bias towards patients with milder disease. Bronchoscopy is also not a viable option during exacerbations or when repeated measures are required, such as during clinical trials. Another common issue is the inability to recruit and retain an adequate number of willing volunteers (Martinsen et al., 2016). BAL also results in an unknown dilution factor with many inflammatory mediators being below the detection limits of current analytical assays (Kavuru et al., 1999, Rennard et al., 1998).

Synthetic absorptive matrix (SAM) might provide an alternative means of sampling the bronchial ELF. It is akin to blotting paper and when placed against a mucosal surface absorbs concentrated ELF. This can then be eluted in a known volume of buffer and subsequently analysed for inflammatory mediators, without the need for DTT. As there is less dilution, SAM also allows for the detection of lower levels of soluble proteins (Leaker et al., 2015). A novel bronchosorption™ device, which contains SAM at its tip, has been developed (Hunt developments, UK) (Figure 2.3) It can pass down the operating channel of any standard bronchoscope and directly absorbs neat bronchial ELF (≈ 20 μl) (Figure 2.4). The feasibility of this technique has already been demonstrated in asthmatic patients (Jackson et al., 2013) and healthy smokers (Leaker et al., 2015). It might also prove to be a safe methodology to quantify soluble mediators in COPD. However, bronchoscopy is still required and difficulties with procedural complications and/or recruitment may still arise.

The sinonasal airways may represent a surrogate sampling site for the bronchi. These two airways are anatomically and histologically related. They also possess similar innate and
adaptive immune responses that are designed to combat the same inhaled pathogens, allergens and particles that are capable of eliciting an inflammatory response in both respiratory tracts (Hox et al., 2015). An association between pulmonary and sinonasal inflammation is strongly suspected for COPD (Hurst, 2010, Burgel, 2015). In their role as sentinel, the sinonasal airways are exposed to the same noxious substances in tobacco smoke that are known to activate chronic inflammation within the lungs of COPD patients. It therefore stands to reason that an analogous inflammatory response is observed within the sinonasal airways (Hurst, 2010, Hox et al., 2015). Indeed, patients with COPD do report a higher incidence of chronic nasal symptoms (Montnemery et al., 2001, van Manen et al., 2001, Chen et al., 2003), which are worse during acute exacerbations (Huerta et al., 2015), heavily impact on their quality of life (Hurst et al., 2004) and correlate with pulmonary symptoms, such as chronic sputum production (Roberts et al., 2003). Furthermore, elevated concentrations of CXCL8 have been reported in the nasal lavage fluid of COPD patients and shown to correlate with concentrations in paired sputum samples (Hurst et al., 2005).

Sampling the nasal ELF might provide a surrogate measure of bronchial inflammation in COPD. This is an attractive hypothesis, as the nose offers comparatively easy access to a mucosal surface, without the need for invasive procedures. Traditionally, the nasal ELF is sampled by lavage. As is the case with BAL, this technique can be hindered by poor fluid recovery (from posterior loss), cell activation (especially with repeated sampling) and an unknown or excessive dilution factor (Howarth et al., 2005). Nasal devices that use SAM to absorb neat ELF have already been developed (Nasosorption™) (Hunt Developments, UK) (Figure 2.2). Pioneering work conducted by the Imperial Clinical Respiratory Research Unit (ICRRU) and its collaborators has demonstrated the feasibility of nasosorption for studying several conditions known to be associated with sinonasal inflammation, such as asthma (Jackson et al., 2013),
allergic rhinitis (Chawes et al., 2010, Scadding et al., 2012, Nicholson et al., 2011) and atopy (Folsgaard et al., 2012). Correlations between upper and lower airway inflammation have already been reported in mild to moderate asthmatics using SAM (Jackson et al., 2013) and repeated nasosorption has been used to demonstrate that nasal inflammation can be modulated by therapies (Nicholson et al., 2011, Dhariwal et al., 2015, Leaker et al., 2016).

Nasosorption is non-invasive and only associated with very mild adverse effects, such as nasolacrimal reactions and epistaxis. Moreover, it has already been formally validated in healthy subjects and asthmatics. The current device consistently absorbs 40 μl of nasal ELF from healthy controls (Jochems et al., 2017), permits an adequate recovery of a wide range of inflammatory proteins of varying molecular weights (Jackson et al., 2010) and is more sensitive than nasal lavage in asthmatics (Jochems et al., 2017). Results are also highly reproducible. Indeed, nasosorption can be employed every 10 minutes for 1 hour to obtain reproducible concentrations of a variety of cytokines and chemokines in healthy controls (ICRRU unpublished data). In contrast, the reproducibility of bronchosorption, with the current FX device, is yet to be reported. This thesis will explore, for the first time, the validity of nasosorption for measuring inflammatory mediators within the nasal ELF of COPD patients.

6.2. Hypothesis

The hypothesis for this chapter is: ‘CXCL8, TNF-α and IL-6 are elevated in the nasal ELF of COPD patients, are representative of bronchial concentrations and can be used as reliable biomarkers of inflammation’.
6.3. Aims

The following aims were adopted in order to investigate the above hypothesis:

- Use nasosorption to sample the nasal ELF of non-smokers, smokers and stable COPD patients, in order to measure the concentrations of CXCL8, TNF-α and IL-6 within the resulting eluates and compare levels between the three subject groups.

- Obtain paired nasosorption and bronchosorption samples from COPD patients in order to correlate nasal and bronchial concentrations of CXCL8, TNF-α and IL-6.

- Obtain serial nasosorption from COPD patients to determine whether concentrations of CXCL8, TNF-α and IL-6 fluctuate, or are stable, over time (28 days).

- Sample nasal CXCL8, TNF-α and IL-6 from a larger COPD cohort, in order to establish whether concentrations are predictive of disease severity (by correlating with spirometry), symptom burden (mMRC and CAT scores) and specific disease phenotypes (comparing concentrations between ‘frequent’ and ‘infrequent’ exacerbators).

- Determine whether concentrations of nasal CXCL8, TNF-α and IL-6 are upregulated during exacerbation by collecting nasosorption samples at baseline and during exacerbation, in order to compare concentrations of inflammatory mediators.

6.4. Methods

6.4.1. Subject selection

COPD patients, smoking controls and healthy non-smokers were recruited as described in Section 2.2.2.1. All subjects underwent spirometry (Section 2.2.2.1), airway reversibility (Section 2.2.2.1) and skin prick testing to common aeroallergens (Section 2.2.2.3). Subjects with evidence of reactive airways, atopy or symptoms suggestive of an upper or lower respiratory tract infection within the last 6 weeks were excluded.
6.4.2. **Symptom assessment**

6.4.2.1. **Nasal symptoms**

All volunteers were asked about the presence of five nasal symptoms, during the preceding three months. These symptoms were rhinorrhea, sneeze, nasal congestion, postnasal drip and anosmia.

6.4.2.2. **COPD symptoms**

All COPD patients completed a CAT questionnaire at the time of nasosorption and reported their degree of breathlessness according to the mMRC dyspnoea scale. Examples can be found in Appendix B and Appendix A, respectively. These data were used to stratify the COPD cohort according to the combined GOLD assessment (Figure 1.1).

6.4.2.3. **COPD exacerbations**

All COPD patients reported on the number of exacerbations that they had experienced during the preceding year. These data were used to divide the cohort into infrequent or frequent exacerbators (< 2 or ≥ 2 exacerbations respectively). Patients who exacerbated after the baseline measures were recorded, were invited to an additional study visit (within 2 days of symptom onset), during which spirometry was repeated and nasosorption and venous blood samples were taken. The blood samples were analysed by the RBHT laboratory for serum concentrations of CRP and fibrinogen.

6.4.3. **Nasosorption**

Nasal ELF was sampled at baseline in non-smokers, smokers and COPD patients. A detailed description of the methodology is available in Section 2.2.5.3, which is in accordance with the methodology employed by the ICRRU. Both nostrils were sampled simultaneously using two separate Nasosorption™ FX:i devices (Hunt Developments, UK). These samples were kept on
ice and promptly processed (Section 2.2.7.2). The eluates were then pooled and stored at -80°C prior to ELISA. In a subgroup of COPD patients, samples were also collected at 1 h, 6 h, 24 h, 7 (± 1) days and 28 (± 2) days. Additional nasosorption samples were collected from COPD patients during exacerbations (≤ 2 days from symptom onset).

6.4.4. Bronchosorption

Bronchosorption samples were only collected from patients with mild or moderate COPD (FEV₁ ≥ 50 %). Patients with severe COPD (FEV₁ < 50 %) have a higher risk of bronchoscopy complications and were therefore excluded from bronchosorption. Suitable subjects underwent bronchoscopy as described in Section 2.2.5.5 and the ELF of the right middle lobe was sampled using a Bronchosorption™ FX device (Hunt Developments, UK), in accordance with the methodology employed by the ICRRU. The SAM was kept on ice and promptly processed using the methodology described in Section 2.2.7.2. Paired nasosorption samples were collected immediately prior to intubation with the bronchoscope.

6.4.5. Measurement of inflammatory mediators

Concentrations of CXCL8, TNF-α and IL-6 were measured in the SAM eluates by ELISA, according to the manufacturer’s instructions. All antibodies were purchased from Invitrogen, UK. For a detailed description of the methodology refer to Section 2.2.14. All samples were run in triplicate and a single 96 well plate was used for any samples that would later be compared (whenever possible). The lower limit of detection (i.e. sensitivity) was 31.25 pg/ml for all assays.

6.4.5.1. Measurement of CXCL8 by ELISA

ELISA was performed with CXCL8 mouse anti-human capture (0.5 μg/ml) and detection (0.4 μg/ml) antibodies. Prior to ELISA, the nasosorption and bronchosorption eluates were diluted
1:20 and 1:5 respectively with 0.5% (w/v) BSA in D-PBS. Concentrations of CXCL8 within each sample were interpolated from the standard curve and corrected according to the dilution factor. The intra-assay CV (i.e. the mean CV for sample triplicates) was < 10% for CXCL8 concentrations (all subject groups).

6.4.5.2. Measurement of TNF-α by ELISA

ELISA was performed with TNF-α mouse anti-human capture (2 μg/ml) and detection (0.1 μg/ml) antibodies. Prior to ELISA, both the nasosorption and bronchosorption eluates were diluted 1:4 with 0.5% (w/v) BSA in D-PBS. Concentrations of TNF-α were interpolated from the standard curve.

6.4.5.3. Measurement of IL-6 by ELISA

ELISA was performed with IL-6 mouse anti-human capture (1 μg/ml) and detection (0.2 μg/ml) antibodies. Prior to ELISA, both the nasosorption and bronchosorption eluates were diluted 1:4 with 0.5% (w/v) BSA in D-PBS. Concentrations of IL-6 were interpolated from the standard curve.

6.4.6. Data analysis

In this chapter, demographic data were compared using a Kruskal-Wallis with Dunn’s post-hoc test. Differences in the proportions of subjects with nasal symptoms were compared using two-proportion Z-tests and corresponding p values. Concentrations of inflammatory mediators were interpolated from the appropriate standard curve (Figures 2.9, 2.10 and 2.11). These data were not normally distributed and further analysis was therefore performed using non-parametric tests. Comparisons between 2 groups were made using either Mann-Whitney U tests (unpaired) or Wilcoxon signed rank tests (paired). Comparisons between ≥ 3 groups were made using a Kruskal-Wallis with Dunn’s post-hoc test. Correlations were performed using a
Spearman’s rank correlation coefficient. In all instances, the null hypothesis (i.e. no difference or an absent correlation) was rejected at $p < 0.05$. All analyses were performed using GraphPad Prism software (USA).

6.5. Results

6.5.1. Subject demographics

Demographic data for the non-smoking, smoking and COPD subjects are shown in Table 6.1. The three groups were matched for age. There were an equal number of males and females in the non-smoking and smoking groups. In the COPD group, female patients out-numbered males. Smokers and COPD patients were matched for smoking history. The COPD group comprised both current ($n = 5$) and ex-smokers ($n = 8$). While there were no differences in spirometry between the non-smokers and smokers, the COPD group had poorer lung function. The mean $\text{FEV}_1$ (L), $\text{FEV}_1$ (% predicted) and $\text{FEV}_1$/FVC ratio were lower relative to the non-smokers. The mean $\text{FEV}_1$ (% predicted) was also significantly less when compared to the smoking controls. All COPD patients were categorised as GOLD Stage II according to spirometric criteria.
<table>
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<th></th>
<th>Non-Smoker (n = 12)</th>
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<tr>
<td><strong>Age (years)</strong></td>
<td>60.2 ± 2.9</td>
<td>60.2 ± 2.2</td>
<td>62.4 ± 1.8</td>
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<td><strong>Gender (male : female)</strong></td>
<td>6:6</td>
<td>5:5</td>
<td>5:8</td>
</tr>
<tr>
<td><strong>Smoking history (pack years)</strong></td>
<td>0.0 ± 0.0</td>
<td>28.4 ± 5.8***</td>
<td>30.1 ± 3.8***</td>
</tr>
<tr>
<td><strong>Current smoker (yes : no)</strong></td>
<td>-</td>
<td>10:0</td>
<td>5:8</td>
</tr>
<tr>
<td><strong>FEV₁ (L)</strong></td>
<td>3.29 ± 0.31</td>
<td>2.58 ± 0.18</td>
<td>1.84 ± 0.13**</td>
</tr>
<tr>
<td><strong>FEV₁ (% predicted)</strong></td>
<td>111.1 ± 5.2</td>
<td>92.7 ± 4.4</td>
<td>68.3 ± 2.1***#</td>
</tr>
<tr>
<td><strong>FVC (L)</strong></td>
<td>4.19 ± 0.45</td>
<td>3.50 ± 0.33</td>
<td>3.13 ± 0.27</td>
</tr>
<tr>
<td><strong>FEV₁/FVC</strong></td>
<td>0.76 ± 0.02</td>
<td>0.75 ± 0.03</td>
<td>0.63 ± 0.04*</td>
</tr>
</tbody>
</table>

Table 6.1. Subject demographics. Participants underwent spirometry and the FEV₁ and FVC were recorded. A single pack year is equivalent to 20 cigarettes smoked every day for 1 year. Data presented are mean ± SEM. * represents p < 0.05, ** p < 0.01, *** p < 0.001 relative to non-smokers. # represents p < 0.05 relative to smokers. Note: All COPD patients (n = 13) were categorised as GOLD stage II.

6.5.2. Nasal symptoms in non-smokers, smokers and COPD patients

Table 6.2 presents the number of non-smokers, smokers and COPD patients that reported nasal symptoms in the 3 months preceding nasosorption. Nasal symptoms were more prevalent in both smokers and COPD patients when compared to non-smoking controls. No differences were observed in the number of subjects reporting any of the individual nasal symptoms (i.e. rhinorrhoea, sneeze, nasal congestion, postnasal drip or anosmia).
Table 6.2. Nasal symptoms in non-smokers, smokers and COPD patients. Participants were asked about the presence of nasal symptoms in the 3 months preceding nasosorption. The following 5 symptoms were recorded: rhinorrhoea, sneeze, nasal congestion, postnasal drip and anosmia. Data presented are numbers and percentages. * represents p < 0.05 relative to non-smokers.

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Non-Smoker (n = 12)</th>
<th>Smoker (n = 10)</th>
<th>COPD (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any nasal symptom</td>
<td>3 (25%)</td>
<td>7 (70%)*</td>
<td>9 (69%)*</td>
</tr>
<tr>
<td>Rhinorrhoea</td>
<td>2 (17%)</td>
<td>4 (40%)</td>
<td>6 (46%)</td>
</tr>
<tr>
<td>Sneezing</td>
<td>1 (8%)</td>
<td>1 (10%)</td>
<td>2 (15%)</td>
</tr>
<tr>
<td>Nasal congestion</td>
<td>2 (17%)</td>
<td>3 (30%)</td>
<td>3 (23%)</td>
</tr>
<tr>
<td>Postnasal drip</td>
<td>1 (8%)</td>
<td>3 (30%)</td>
<td>4 (31%)</td>
</tr>
<tr>
<td>Anosmia</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>2 (15%)</td>
</tr>
</tbody>
</table>

6.5.3. Nasal CXCL8 in non-smokers, smokers and COPD patients

Figure 6.1 shows the concentrations of CXCL8 within the nasal ELF of non-smokers, smokers and COPD patients. CXCL8 was detected in all of the eluates. Concentrations were higher in COPD when compared to non-smokers. No differences were observed between the non-smokers and smokers, or the smokers and COPD patients.

6.5.4. Nasal TNF-α in non-smokers, smokers and COPD patients

Concentrations of TNF-α within the nasal ELF of non-smokers, smokers and COPD patients were below the limit of detection of the assay.

6.5.5. Nasal IL-6 in non-smokers, smokers and COPD patients

Concentrations of IL-6 within the nasal ELF of non-smokers, smokers and COPD patients were below the limit of detection of the assay.
Figure 6.1. Concentrations of CXCL8 in the nasal ELF of non-smokers (NS), smokers and COPD patients. Non-smokers (▲), smokers (▼) and COPD patients (●) underwent nasosorption (n = 12, n = 10 and n = 13 respectively). Concentrations of CXCL8 within the eluates were measured by ELISA. Data presented are the medians ± IQR. ** p < 0.01.

6.5.6. Correlation between nasal and bronchial CXCL8 in COPD

Having shown that CXCL8 could be measured in nasal ELF, it was important to determine whether these levels reflected those observed in the lower airways. To this end, paired nasosorption and bronchosorption samples were collected from 8 COPD patients. CXCL8 was detected in all of the resulting eluates. Concentrations were greater in the nasal ELF when compared to the bronchial ELF (median ± IQR were 2.41 ± 1.84 ng/ml and 1.06 ± 1.18 ng/ml respectively, p < 0.01) but comparable to those sampled from the COPD patients in Section 6.5.3 (median ± IQR were 2.41 ± 1.84 ng/ml and 3.81 ± 2.55 ng/ml respectively, p = 0.22). A strong correlation was observed between nasal and bronchial concentrations of CXCL8 in COPD (Figure 6.2).
6.5.7. Concentrations of nasal CXCL8 in COPD over a 28-day time course

Figure 6.3 demonstrates the concentrations of CXCL8 measured in the nasal ELF of COPD patients at baseline, 1h, 6h, 1 day, 7 (± 1) days and 28 (± 2) days. Concentrations were unchanged over this time-course and comparable to those sampled from the COPD patients in Section 6.5.3 (median ± IQR were 3.35 ± 0.35 ng/ml and 3.81 ± 2.55 ng/ml respectively, p = 0.82).

6.5.8. Effect of gender on concentrations of nasal CXCL8

The COPD group contained more females than males (Table 6.1). Therefore, any effect of gender on the concentration of nasal CXCL8 was examined (Figure 6.4). There was no difference in nasal CXCL8 between males and females across all 3 subject groups. When analysing each group independently, concentrations of nasal CXCL8 were unaffected by gender.
Figure 6.3. Concentrations of CXCL8 in the nasal ELF of COPD patients sampled over 28 days. COPD patients (●) underwent nasosorption at baseline (B), 1h, 6h, 1 day, 7 (± 1) days and 28 (± 2) days (n = 5). Concentrations of CXCL8 were measured simultaneously by ELISA. Data presented are mean ± SEM.

Figure 6.4. Concentrations of CXCL8 in the nasal ELF of all male and female subjects. Non-smokers (▲), smokers (■) and COPD patients (●) underwent nasosorption (n = 12, n = 10 and n = 13 respectively). Concentrations of CXCL8 were measured by ELISA. Data presented are medians ± IQR.
6.5.9. **Effect of age on concentrations of nasal CXCL8**

To determine whether there was a relationship between age and the concentrations of CXCL8 in the nasal ELF, a correlation was performed (Figure 6.5). No correlation was observed between age and levels of nasal CXCL8, across all the three groups or when analysing each group independently.

![Figure 6.5. Correlation between age and the concentrations of CXCL8 in the nasal ELF of all subjects.](image)

Non-smokers (▲), smokers (■) and COPD patients (●) underwent nasosorption (n = 12, n = 10 and n = 13 respectively). Concentrations of CXCL8 were measured by ELISA. Data shown are Spearman rank correlation coefficients (subject groups analysed together).

6.5.10. **Effect of lung function on concentrations of nasal CXCL8**

To determine whether concentrations of nasal CXCL8 were related to lung function, correlations were performed with FEV₁ (L and % predicted), FVC and the FEV₁/FVC ratio (Figure 6.6). No correlation was observed between nasal CXCL8 and FVC or FEV₁/FVC. Similar data were obtained when each subject group was analysed independently or when all subjects were analysed as a whole. FEV₁ (L) did however weakly and negatively correlate with the
concentration of nasal CXCL8, but only when all groups were combined. A strong negative correlation was also observed with FEV$_1$ (% predicted). This was especially strong when independently analysing the COPD group ($r = -0.83$, $p < 0.001$), but no correlations were observed with either non-smokers or smokers.

Figure 6.6. Correlation between the concentrations of CXCL8 in the nasal ELF and (A) FEV$_1$ (L) (B) FEV$_1$ (% predicted) (C) FVC (D) FEV$_1$/FVC ratio. Non-smokers (▲), smokers (■) and COPD patients (●) underwent nasosorption ($n = 12$, $n = 10$ and $n = 13$ respectively). Concentrations of CXCL8 were measured by ELISA. Spirometry was performed to determine FEV$_1$ and FVC. Data shown are Spearman rank correlation coefficients (subject groups analysed together).
6.5.11. **Effect of smoking on nasal CXCL8 in smokers and COPD patients**

To determine whether concentrations of nasal CXCL8 were related to smoking (COPD patients and smokers), correlations were performed with the number of pack years smoked (Figure 6.7). When smokers and COPD patients were analysed together, no correlation was observed between nasal CXCL8 and pack years (Figure 6.7; Panel A). However, a strong correlation was present when analysing smokers independently (Figure 6.7; Panel B). In contrast, pack years did not correlate with concentrations of nasal CXCL8 in COPD (Figure 6.7; Panel C).

The concentrations of nasal CXCL8 in COPD patients who currently smoke and those who used to smoke, were also compared (Figure 6.7; panel D). There was no difference in concentrations between current and ex-smoking COPD patients.

6.5.12. **Effect of lung function on nasal CXCL8 in a larger COPD cohort**

The data presented in Figure 6.6 (Panel B) demonstrated a strong correlation between FEV₁ % predicted, an important marker of disease severity, and nasal CXCL8 in COPD. In order to confirm this relationship, nasosorption was used to sample the nasal ELF from a larger COPD cohort (n = 32) and concentrations of nasal CXCL8 were correlated with spirometry. Data from earlier sections of this chapter were included. No correlations were observed between nasal CXCL8 and FEV₁ (L), FEV₁ (% predicted), FVC or FEV₁/FVC (Figure 6.8). However, the median concentration of nasal CXCL8 (± IQR) were similar for the additional COPD patients newly sampled for this section, when compared to those sampled in Section 6.5.3 (4.75 ± 3.82 ng/ml (n = 19) and 3.81 ± 2.55 ng/ml (n = 13) respectively; p = 0.20).
Figure 6.7. Effect of smoking on CXCL8 concentrations in the nasal ELF of smokers and COPD patients: (A) Correlation between pack years and nasal CXCL8 in smokers and COPD patients (B) Correlation between pack years and nasal CXCL8 in smokers only (C) Correlation between pack years and nasal CXCL8 in COPD patients (D) Concentrations of nasal CXCL8 in current and ex-smoking COPD patients. Smoking controls (■) and COPD patients (●) underwent nasosorption (n = 10 and n = 13 respectively). COPD patients were either current (n = 5) or ex-smokers (n = 8). Concentrations of CXCL8 were measured by ELISA. A single pack year is equivalent to 20 cigarettes smoked every day for 1 year. Data presented are Spearman rank correlation coefficients (subject groups analysed together) or medians ± IQR.
Figure 6.8. Correlation between CXCL8 concentrations in the nasal ELF of COPD patients and spirometry. (A) FEV₁ (L) (B) FEV₁ (% predicted) (C) FVC (L) (D) FEV₁/FVC ratio. A larger cohort of COPD patients (●) underwent nasosorption (n = 32). Concentrations of CXCL8 were measured by ELISA. FEV₁ and FVC were assessed using spirometry. Data presented are Spearman rank correlation coefficients.

6.5.13. Effect of smoking on nasal CXCL8 in a larger COPD cohort

The data presented in Figure 6.7 (Panels C & D) demonstrated that concentrations of nasal CXCL8 in COPD patients were not related to pack years or smoking status. In order to confirm this finding, data from the larger cohort (n = 32) was analysed and concentrations of nasal
CXCL8 were correlated with pack years (Figure 6.9; Panel A) and compared between current and ex-smokers (Figure 6.9; Panel B). Nasal CXCL8 did not correlate with the number of pack years smoked. No difference was observed in nasal concentrations of CXCL8 when comparing current and ex-smoking COPD patients.

**Figure 6.9. Effect of smoking on CXCL8 concentrations in the nasal ELF of COPD patients.** (A) Correlation between pack years and concentrations of nasal CXCL8 in COPD patients (B) Effect of smoking status on nasal CXCL8 in COPD patients. COPD patients (●) underwent nasosorption (n = 32). The patients were either current (n = 6) or ex-smokers (n = 26). Concentrations of CXCL8 were measured by ELISA. A single pack year is equivalent to 20 cigarettes smoked every day for 1 year. Data presented are Spearman rank correlation coefficients or medians ± IQR.

### 6.5.14. Relationship between symptoms and nasal CXCL8 in COPD

The severity of COPD symptoms can be graded according to the mMRC dyspnoea scale or CAT questionnaire. According to current guidelines, patients can be categorised as more breathless with mMRC ≥ 2 and more symptomatic with CAT ≥ 10 (GOLD, 2015). To determine whether symptom severity was related to concentrations of nasal CXCL8, the COPD cohort was divided...
according to these suggested mMRC and CAT score cut-offs. No difference in CXCL8 concentrations was observed between patients with mMRC ≥ 2 and those with mMRC < 2 (Figure 6.10, panel A). Patients with CAT scores ≥ 10 did not have different concentrations of nasal CXCL8 than those with CAT scores < 10 (Figure 6.10, panel B).

![Figure 6.10. Concentrations of CXCL8 in the nasal ELF of COPD patients according to symptom severity: (A) mMRC dyspnoea scale (B) CAT scores. COPD patients underwent nasosorption (n = 32). All patients completed a CAT questionnaire. The degree of dyspnoea was also scored using the mMRC scale. Patients with more symptoms and those who were most breathless were categorised as those with a CAT score ≥ 10 or mMRC ≥ 2 respectively. Concentrations of CXCL8 were measured by ELISA. Data presented are medians ± IQR.](image)

### 6.5.15. Relationship between nasal CXCL8 and COPD exacerbation frequency

COPD 'frequent exacerbators' are defined as patients who experienced ≥ 2 exacerbations per year (Wedzicha et al., 2013). To determine whether there was a relationship between exacerbation frequency and concentrations of nasal CXCL8, the entire COPD cohort (i.e. all patients used in this chapter; n = 32) was first divided into frequent (≥ 2 / year) and infrequent...
exacerbators (< 2 / year) on the basis of self-reported exacerbation frequency during the preceding year. Demographic data for frequent and infrequent exacerbators are shown in Table 6.3. The mean number of exacerbations were 2.9 ± 0.2 and 0.5 ± 0.1 respectively (p < 0.0001). Both groups were matched for age, smoking history, FEV₁ (L and % predicted) and FEV₁/FVC. Frequent exacerbators had a lower FVC but previous data has demonstrated that FVC has no effect on nasal CXCL8 (Figure 6.8; Panel C). Infrequent exacerbators were predominantly male, while females outnumbered males in the frequent exacerbator group (Figure 6.4 confirms that nasal CXCL8 is not influenced by gender).

<table>
<thead>
<tr>
<th></th>
<th>Infrequent Exacerbators (n = 16)</th>
<th>Frequent Exacerbators (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>67.0 ± 1.6</td>
<td>66.4 ± 1.5</td>
</tr>
<tr>
<td>Gender (male : female)</td>
<td>10:6</td>
<td>5:11</td>
</tr>
<tr>
<td>Smoking history (pack years)</td>
<td>35.2 ± 2.5</td>
<td>27.8 ± 3.4</td>
</tr>
<tr>
<td>FEV₁ (L)</td>
<td>1.55 ± 0.13</td>
<td>1.41 ± 0.13</td>
</tr>
<tr>
<td>FEV₁ (% predicted)</td>
<td>56.2 ± 4.2</td>
<td>58.9 ± 2.9</td>
</tr>
<tr>
<td>FVC (L)</td>
<td>3.17 ± 0.16</td>
<td>2.49 ± 0.23**</td>
</tr>
<tr>
<td>FEV₁/FVC</td>
<td>0.53 ± 0.04</td>
<td>0.56 ± 0.04</td>
</tr>
<tr>
<td>Exacerbations (preceding yr)</td>
<td>0.5 ± 0.1</td>
<td>2.9 ± 0.2****</td>
</tr>
</tbody>
</table>

Table 6.3. Subject demographics for infrequent and frequent exacerbators. COPD patients were categorised as infrequent (< 2 / year) or frequent (≥ 2 / year) exacerbators. All patients underwent spirometry, with FEV₁ and FVC being recorded. A single pack year is equivalent to 20 cigarettes smoked every day for 1 year. Data presented are mean ± SEM. ** represents p < 0.01, **** p < 0.0001 relative to infrequent exacerbators.
After dividing the COPD cohort into frequent and infrequent exacerbators, concentrations of nasal CXCL8 were then compared (Figure 6.11). Concentrations of nasal CXCL8 were greater in patients who exacerbated ≥ 2 times per year.

**Figure 6.11. Concentrations of CXCL8 in the nasal ELF of COPD patients with infrequent (< 2 / year) and frequent (≥ 2 / year) exacerbations.** COPD patients (●) underwent nasosorption. Concentrations of CXCL8 were measured by ELISA. Data presented are medians ± IQR. * represents p < 0.01

### 6.5.16. Effect of COPD exacerbations on nasal CXCL8

COPD exacerbations are associated with upregulated pulmonary inflammation, including levels of bronchial CXCL8 (Crooks et al., 2000, Aaron et al., 2001). As concentrations of nasal CXCL8 were shown to correlate with bronchial levels (Figure 6.2), the effect of exacerbations on nasal CXCL8 was examined. COPD patients who had acutely exacerbated were asked to re-present within 2 days of symptom onset for clinical assessment (history, examination, spirometry and venous blood samples) and repeat nasosorption. Concentrations of CXCL8 within these eluates...
were then compared to baseline. Only four patients reported exacerbations. The characteristics of these subjects at baseline and during exacerbation are shown in Table 6.4. No changes in FEV\(_1\) or FEV\(_1\) % predicted were observed. However, concentrations of serum CRP and fibrinogen were greater during exacerbation.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Exacerbation</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV(_1) (L)</td>
<td>1.33 ± 0.20</td>
<td>1.19 ± 0.22</td>
</tr>
<tr>
<td>FEV(_1) (% predicted)</td>
<td>57.8 ± 8.8</td>
<td>51.3 ± 9.8</td>
</tr>
<tr>
<td>Serum CRP (mg/L)</td>
<td>4.8 ± 3.4</td>
<td>77.5 ± 9.8*</td>
</tr>
<tr>
<td>Serum fibrinogen (g/L)</td>
<td>3.3 ± 0.4</td>
<td>5.3 ± 0.6*</td>
</tr>
</tbody>
</table>

Table 6.4. Characteristics of COPD patients at baseline and during exacerbation. COPD patients \(n = 4\) underwent spirometry and provided venous blood prior to nasosorption (baseline). FEV\(_1\) (L and % predicted) was recorded and concentrations of serum CRP and fibrinogen were analysed by the RBHT clinical laboratories. Spirometry and further blood samples were also taken during exacerbation (\(\leq 2\) days from symptom onset). * represent \(p < 0.05\).

There was no statistical difference between the median concentration of nasal CXCL8 at baseline and during exacerbation (Figure 6.12). This was despite the exacerbations being associated with significant rises in systemic inflammatory markers (CRP and fibrinogen). A trend was visible however, with 3 out of the 4 subjects appearing to have greater concentrations during exacerbation (approximately 1.6, 1.9 and 4.4-fold greater, respectively).
Figure 6.12. Concentration of CXCL8 in the nasal ELF of COPD patients at baseline and during exacerbation. COPD patients underwent nasosorption at baseline. A further sample was taken during exacerbation (≤ 2 days from symptom onset) (n = 4). Concentrations of CXCL8 were measured simultaneously by ELISA. Data presented are individual COPD subjects (○●●○●●○) and the mean ± SEM (○).

6.5.17. Relationship between GOLD combined assessment and nasal CXCL8

COPD patients can be categorised according to the combined GOLD assessment (Groups A – D) (Figure 1.1). To determine whether there was a relationship between the GOLD combined assessment and concentrations of nasal CXCL8, the entire COPD cohort (i.e. all patients used in this chapter; n = 32) were divided into these 4 groups and comparisons were made. No differences in concentrations of nasal CXCL8 were observed between the groups (Figure 6.13).
Figure 6.13. Concentration of CXCL8 in the nasal ELF of COPD patients according to the combined GOLD assessment. COPD patients (●) underwent nasosorption (n = 32). They were classified according GOLD: A (n = 5), B (n = 5), C (n = 8) and D (n = 14). Concentrations of CXCL8 were measured by ELISA. Data presented are medians ± IQR.

6.6. Discussion

The data presented in this chapter has shown that COPD patients report more frequent nasal symptoms and have greater concentrations of CXCL8 in their nasal ELF than non-smokers. These findings support the current literature, which strongly suggests that COPD is associated with sinonasal inflammation. Large epidemiological studies have reported a higher prevalence of nasal symptoms in patients with COPD, relative to both the general population and healthy smokers (Montnemery et al., 2001, van Manen et al., 2001, Chen et al., 2003). These studies can be criticised however, for not using spirometry or reversibility testing to confirm COPD, rather a reliance on patient self-reporting (Hurst, 2010). The data presented above strengthens the argument that COPD is associated with a pan-airway inflammatory response. Not only was airflow obstruction demonstrated in all COPD patients, but any potential bias from asthma or
atopy was eliminated by excluding subjects with airway reversibility or positive skin prick tests. Nasal symptoms were experienced by almost three times as many stable COPD patients (69%) and healthy smokers (70%) than non-smoking controls (25%). These proportions seem quite high and it might be argued that this cohort was small and therefore an unrepresentative sample. The data however, is strikingly similar to other studies. Roberts et al. (2003) recruited 61 patients with confirmed COPD and reported nasal symptoms in 75%. As with the data presented herein, rhinorrhoea was most common (53% vs 46%). Therefore, although this pilot study is undoubtedly quite small, it would appear to be representative.

Nasosorption proved sufficiently sensitive to detect CXCL8 in the nasal ELF of non-smokers, smokers and COPD patients. The median concentration of nasal CXCL8 for the non-smoking controls (± IQR; range) was 0.92 ng/ml (± 0.91 ng/ml; 0.13 – 7.48 ng/ml). These data are consistent with previously published studies that have used nasosorption to sample non-smoking healthy control subject. These larger studies incorporated more subjects (n = 15 – 41) and reported nasal CXCL8 to range between 0.25 – 1.5 ng/ml (Dhariwal et al., 2015), 0.74 – 14.3 ng/ml (Chawes et al., 2010) and 0.8 – 14.0 ng/ml (Jochems et al., 2017). Moreover, the results presented herein provide some evidence of reproducibility in COPD, with similar median concentrations of nasal CXCL8 measured across the different COPD cohorts within this chapter; median concentrations of nasal CXCL8 (± IQR) were 3.81 ± 2.55 ng/ml (n = 13), 2.41 ± 1.84 ng/ml (n = 8), 3.35 ± 0.35 ng/ml (n = 5) and 4.75 ± 3.83 ng/ml (n = 19; newly-sampled patients only) for Sections 6.5.3, 6.5.6, 6.5.7 and 6.5.12 respectively.

Relative to non-smokers, the concentration of nasal CXCL8 was elevated in this mild to moderate COPD cohort by approximately four-fold. Such elevated concentrations have been reported in COPD before (Hurst et al., 2005). However, this is the first time that nasosorption has been used. Hurst et al. (2005) found CXCL8 to be greater in the nasal lavage of COPD
patients when compared to a control group (156.1 pg/ml vs 58.9 pg/ml). Their COPD cohort had more severe disease and the control group was recruited from an otolaryngology clinic, including patients with sinonasal pathology and ex-smokers. As such, the present study provides more robust evidence that CXCL8 is indeed upregulated in the nasal ELF of COPD patients, including those with only mild or moderate disease. This may be indicative of a more global underlying inflammatory response within the nasal ELF of COPD patients, with increased leukocyte recruitment and an upregulation in a wider range of cytokines, chemokines and growth factors. Such changes might account for the higher prevalence of sinonasal symptoms in COPD and ultimately result in pathology.

Although not statistically significant, there was a trend towards elevated concentrations of nasal CXCL8 in smokers without COPD. This pilot study did prove to be underpowered however, which might explain why the median concentration from smokers was indistinguishable from both non-smokers and COPD patients. Subsequent power calculations have suggested that 75 samples in total would be required to statistically address this important observation. Nevertheless, it seems likely that nasal CXCL8 would be elevated in smokers but it remains unclear as to whether these concentrations would be upregulated to the same degree as COPD.

The current study can only speculate that the increased concentrations of CXCL8 contribute to the higher prevalence of nasal symptoms in COPD patients. Nor was it designed to explore the mechanisms underpinning this upregulation of CXCL8. It can be hypothesised however, that exposure of the nasal epithelium to tobacco smoke, either from the cigarette’s side-stream (i.e. originating from its tip) or via exhalation through the nose (Hurst et al., 2010) drives the upregulation of CXCL8 and generates nasal symptoms. The effects of tobacco smoke on the upper airways have been poorly studied relative to the wealth of literature examining its
impact on the lung epithelium. Nonetheless, recent studies have shown that smoking is associated with an increased risk of chronic rhinitis and rhinosinusitis (Eriksson et al., 2013, Hastan et al., 2011) and that this risk is dose-dependent, i.e. positively correlates with the number of pack years smoked (Eriksson et al., 2013). In vitro, experiments have also demonstrated that nasal epithelial cells from COPD patients can be stimulated to release CXCL8 after incubation with cigarette smoke extract or acrolein, an important constituent of tobacco smoke (Comer et al., 2012). An alternative hypothesis might be that nasal inflammation arises indirectly from the lower airway. It seems likely that inflammatory cells and mediators are shared between the two indistinct airway ‘compartments’ and that a stimulus applied to the bronchi will elicit a response in the sinonasal airways, or vice versa. Indeed, nasal inflammatory responses have been observed as soon as 24h after the instillation of grass pollen into the bronchi of subjects with allergic rhinitis (Braunstahl et al., 2001). Similar mechanisms might exist in COPD.

In the smoking control group, a correlation was observed between the number of pack years smoked and the concentration of nasal CXCL8. This dose-dependent relationship supports the hypothesis that cigarette smoke elicits an inflammatory response in the sinonasal airways, which includes the upregulation of CXCL8. Interestingly however, no correlation was found between pack years and the concentration of nasal CXCL8 in COPD patients. It is also worth highlighting that a significant proportion of the COPD cohort were ex-smokers (62%), yet the median concentration of CXCL8 was still greater than the non-smoking controls. Moreover, no differences were observed between the concentrations of nasal CXCL8 from current and ex-smoking COPD patients. When taken together, these data strongly suggest that smoking does initiate nasal inflammation but this response is self-perpetuating in COPD. Roberts et al. (2003) arrived at the same conclusion after observing that nasal symptoms were no less prevalent in
ex-smoking than currently-smoking COPD patients. This is analogous to the current understanding of pulmonary inflammation in COPD, which is also considered to be self-perpetuating. Indeed, cellular infiltrates and protein markers are reported to be similar in COPD patients who continue to smoke and those who have stopped (Gamble et al., 2007).

Bronchosorption was successfully used to sample the bronchial ELF of 8 COPD patients, with CXCL8 reliably detected in all of the resulting eluates. The median concentration (± IQR) of CXCL8 was 1.06 (± 1.18) ng/ml, which was approximately half of that measured in the nasal ELF of the same subjects (2.41 (± 1.04) ng/ml). This might simply reflect differences in the absorption potential of the two devices. This bronchial concentration was however, similar to levels reported within the BALF of COPD patients (range from the literature of 0.12 – 2.5 ng/ml) (Leaker et al., 2015) and concentrations within induced sputum supernatants from other COPD cohorts, e.g. as reported by Daldegan et al. (2005) (median (± IQR) of 0.82 (± 0.11) ng/ml). Few studies have reported on the use of bronchosorption (Jackson et al., 2013, Leaker et al., 2015) and none (current study excluded) have recruited COPD patients. Only Leaker et al. (2015) have published on the concentrations of CXCL8 within bronchosorption eluates and these samples were taken from a small group of 8 smokers. The range in this study was 0.46 – 5.40 ng/ml (median 2.50 ng/ml), which is also comparable to the data presented herein for COPD patients.

Despite only collecting paired nasosorption and bronchosorption samples from a total of 8 COPD patients, a strong correlation was still observed between the concentrations of nasal and bronchial CXCL8. This is the first time such correlations have been reported in COPD, where SAM was used to sample both the nasal and bronchial ELF. This supports the hypothesis that sampling of the nasal mucosa can provide a surrogate measure of bronchial inflammation in COPD. Confirmation in a larger COPD cohort is still required and any future studies should also
establish whether similar relationships exist for a wider range of inflammatory mediators. However, the data presented herein suggests that the ‘one airway, one disease’ concept, that is commonly adopted for asthma (Grossman, 1997), might also be applied to COPD. Correlations between nasal and bronchial CXCL8 have been reported in COPD before, albeit using different sampling techniques. Hurst et al. (2005) collected induced sputum and nasal lavage samples from 47 stable COPD patients and found a correlation between the concentrations of CXCL8 in both samples. Outside of COPD, SAM has been used to simultaneously sample the nasal and bronchial ELF, and relationships have been identified between inflammatory profiles in asthma. Jackson et al. (2013) induced exacerbations in asthmatics by intra-nasal inoculation with rhinovirus. They subsequently measured IL-33, IL-13 and IL-5 in paired nasosorption and bronchosorption samples, and found correlations between the nasal and bronchial concentrations of these mediators.

An ideal COPD biomarker should be easy to sample and inexpensive to measure. To this end, nasal CXCL8 may be suitable. Not only were the concentrations elevated in COPD relative to non-smokers, but they were representative of bronchial levels and reliably detected by ELISA. Importantly, nasal CXCL8 was also found to be unaffected by potential confounding variables, such as gender or age. The levels of nasal CXCL8 were also unchanged over 28 days in stable COPD patients. This is an important finding, as it demonstrates a consistent baseline against which acute changes could be reliably detected (e.g. during exacerbation) and the effects of interventions assessed (e.g. novel anti-inflammatory treatments). Nasosorption also proved to be a relatively inexpensive, non-invasive and safe sampling technique. Adverse effects were infrequent and typically mild. They included temporary discomfort, sneezing, nasolacrimal reactions and brief self-limiting epistaxis. None of these adverse effects resulted in the withdrawal of consent for repeated sampling.
Exacerbations are important determinants of morbidity and mortality in COPD ([Sections 1.4.3 and 1.4.4](#)). As such, their effects on nasal CXCL8 were also examined in this chapter. Exacerbations were determined by changes in symptoms ([Seemungal et al., 1998](#)) and confirmed by an upregulation of serum CRP and fibrinogen. Unfortunately, only 4 patients presented following an exacerbation and no definitive conclusions can therefore be made. Nevertheless, nasal CXCL8 appeared to rise above baseline values for 3 out of the 4 patients, suggesting that further data collection is certainly warranted. A larger sample size would also allow for an evaluation of nasal CXCL8 during different types of exacerbation (e.g. viral, bacterial or non-infective) and during exacerbations with and without nasal symptoms.

Another related research question that is worth addressing is whether concentrations of nasal CXCL8 can help detect those patients at risk of frequent exacerbations. Within this COPD cohort, frequent exacerbators (≥ 2 / year) had greater concentrations of nasal CXCL8 than those who exacerbated infrequently (approximately 1.5-fold greater). This is not an unexpected finding, especially given that nasal symptoms (specifically discharge) are reported more commonly by frequent exacerbators ([Huerta et al., 2015](#)). The mechanisms underpinning the upregulation of CXCL8 in the nasal ELF of frequent exacerbators was not addressed by the present study. It can be hypothesised however, that it reflects a heightened pan-airway inflammatory response resulting from global dysfunctional immune responses, a greater bacterial load and/or microbial colonisation of both the upper and lower respiratory tract. Indeed, concentrations of CXCL8 are known to be elevated in induced sputum supernatants taken from frequent exacerbators ([Bhowmik et al., 2000](#)).

Another important characteristic for any potential COPD biomarker would be an ability to predict disease severity. However, in the larger COPD cohort (n = 32), nasal CXCL8 did not correlate with any spirometric measure, including FEV₁ (L) and FEV₁ (% predicted). Moreover,
when these patients were separated according to symptom burden (CAT ≥ 10 vs CAT < 10) or degree of breathlessness (mMRC ≥ 2 vs mMRC < 2), no differences were seen between the median concentrations of nasal CXCL8. Similarly, when the comprehensive combined GOLD assessment was used to categorise patients as A, B, C or D, no differences were observed between the median concentrations of nasal CXCL8. Taken together, these data strongly suggest that although CXCL8 is elevated in the nasal ELF of COPD patients, individual patient concentrations are unable to predict disease severity. Future studies may wish to include a larger sample size and examine whether nasal CXCL8 correlates with more direct measures of small airway disease, such as those obtained during IOS (e.g. R5-20). Longitudinal studies may also help ascertain whether nasal CXCL8 changes over time and whether this reflects the progression of lung disease.

In isolation, it is highly unlikely that nasal CXCL8 would act as a sensitive and specific biomarker for COPD. Indeed, nasal CXCL8 will be elevated with other pan-airway inflammatory conditions (e.g. asthma) and affected by other common upper airway pathologies or systemic inflammatory conditions. Measurements of TNF-α and IL-6 were also performed in non-smokers, smokers and COPD patients, with the expectation that these would allow for more a detailed assessment and comparison of differing nasal inflammatory profiles. However, levels of both TNF-α and IL-6 were below the detection limit of a standard ELISA for all subject groups. Multiplex ELISA (e.g. MesoScale Discovery (MSD) or Luminex®) are more sensitive assays that have been used before to measure proteins within the eluates of nasosorption and bronchosorption samples. Using MSD and Luminex®, the unstimulated levels of TNF-α and IL-6 have been detected in the nasal ELF of healthy control subjects (Chawes et al., 2010, Folsgaard et al., 2012, Dhariwal et al., 2015) and IL-6 has been measured in the bronchial ELF of smokers (Leaker et al., 2015). The nasosorption and bronchosorption eluates collected
during the current study could be analysed with multiplex ELISA for TNF-α and IL-6, as well as for a broader panel of proteins, to thereby generate a more detailed nasal inflammatory profile. This might help determine a specific biomarker panel for COPD and/or different COPD phenotypes (e.g. rapidly progressive disease or frequent exacerbators). Nasosorption and multiplex ELISA may also help address some other important research questions, e.g. can those smokers at risk of developing COPD be identified? Can a response to therapy be predicted? Can bacterial and viral exacerbations be differentiated, or can species-specific bacterial exacerbations be identified to help guide appropriate antibiotic use? The bronchosorption samples should also be analysed with multiplex ELISA to potentially prove (or disprove) the correlation between nasal and bronchial inflammation in COPD.

Another limitation of this study is the absence of any detailed clinical evaluation of the upper respiratory tract. CT imaging of the sinuses and/or nasal endoscopy would have identified specific sinonasal pathologies, that may have occurred more commonly in COPD. Rhinomanometry and acoustic rhinometry could also have been used to collect detailed measures of nasal airflow, resistance and patency. Values could have been compared between the three groups and correlated with the corresponding measures of pulmonary airflow and nasal CXCL8. This might have helped to further strengthen the argument that COPD is associated with nasal inflammation and that the pathological processes affecting the upper and lower airway are closely related. Indeed, in one small study (42 COPD patients and 12 healthy volunteers), nasal symptoms were not only more prevalent in COPD but were also associated with an increase in nasal resistance, reduction in nasal airflow and more frequent pathology at nasal endoscopy (Celakovsky et al., 2015). Furthermore, Hurst et al. (2006b) were able to demonstrate a correlation between nasal patency and pulmonary airflow obstruction, as assessed by acoustic rhinometry and FEV₁ % predicted respectively. A validated
questionnaire, such as the SNAQ-11 (Sino-Nasal Assessment Questionnaire) or SNOT-20 (Sino-Nasal Outcome Test) could also have helped quantify nasal symptoms in the participants, assess any impact on their quality of life and allowed for more detailed comparisons between the three groups.

The hypothesis for this chapter was ‘CXCL8, TNF-α and IL-6 are elevated in the nasal ELF of COPD patients, are representative of bronchial concentrations and can be used as reliable biomarkers of inflammation’. This hypothesis can only be partially accepted. CXCL8 was elevated in the nasal ELF of COPD patients relative to non-smokers but not smokers, and concentrations strongly correlated with bronchial levels. It was unaffected by potential confounding variables such as gender and age, was stable over 28 days and was elevated in frequent exacerbators. However, it did not correlate with any parameters of disease severity, including lung function, symptom scores or the GOLD combined assessment. TNF-α and IL-6 were not detectable in the nasal ELF using standard ELISA. As such, no conclusions can be made regarding these inflammatory mediators with respect to the above hypothesis.

This chapter has shown that concentrations of nasal CXCL8 are representative of bronchial levels and provided some evidence that nasal CXCL8 can act as a biomarker for COPD. As such, the next chapter uses nasosorption to non-invasively sample the nasal ELF for CXCL8, during treatment with a potentially novel anti-inflammatory.
CHAPTER 7

A Clinical Trial Evaluating the Effect of Solithromycin on Airway Inflammation in COPD
7.1. Introduction

Novel anti-inflammatory treatments are urgently required for COPD. Currently, there are no effective drugs, capable of downregulating inflammation, slowing disease progression and reducing mortality (Barnes, 2015). Even corticosteroids, which are the mainstay of treatment for many other chronic inflammatory conditions, are largely ineffective and potentially harmful (Suissa and Barnes, 2009). In COPD, oxidative stress leads to a reduction in the expression and activity of HDAC2, which would otherwise mediate corticosteroid-induced transrepression of pro-inflammatory genes (Ito et al., 2005, Ito et al., 2006).

Macrolide antibiotics could potentially act as anti-inflammatories in COPD as they are immunomodulatory at doses lower than their MIC (Kanoh and Rubin, 2010) and capable of suppressing inflammatory gene expression in vitro, through the inhibition of transcription factors such as NF-κB or AP-1 (Desaki et al., 2000, Kikuchi et al., 2002, Li et al., 2012), which are activated in COPD (Di Stefano et al., 2002). They are also capable of inhibiting neutrophil chemotaxis (Oda et al., 1994), downregulating adhesion molecules (Kawasaki et al., 1998) improving bacterial phagocytosis (Hodge and Reynolds, 2012), augmenting efferocytosis (Hodge et al., 2006b), reducing fibroblast proliferation (Nonaka et al., 1999) and decreasing mucus hypersecretion (Tamaoki et al., 1995). In vivo, 2 weeks of clarithromycin (500 mg twice daily) reduced total sputum leukocyte counts and concentrations of sputum and serum CXCL8 and TNF-α in 30 male COPD patients (Basyigit et al., 2004). Banerjee et al. (2004a) also found that 500 mg of sustained-release clarithromycin (once daily for 3 months) inhibited neutrophil chemotaxis and reduced sputum neutrophilia in 31 COPD patients.

Several randomised control trials (Suzuki et al., 2001, Seemungal et al., 2008, Blasi et al., 2010, He et al., 2010, Albert et al., 2011, Uzun et al., 2014) and meta-analyses (Donath et al., 2013,
Ni et al., 2015) have shown that the principle advantage of long-term macrolide treatment in COPD is a reduction in the frequency of exacerbations. It remains unclear however, whether this directly results from immunomodulation or indirectly through reductions in bacterial load, as would be expected from administering an antibiotic to COPD patients, of whom at least 30% will be colonised with bacteria (Rosell et al., 2005). Bacteria are not only a common cause for exacerbation (Papi et al., 2006) but also an important pro-inflammatory stimulus (Hill et al., 2000, Banerjee et al., 2004b, Sethi et al., 2006, Marin et al., 2012). As such, airway sterilisation with macrolide antibiotics might explain any reductions in exacerbation frequency and the anti-inflammatory effects reported by Basyigit et al. (2004) and Banerjee et al. (2004a). A study that excluded patients with bacterial colonisation, would be best placed to demonstrate the ‘pure’ anti-inflammatory effects of a macrolide in vivo.

There are potential risks associated with macrolide therapy. These agents are motilin receptor agonists and are associated with gastrointestinal side-effects (Catnach and Fairclough, 1992). They are also capable of hepatotoxicity (Andrade and Tulkens, 2011), cardiac arrhythmias (Cheng et al., 2015), QTc prolongation (Iannini, 2002), ototoxicity (Wallace et al., 1994) and antagonism of acetylcholine receptors (Bertrand et al., 2010). Such adverse effects frequently lead to the discontinuation of treatment (Donath et al., 2013) and are especially pertinent to COPD, which has a high prevalence of comorbidity (van Manen et al., 2001). The risk of harm from macrolides is not insignificant and best highlighted by the restrictions imposed on telithromycin by the FDA, following cases of fatal liver failure, myasthenia gravis, visual disturbance and loss of consciousness during post-marketing surveillance (Ross, 2007, Georgopapadakou, 2014).

Long-term macrolide treatment is also associated with high rates of microbial resistance (Serisier, 2013), particularly amongst important respiratory pathogens such as S. pneumoniae.
Indeed, long-term azithromycin treatment is associated with a 2.7-fold increase in macrolide resistance (Li et al., 2014) and might contribute to the already high incidence of macrolide-resistant *S. pneumoniae* (31%) that is reported by global surveillance studies (Felmingham et al., 2002). Macrolides bind to a single site on the 50s subunit of the bacterial ribosome, thereby inhibiting protein synthesis (Zuckerman, 2004). Modification of this solitary binding site, either through methylation or mutation, is a major mechanism through which macrolide resistance can be generated with relative ease (Leclercq and Courvalin, 2002).

Solithromycin is a novel, fourth generation macrolide that is manufactured by Cempra Pharmaceuticals (Chapel Hill, USA). It is a semi-synthetic erythromycin derivative, *i.e.* a ketolide. It differs structurally from other ketolides, *e.g.* telithromycin, by the presence of a fluorine group in the macrolactone ring (Fernandes et al., 2016). As a fluoroketolide, it binds to at least three bacterial ribosomal sites (Llano-Sotelo et al., 2010) and therefore has a much lower potential to induce microbial resistance than earlier generations of macrolide. Indeed, in one study involving eight pneumococcal strains, most of which possessed genotypic resistance to first and second generation macrolides, only one developed solithromycin resistance and this required eighteen repeated exposures to sub-inhibitory concentrations (McGhee et al., 2010).

Solithromycin is highly active against Gram-positive, Gram-negative, atypical and non-tuberculous mycobacteria (Fernandes et al., 2011), including macrolide-resistant strains (McGhee et al., 2010). It is a more potent antibiotic than azithromycin and clarithromycin *in vitro* and comparable to telithromycin (Rodgers et al., 2013). It is in clinical development for the treatment of bacterial community-acquired pneumonia, with phase 2 and 3 randomised control trials demonstrating non-inferiority to levofloxacin (Oldach et al., 2013) and
moxifloxacin (Barrera et al., 2016). The latter included COPD patients, in whom the response to solithromycin was possibly superior (11% of all subjects) (Barrera et al., 2016).

Like other macrolide antibiotics, solithromycin has also been shown to be immunomodulatory in vitro. In Chapter 5, it inhibited the release of TNF-α from LPS-stimulated MDM, with a comparable efficacy but potentially-greater potency than clarithromycin. Kobayashi et al. (2013b) also demonstrated that solithromycin suppresses TNF-α, CXCL8 and MMP-9 release and activity from human macrophage cell lines and monocytes isolated from COPD patients. This was achieved via the inhibition of NF-κB, and solithromycin was considerably more potent than other macrolide comparators (Kobayashi et al., 2013b). The same authors also found that solithromycin improved HDAC2 activity to restore dexamethasone sensitivity in H2O2-treated corticosteroid-insensitive leukocytes (Kobayashi et al., 2013a). These findings were subsequently confirmed in vivo; solithromycin reduced both pulmonary neutrophilic inflammation (50%) and pro-MMP-9 concentrations, while improving dexamethasone responses in cigarette-exposed mice (Kobayashi et al., 2013b, Kobayashi et al., 2013a).

Studies have shown that oral solithromycin is safe, well-tolerated and has a side-effect profile that is similar to other macrolides (Fernandes et al., 2016). In phase 1 studies, adverse effects were mild, none were serious and no deaths were reported (Oldach et al., 2012). Solithromycin also lacks motilin receptor activity, which could result in fewer gastrointestinal side-effects (Fernandes et al., 2010). It is also inactive against nicotinic acetylcholine receptors in vitro (Bertrand et al., 2010) and is therefore less likely than telithromycin to result in visual disturbances, exacerbations of myasthenia gravis or other neurological side-effects. Thorough cardiac studies have also demonstrated a low potential for arrhythmia and QTc prolongation (Darpo et al., 2015). There are reports of elevations in liver transaminases however, albeit asymptomatic, transient, modest and reversible (Oldach et al., 2012). Solithromycin is oxidised
by the cytochrome CYP450 isoenzymes (almost exclusively CYP3A4) and predominantly excreted via bile and faeces (> 70%) (Jamieson et al., 2015). It is also a substrate for p-glycoprotein. Moreover, it is a potent inhibitor of both these systems, with effects on the metabolism and transport of several concomitant drugs and itself (auto-inhibition) (Jamieson et al., 2015). As such, there is a risk of drug interactions.

When taken together, the data presented in this introduction suggest that oral solithromycin is a safe treatment that could work as an anti-inflammatory in COPD, via direct effects on leukocytes and restoring sensitivity to inhaled corticosteroids. Therefore, this chapter will examine the anti-inflammatory effects of solithromycin in vivo by reporting on a phase 2 randomised control trial in COPD. Outcome measures include effects on sputum neutrophilia, pulmonary, nasal and systemic inflammatory markers, spirometry, IOS and patient symptoms. These were explored in a group of patients without bacterial colonisation, thereby avoiding any potential confounding effects from administering an antibiotic (i.e. reductions in bacterial load). A once-daily dose of 400 mg was chosen, as this is being evaluated for the treatment of bacterial pneumonia and the pharmacokinetics (PK) of 400 mg have been characterised in human plasma up to 7 days. This was an early phase 2 trial in a vulnerable patient population, so subject numbers were limited to 30, treatment was for 28 days only, equal emphasis was placed upon evaluating safety and pharmacokinetics were examined from days 7 to 28. A crossover design was therefore adopted to limit the variability from this small patient population.

7.2. Hypothesis

The hypothesis for this chapter is: ‘solithromycin is immunomodulatory in vivo and capable of downregulating airway inflammation in stable COPD patients’.
### 7.3. Aims

The following aims were adopted in order to investigate the above hypothesis:

- Undertake a double-blind, randomised, placebo-controlled, cross-over study in COPD patients without evidence of bacterial infection or colonisation, to evaluate the direct effects of 28 days of 400 mg oral solithromycin on airway inflammation (CE01-204 clinical trial). Specifically, to determine any effects on sputum neutrophilia, sputum inflammatory mediators (CXCL8, IL-6, MPO, MMP-9, CCL2 and TNF-α), nasal CXCL8, spirometry, small airway resistance (R_{5-20} using IOS), systemic biomarkers (C-reactive protein and fibrinogen) and symptoms (CAT scores).

- Assess the safety and tolerability of oral solithromycin in COPD patients. Specifically, to determine any hepatic, cardiac, gastrointestinal, renal, haematological or neurological adverse effects during CE01-204.

- Better understand the PK of 400 mg of oral solithromycin in COPD patients, including treatment after 7 days. Specifically, to evaluate plasma concentrations of drug, N-acetyl CEM-101 and CEM-214 during CE01-204.

### 7.4. Methods

#### 7.4.1. CE01-204 study design

#### 7.4.1.1. General study design

CE01-204 was a phase 2, single-centre (Harefield Hospital), double-blind, randomised, placebo-controlled, crossover study (Figure 7.1). COPD patients were screened (Section 7.4.4.1) according to inclusion (Section 7.4.1.3) and exclusion (Section 7.4.1.4) criteria, with all suitable subjects crossing over to receive 400 mg of oral solithromycin and placebo. The order in which the patients received the study treatments was randomised (Section 7.4.2),
with both the patient and investigator blinded (Section 7.4.2). The two treatment periods were 28 days in duration and separated by 28 days of washout, during which no investigational drugs were taken. Patients attended at baseline and on days 1, 7 (± 2), 14 (± 2) and 28 for repeated measures to assess the tolerability and safety of oral solithromycin (Section 7.4.1.7), as well as the primary (Section 7.4.1.5) and secondary (Section 7.4.1.6) outcomes. A detailed schedule of the visits, assessments and procedures can be found in subsequent sections of this chapter and Table 7.1. Patients were also contacted by telephone midway through the washout and 14 days after completing both treatments.
Figure 7.1. **CE01-204 study design.** CE01-204 was a phase 2, single-centre, double-blind, randomised, placebo-controlled, crossover study. COPD patients attending pulmonary rehabilitation at Harefield hospital were screened. Suitable patients were randomised (1:1) to receive either 400 mg of oral solithromycin followed by placebo, or placebo followed by 400 mg of oral solithromycin. Both the patient and investigator were blinded to the treatment order. Treatment periods were 28 days and separated by a washout of similar duration. Patients attended at baseline and on days 7, 14 and 28 for repeated measures. They were also contacted by telephone midway through the washout period (WoP TC) and 2 weeks after the end of the study (EoS TC).
7.4.1.2. Patient recruitment

CE01-204 aimed to recruit 30 COPD patients by enrolling approximately 40, allowing for a 25% dropout. This sample size was chosen empirically in consultation with a medical statistician. It was estimated that this would offer adequate statistical power while providing safety and satisfying the ethical considerations for this phase 2 study.

Male and female patients with stable COPD (GOLD stage 2 or 3), who reported symptoms compatible with chronic bronchitis (i.e. cough and sputum production for ≥ 3 months for 2 consecutive years) were approached (GOLD, 2015). Patients recovering from a recent exacerbation (≤ 60 days) and those with evidence of bacteria in their sputum (qPCR analysis) were excluded. Patient suitability was decided upon by the study doctor (Dr Craig Batista) and concomitant medication discussed with Cempra Inc., prior to enrolment.

7.4.1.3. Inclusion criteria

The following inclusion criteria were adopted:

- Male and female patients ≥ 45 years of age.
- History of cigarette smoking >10 pack-years.
- Post-bronchodilator FEV₁/FVC ratio < 0.7 and FEV₁ = 30-79 % of predicted.
- Patients prescribed inhaled corticosteroids were enrolled.
- Females of non-childbearing potential were enrolled, i.e. surgically sterile or ≥ 2 years after menopause.
- Females of childbearing potential were recruited provided a serum pregnancy test was negative at enrolment and they agreed to use highly effective methods of birth control until 30 days after the last dose.
- Males who wished to father children were enrolled.
Only patients who were able and willing to comply with all study visits and procedures were recruited.

Only patients who were suitable candidates for oral therapy and able to swallow capsules intact were enrolled.

Written informed consent was required.

7.4.1.4. Exclusion criteria

The following exclusion criteria were adopted:

- Acute exacerbation within the previous 60 days or during washout.
- Evidence of active bacterial infection in sputum by qPCR evaluation (equivalent to < $10^4$ gene copies/ml).
- Any condition that could possibly affect oral drug absorption.
- Use of medication for HIV, chronic hepatitis B, or hepatitis C.
- Use of theophylline or other xanthine medication.
- Use of warfarin.
- Concomitant infection (pulmonary or otherwise) that could require additional systemic antibiotics.
- QTc > 450 ms in males or females (as corrected by the Fridericia formula).
- Use of drugs known to prolong the QT interval. Examples provided in Appendix D.
- Concomitant use of drugs, foods, or herbal products known to be moderate or potent inhibitors of CYP3A4 isozymes. Examples provided in Appendix D.
- Use within the previous 7 days of drugs or herbal products known to be moderate to potent inducers of CYP3A4 isozymes. Examples provided in Appendix D.
- Use of drugs with narrow therapeutic indices that are principally metabolized by CYP3A4 or transported by P-glycoprotein, for which a drug interaction with solithromycin could
result in higher and possibly unsafe exposures to these drugs. Examples provided in Appendix D.

- History of organ transplant.
- Cytotoxic chemotherapy or radiation therapy within the previous 3 months.
- Known neuromuscular disorder (e.g. myasthenia gravis or Parkinson’s disease).
- Significant renal, hepatic or hematologic impairment.
- Women who are pregnant or breast feeding.
- Prior participation in the CE01-204 protocol.
- Use of any investigational drugs or devices within 4 weeks.
- History of intolerance or hypersensitivity to macrolide antibiotics.
- Any concomitant condition that would preclude an evaluation of a response, or make it unlikely that the therapy and follow-up would be completed (e.g. life expectancy < 30 days).

7.4.1.5. Primary outcome measure

The primary outcome measure was the effect of solithromycin on the number of neutrophils per ml of sputum compared to any effect with placebo.

7.4.1.6. Secondary outcome measures

The secondary outcome measures were:

- The effect of solithromycin on concentrations of sputum CXCL8, IL-6, MPO, MMP-9, CCL2 and TNF-α compared to any effect with placebo.
- The effect of solithromycin on concentrations of nasal CXCL8 compared to any effect with placebo.
- The effect of solithromycin on FEV₁ and R₅₋₂₀, compared to any effects with placebo.
• The effect of solithromycin on CAT scores compared to any effect with placebo.

• The effect of solithromycin on concentrations of serum biomarkers (C-reactive protein and fibrinogen) compared to any effect with placebo.

7.4.1.7. Safety and tolerability of oral solithromycin in COPD

The safety and tolerability of oral solithromycin was assessed in COPD patients by recording and comparing all the adverse events (Chapter 7.4.7), clinical examination findings (including vital signs) and clinical laboratory results from the two treatment periods.

7.4.1.8. Pharmacokinetics of oral solithromycin in COPD

No PK data is specifically available for COPD patients or following exposure to solithromycin for > 7 days. As such, blood samples were taken on days 7 (± 2), 14 (± 2) and 28 for PK analysis (Chapter 7.4.7).

7.4.2. Randomisation, blinding and emergency unblinding

Patients received both solithromycin and placebo during the study. However, the treatment order was randomised in a 1:1 ratio. Randomisation was performed using a schedule generated by a medical statistician (Mr Winston Banya) with Stata software (StataCorp LLC). CE01-204 was double-blind, meaning that the investigators and patients did not have access to this schedule. Patients were assigned to the schedule according to their order of enrolment. In other words, if the treatment schedule for ‘subject 1’ stated that they should receive solithromycin during treatment period 1 followed by placebo during treatment period 2, then the first patient to be enrolled in the study would be assigned the status of subject 1 and receive the investigational products in this order. With this in mind, the randomisation schedule was held by Catalent (USA), who were responsible for drug packaging. Microconstants (USA), who analysed the PK samples, also had access. However, Cempra Inc.
and the hospital pharmacy were blinded. The latter had access to concealed, individual randomisation codes, which could be used for emergency unblinding.

7.4.3. **Study drugs and dosing**

7.4.3.1. **Solithromycin**

Solithromycin was provided in size ‘0’, hard gelatin capsules, which were filled with white to off-white powder. Each capsule contained 200 mg of formulated drug. Patients received a blister pack containing 28, once daily doses of 400 mg (i.e. 2 capsules / day).

7.4.3.2. **Placebo**

Placebo capsules were identical in appearance, but contained methylcellulose and no active ingredient. Patients received a blister pack containing 28, once daily doses of placebo (2 capsules / day).

7.4.3.3. **Blister pack cards**

Solithromycin and placebo were packaged by Catalent (USA) into two separate, but identical, blister pack cards. These were then collated according to the randomisation schedule, labelled as subjects 1 - 40 and delivered to the pharmacy at Harefield hospital. The relevant blister pack cards were only dispensed at the start of each treatment period.

7.4.3.4. **Study drug accountability**

The investigational drugs were delivered directly to the Harefield hospital pharmacy, where they were kept at room temperature, in a dedicated secure location. Only the trial pharmacists had access. On the day of dispensing, the appropriate blister pack cards for both treatment periods were labelled with the patient’s details and instructions. The study doctor completed a prescription and collected the blister pack card for treatment period 1, double-checking the details and that the blisters were unopened. The card for treatment period 2 was retained by
the pharmacy until the patient entered this phase of the study. The prescription instructions were then explained to the patient, before they took the first dose on site. Subsequent doses were taken at home and the patients were asked to record the dates and times on the card. Patients were also instructed to bring their blister packs to each study visit. Pill counts were performed and all blister packs were collected upon completion of the treatment period. The patients were also asked to return any unused study drug.

7.4.3.5. Study drug discontinuation criteria

Study drug discontinuation criteria were predetermined and detailed in the protocol. These enabled the investigators to decide whether solithromycin should be discontinued in the event of an adverse effect, e.g. hepatotoxicity, QTc prolongation or neurological deterioration. These can be viewed in Appendix E.

7.4.4. Study visits

7.4.4.1. Screening

All potentially-suitable COPD patients were initially contacted by telephone. They had been identified from a database containing the details and clinical information of all patients that had attended the pulmonary rehabilitation service at Harefield hospital under the care of Dr William Man. Their willingness to participate and potential suitability was explored on the telephone prior to inviting them to screening. Screening was combined with the baseline visit (Table 7.1).

7.4.4.2. Study schedule

Suitable COPD patients were asked to sign the informed consent form, before being randomised. They were then invited to a series of study visits according to a pre-determined schedule. This is shown in Table 7.1, which also details the assessments and procedures that
were undertaken at each visit. In short, patients attended at baseline and then on day 1 for the on-site administration of the first dose (within 21 days of screening). This was then followed by visits on day 7 (± 2), day 14 (± 2) and day 28. The two treatment periods were identical. Patients were telephoned during washout (day 14 ± 2) and 2 weeks after completing the study protocol. The duration of each individual’s participation was approximately 119 days, including screening, the two treatment periods, washout and concluding telephone call.

7.4.4.3. Case report forms

All patient data were captured in electronic case report forms (eCRF), using the InForm™ software and system (Oracle, USA). Support for the eCRF was provided by the InForm™ team at the Clinical Trials Unit, Imperial College. Source data (e.g. clinical notes or blood results) were input into the appropriate eCRF by the study doctor.
Table 7.1. CE01-204 schedule of visits, assessments and procedures. Patients were screened within 21 days of starting treatment. They attended visits at baseline, Day 1, Day 7 (± 2), Day 14 (± 2) and Day 28 (identical for both treatment periods (TP)). The assessments and procedures that took place are denoted by ‘X’. a baseline for TP 1 was the same as screening, while baseline for TP 2 occurred during washout (within 7 days of drug administration); b telephone call at 14 (± 2) days of washout; c telephone call at 14 (± 2) days following TP 2; d heart rate, blood pressure, temperature, respiratory rate and pulse oximetry; e a portion was sent for qPCR at screening only; f FEV$_1$, FVC and $R_{5} - R_{20}$; g full blood count, urea and electrolytes, liver function tests, glucose, bone profile and creatinine kinase; h females of childbearing potential; i taken 4 h after dose.
7.4.5. **Clinical measurements and assessments**

7.4.5.1. **Medical history**

A detailed medical history was taken, including any current symptoms, the patient’s past medical and surgical history, concomitant and recent treatments, allergies, smoking, alcohol intake and a system review.

7.4.5.2. **Physical examination & vital signs**

A physical examination of the heart, lungs and abdomen was performed. This included recording the heart rate, blood pressure, temperature, respiratory rate and pulse oximetry.

7.4.5.3. **COPD assessment test**

The CAT was undertaken without any assistance or reference to previously completed questionnaires. An example can be viewed in Appendix B.

7.4.5.4. **Impulse Oscillometry**

IOS was performed using the JAEGER® Vyntus™ IOS system and SentrySuite® software (CareFusion, UK), as described in Section 2.2.2.4. Three reproducible attempts were performed and the mean values recorded. Measures of \( R_5 \), \( R_{20} \), \( X_5 \), AX and RF were calculated by the software, while \( R_{5:20} \) was manually calculated by subtracting \( R_{20} \) from \( R_5 \). IOS was always undertaken prior to spirometry, to avoid the possible recruitment of collapsed airways during the forced manoeuvre. An example of results with non-smokers and COPD patients, and correlations with spirometry values can be viewed in Appendix C.

7.4.5.5. **Spirometry and reversibility testing**

Spirometry was performed as described in Section 2.2.2.1, with the exception that the JAEGER® Vyntus™ IOS system and SentrySuite® software (CareFusion, UK) were used. This system is capable of performing both IOS and spirometry with separate manoeuvres (i.e. tidal
breathing for the former and forced expiration for the later). Three reproducible attempts were made, but only the best values for FEV₁ and FVC were recorded. Reversibility testing was then performed, as described in Section 2.2.2.2. This was always performed after IOS and baseline spirometry, to avoid the effects of bronchodilation.

7.4.5.6. Electrocardiogram

A standard 12-lead electrocardiogram was performed and interpreted. The QT was corrected by the Fridericia formula (*i.e.* QTc = QT/RR¹/₃).

7.4.5.7. Recording adverse events

An adverse event (AE) was ‘any untoward medical occurrence associated with the use of solithromycin or placebo, regardless of causal relationship.’ This included any clinical signs, symptoms, new conditions, physiological observations or significant laboratory abnormalities that required intervention (*e.g.* study drug interruption or discontinuation). A serious adverse event (SAE) was ‘any AE that could have, or did, result in death, a life-threatening experience, inpatient hospitalisation, a congenital anomaly or a persistent or significant incapacity / substantial disruption of the ability to conduct normal life functions.’ Suspected Unexpected Serious Adverse Reactions (SUSAR) were ‘SAEs that were unexpected (*i.e.* not consistent with the applicable product information)’. Details of all AEs, SAEs and SUSARS were recorded in the eCRF (*e.g.* duration, severity, association with study drug, action taken, and outcome). In addition, all SAEs and SUSARS were reported to the sponsor, Cempra Inc. and the Medicines and Healthcare products Regulatory Agency (MHRA).
7.4.6. **Sampling techniques**

7.4.6.1. **Venepuncture and clinical blood tests**

Venepuncture was performed using a standard technique, 21-gauge butterfly needle and vacutainer tubes. Blood samples were taken according to the study schedule (Table 7.1) and a full blood count, urea and electrolytes, liver function tests, glucose, bone profile, creatinine kinase, serum pregnancy test (where appropriate) and serum biomarkers (fibrinogen and C-reactive protein) were performed. All assays were performed by the clinical biochemistry and haematology laboratories at RBHT. Blood samples were also taken for PK. This is described in Section 7.4.7.4.

7.4.6.2. **Sputum induction and processing**

Sputum induction was undertaken as described in Section 2.2.5.2. All of the recovered sputum was immediately stored on ice and processed within 2 h according to Section 2.2.7.1. At screening, a portion ($\geq 1$ ml) was left unprocessed and analysed for bacteria using qPCR (Section 7.4.7.1).

7.4.6.3. **Nasosorption**

The nasal ELF was sampled using two separate Nasosorption™ FX-i devices (Hunt Developments, UK). These were simultaneously applied to the anterior portion of the inferior turbinate of each nostril. A detailed description of the technique and processing is available in Section 2.2.5.3 and Section 2.2.7.2.

7.4.7. **Laboratory techniques**

7.4.7.1. **Quantitative polymerase chain reaction**

Real-time multiplex qPCR assays were performed by the RBHT microbiology department to detect *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in induced sputum. The methodology
is detailed in Section 2.2.15. An Applied Biosystems® 7500 thermocycler (ThermoFisher, UK) and validated, commercially-available kits were used, according to the manufacturer’s instructions (Bacterial Pneumonia CAP, Fast Track Diagnostics, Luxembourg). The minimum limit of detection was $10^4$ gene copies/ml.

7.4.7.2. Quantification of sputum neutrophils

Induced sputum was initially collected and processed, as described in Section 7.4.6.2. Samples were then cytospun, before being stained with a REASTAIN Quick-Diff kit (Section 2.2.8) and viewed under light microscopy. Differential cell counts were performed and adjusted according the volume of sputum. Neutrophils were then quantified as the number of cells per ml of sputum. A minimum of 200 cells were counted on each slide.

7.4.7.3. Measurement of inflammatory mediators

Concentrations of inflammatory mediators were measured in induced sputum supernatants and nasosorption eluates using ELISA and according to the manufacturer’s instructions. Antibodies were purchased from Invitrogen, UK. For a detailed description of the methodology refer to Sections 2.2.14.

7.4.7.4. Pharmacokinetics

Plasma concentrations of solithromycin, N-acetyl CEM-101 and CEM-214 were quantified by mass spectrometry. Venous blood (6 ml) was sampled approximately 4h after the solithromycin dose, using two K$_2$EDTA vacutainers (Becton Dickinson medical technology, USA). The samples were kept on ice and processed within 1 h. They were centrifuged (1500 x g for 10 min at 4°C) to obtain plasma, before being stored at -80 °C. Batches were shipped on dry ice to Microconstants (USA) for mass spectrometry.
7.4.8. Regulation

CE01-204 was approved by a National Research Ethics Committee (Bloomsbury, London; Reference 13/LO/1403) and the MHRA. It was also registered with the European Medicines Agency and European Clinical Trials Database (EudraCT; Reference 2014-003077-42), as well as the U.S. National Institutes of Health website, ClinicalTrials.gov (Identifier NCT02628769). Appropriate clinical trial insurance was held. Study monitoring was undertaken by the Joint Research Compliance Office (JRCO) at Imperial College. All patients were provided with an approved patient information sheet and were asked to sign the accompanying informed consent form.

7.4.9. Sponsor, funding and NHS host

CE01-204 was sponsored by Imperial College and funded by Cempra Inc. Cempra Inc. was not involved in data collection or analysis. The study was hosted by RBHT.

7.4.10. Statistical analysis plan

A statistical analysis plan was developed by a medical statistician (Mr Winston Banya). However, for reasons that will become apparent in Section 7.5.2.2, this plan was not required. Due to the paucity of available data, statistical tests have not been conducted. Graphical data are presented for each individual subject and as means ± SEM. All graphs were created using GraphPad Prism (version 7.02).

7.5. Results

7.5.1. Patient recruitment and progress

7.5.1.1. Screening, randomisation and subject withdrawals

A total of 43 potentially-suitable patients were identified from the Harefield pulmonary rehabilitation database. All were contacted by telephone. Of these patients, 6 were found to
be suitable and willing to participate. All of these patients subsequently met the inclusion and exclusion criteria at their screening visit.

The recruited patients were assigned the subject numbers 1 - 6 before being randomised. CE01-204 has since been unblinded. Subjects 1, 3 and 6 received solithromycin during treatment period 1 followed by placebo in treatment period 2, while subjects 2, 4 and 5 received the study drugs in the reverse order. Subject 3 exacerbated before starting treatment period 1 and was therefore withdrawn (no treatment received). Subject 1 experienced significant hepatotoxicity with solithromycin, leading to emergency unblinding and the early discontinuation of treatment (no measures at day 28). Subject 2 completed placebo treatment but was withdrawn before commencing solithromycin due to an exacerbation (no measures were made). Therefore, only subjects 1, 4, 5 and 6 received solithromycin, with subject 1 not completing 28 days of treatment. An unacceptable risk of hepatotoxicity ultimately led to the early termination of CE01-204. As a result, no further patients were recruited following subject 6. The screening, randomisation and subject withdrawals are summarised in Figure 7.2.
Forty-three COPD patients were screened. Thirty-seven were excluded and six recruited (subjects 1 to 6). These patients were randomised 1:1. Subjects 1, 3 and 6 received solithromycin in treatment period (TP) 1 followed by placebo in TP 2. Subjects 2, 4 and 5 received placebo in TP 1 and solithromycin in TP 2. Subject 3 exacerbated before starting TP 1 and was withdrawn. Subject 1 experienced significant hepatotoxicity with solithromycin and was withdrawn. Subject 2 completed TP 1 but was withdrawn before TP 2 due to an exacerbation. Subjects 4, 5 and 6 completed the trial protocol.

**Figure 7.2. Screening, randomisation and subject withdrawals for CE01-204.** Forty-three COPD patients were screened. Thirty-seven were excluded and six recruited (subjects 1 to 6). These patients were randomised 1:1. Subjects 1, 3 and 6 received solithromycin in treatment period (TP) 1 followed by placebo in TP 2. Subjects 2, 4 and 5 received placebo in TP 1 and solithromycin in TP 2. Subject 3 exacerbated before starting TP 1 and was withdrawn. Subject 1 experienced significant hepatotoxicity with solithromycin and was withdrawn. Subject 2 completed TP 1 but was withdrawn before TP 2 due to an exacerbation. Subjects 4, 5 and 6 completed the trial protocol.
7.5.1.2. Patient demographics

Demographic data for the 6 recruited patients are shown in Table 7.2. The mean age (± SEM) was 69.3 (± 1.5) years. There were an equal number of males and females. All patients were of white British ethnicity. The mean smoking history (± SEM) was 31.5 (± 4.1) pack years and all patients were ex-smokers. The mean (± SEM) FEV$_1$, FEV$_1$ % predicted, FVC and FEV$_1$/FVC ratio were 1.29 L (± 0.14), 55.2 % (± 7.1), 2.87 L (± 0.33) and 0.48 (± 0.06), respectively. These results suggested moderate to severe airflow obstruction on average (GOLD stage 2 and 3). The mean Rs-20 (± SEM) was 0.18 kPa.L$^{-1}$.s$^{-1}$ (± 0.05). The mean CAT score (± SEM) was 17 (± 3) and the patients experienced an average of 2 (± 0.6) exacerbations per year. All the patients (except subject 2) were classified as Group D according to the combined GOLD assessment (i.e. more symptoms and high risk of exacerbation). Table 7.3 details the subjects’ individual characteristics (age, gender and BMI), along with their medical/surgical histories. Two patients had a BMI outside of the normal range. Subject 5 was overweight and subject 6 was obese. None of the patients were underweight.
<table>
<thead>
<tr>
<th></th>
<th>COPD (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>69.3 ± 1.5</td>
</tr>
<tr>
<td><strong>Gender (male : female)</strong></td>
<td>3:3</td>
</tr>
<tr>
<td><strong>Ethnicity (white British)</strong></td>
<td>6</td>
</tr>
<tr>
<td><strong>Smoking history (pack years)</strong></td>
<td>31.5 ± 4.1</td>
</tr>
<tr>
<td><strong>Current smoker (yes : no)</strong></td>
<td>0:6</td>
</tr>
</tbody>
</table>

**Spirometry and IOS:**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td><strong>FEV₁ (L)</strong></td>
<td>1.29 ± 0.14</td>
</tr>
<tr>
<td><strong>FEV₁ (% predicted)</strong></td>
<td>55.2 ± 7.1</td>
</tr>
<tr>
<td><strong>FVC (L)</strong></td>
<td>2.87 ± 0.33</td>
</tr>
<tr>
<td><strong>FEV₁/FVC</strong></td>
<td>0.48 ± 0.06</td>
</tr>
<tr>
<td><strong>R₅-20 (kPa.L⁻¹.s)</strong></td>
<td>0.18 ± 0.05</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean CAT Score</strong></td>
<td>17 ± 3</td>
</tr>
<tr>
<td><strong>Mean number of exacerbations / year</strong></td>
<td>2.0 ± 0.6</td>
</tr>
<tr>
<td><strong>GOLD Classification (A / B / C / D)</strong></td>
<td>0 / 0 / 1 / 5</td>
</tr>
</tbody>
</table>

Table 7.2. Subject demographics and clinical data for CE01-204. Participants underwent spirometry and IOS, with FEV₁, FVC and R₅-20 being recorded. They also completed a CAT and the number of COPD exacerbations during the preceding year were self-reported. All patients were categorised according to the combined GOLD assessment. A single pack year is equivalent to 20 cigarettes smoked every day for 1 year. Data presented are numbers and mean ± SEM.
### Table 7.3. Individual subject characteristics

Heights and weights were recorded and the BMI calculated (normal range 18.5 - 25). Medical histories were taken and past medical conditions / surgery recorded.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Gender</th>
<th>BMI</th>
<th>Past medical / surgical history (year of diagnosis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>73</td>
<td>M</td>
<td>25.0</td>
<td>None.</td>
</tr>
</tbody>
</table>

#### 7.5.1.3. Concomitant medication

Concomitant medications are shown in Table 7.4. All six patients were prescribed at least one inhaler. For five of them, this included a LABA, LAMA and ICS. All patients were taking an ICS. Only subject 6 was not taking any oral medication.
### Table 7.4. Concomitant medication in CE01-204.

Subjects were asked to provide drug histories. Data presented are medications taken by each subject.

OD = once daily, BD = twice daily, OW = once weekly, I.U. = international units.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Oral Medication</th>
<th>Inhalers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Finasteride (5 mg OD)</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Alendronic acid (70 mg OW)</td>
<td>Omeprazole (20 mg OD)</td>
</tr>
<tr>
<td>3</td>
<td>Omeprazole (10 mg OD)</td>
<td>Hydroxychloroquine (200 mg BD)</td>
</tr>
<tr>
<td>4</td>
<td>Omeprazole (20 mg OD)</td>
<td>Simvastatin (40 mg OD)</td>
</tr>
<tr>
<td>5</td>
<td>Levothyroxine (75 mcg OD)</td>
<td>Atorvastatin (20 mg OD)</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
7.5.2. Safety and tolerability of oral solithromycin in COPD

7.5.2.1. Tolerability of oral solithromycin in COPD

No patient reported an AE that led to their voluntary discontinuation of solithromycin or withdrawal from the study.

7.5.2.2. Hepatobiliary effects of oral solithromycin in COPD

As a drug class, macrolide antibiotics are known to be associated with hepatobiliary AEs. As such, the effect of 400 mg of oral solithromycin on bilirubin and liver enzymes was evaluated over the 28 days of treatment (Figures 7.3, 7.4, 7.5 and 7.6). A significant hepatotoxic signal was observed with solithromycin, but not placebo. Three out of the four patients who received solithromycin (subject 1, 5 and 6) went on to develop deranged hepatic enzymes (75%). The changes were most notable for subject 1 and this was reported as a SUSAR. Only subject 4 did not develop deranged hepatic enzymes. Each case is now described.

**Subject 1** had a normal bilirubin, alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) at baseline. However, liver enzymes rose after 14 days of treatment. Bilirubin concentrations were also significantly elevated by day 23. The patient became icteric and reported pruritus but did not require hospitalisation or any treatment. This was also associated with a peripheral blood eosinophilia (**Figure 7.7**). Synthetic function was not affected (*i.e.* INR or albumin). A ‘liver screen’ was normal, including an ultrasound and viral hepatitis serology. Treatment was unblinded at day 23 and solithromycin immediately discontinued. This resulted in a reduction in the bilirubin and liver enzymes (returned to normal). The concomitant finasteride was also temporarily discontinued at day 23 and serum concentrations were retrospectively assayed by Microconstants. Levels of finasteride were 158 ng/ml and 111 ng/ml at days 7 and 14 respectively, which are approximately 3-4 times the
expected values for once daily dosing at 5 mg (reference range of 27-45 ng/ml; from FDA-approved drug label).

**Subject 4** had a normal bilirubin and liver enzymes at baseline. Levels were unaffected by 28 days of 400 mg oral solithromycin.

**Subject 5** essentially had normal liver enzymes and bilirubin at baseline. Although the ALP was slightly elevated at baseline (133 U/L), this was also observed during treatment with placebo (141 U/L) and these raised levels are negligible (upper limit of normal is 130 U/L). However, by day 28 of solithromycin treatment, ALT and AST were elevated. This was associated with mild abdominal bloating, flatulence and gastro-oesophageal reflux. Concomitant medications were not withdrawn, further investigations were not arranged and the study drug was not unblinded. This was the end of the treatment period, so the study drug was stopped in accordance with the protocol (not prematurely withdrawn). Unlike subject 1, no peripheral eosinophilia was observed (**Figure 7.7**). Discontinuation of solithromycin resulted in a reduction of all the liver enzymes. The patient declined follow-up after 37 days.

**Subject 6** had a normal bilirubin and liver enzymes at baseline. However, modest rises in ALP, ALT and AST were observed at day 14. This was associated with mild epigastric discomfort and gastro-oesophageal reflux following meals. Further investigations were not arranged and the study drug was continued without unblinding. Unlike subject 1, no peripheral eosinophilia was observed (**Figure 7.7**). The liver enzymes normalised despite continuing treatment with solithromycin.
Figure 7.3. Effect of solithromycin on total bilirubin. Subject 1 (●), subject 4 (○), subject 5 (○) and subject 6 (○) received solithromycin as part of the CE01-204 protocol. Blood tests were taken as scheduled (baseline (B), days 7 (± 2), 14 (± 2) and 28) or according to clinical need. Bilirubin concentrations were reported by the RBHT clinical biochemistry laboratory. Subjects 4, 5 and 6 received 28 days of solithromycin. Subject 1 was withdrawn (solithromycin discontinued at day 23). Upper and lower limits of normal are represented by grey shading.

Figure 7.4. Effect of solithromycin on alkaline phosphatase (ALP). Subject 1 (●), subject 4 (○), subject 5 (○) and subject 6 (○) received solithromycin as part of the CE01-204 protocol. Blood tests were taken as scheduled (baseline (B), days 7 (± 2), 14 (± 2) and 28) or according to clinical need. ALP concentrations were reported by the RBHT clinical biochemistry laboratory. Subjects 4, 5 and 6 received 28 days of solithromycin. Subject 1 was withdrawn (solithromycin discontinued at day 23). Upper and lower limits of normal are represented by grey shading.
Figure 7.5. Effect of solithromycin on alanine aminotransferase (ALT). Subject 1 (●), subject 4 (○), subject 5 (●) and subject 6 (○) received solithromycin as part of the CE01-204 protocol. Blood tests were taken as scheduled (baseline (B), days 7 (± 2), 14 (± 2) and 28) or according to clinical need. ALT concentrations were reported by the RBHT clinical biochemistry laboratory. Subjects 4, 5 and 6 received 28 days of solithromycin. Subject 1 was withdrawn (solithromycin discontinued at day 23). Upper and lower limits of normal are represented by grey shading.

Figure 7.6. Effect of solithromycin on aspartate aminotransferase (AST). Subject 1 (●), subject 4 (○), subject 5 (●) and subject 6 (○) received solithromycin as part of the CE01-204 protocol. Blood tests were taken as scheduled (baseline (B), days 7 (± 2), 14 (± 2) and 28) or according to clinical need. AST concentrations were reported by the RBHT clinical biochemistry laboratory. Subjects 4, 5 and 6 received 28 days of solithromycin. Subject 1 was withdrawn (solithromycin discontinued at day 23). Upper and lower limits of normal are represented by grey shading.
Figure 7.7. Effect of solithromycin on peripheral blood eosinophils. Subject 1 (●), subject 4 (○), subject 5 (○) and subject 6 (□) received solithromycin as part of the CE01-204 protocol. Blood tests were taken as scheduled (baseline (B), days 7 (±2), 14 (±2) and 28) or according to clinical need. Peripheral blood eosinophil counts were reported by the RBHT haematology laboratory. Subjects 4, 5 and 6 received 28 days of solithromycin. Subject 1 was withdrawn (solithromycin discontinued at day 23). Upper and lower limits of normal are represented by grey shading.

7.5.2.3. **Adverse effects of oral solithromycin in COPD**

Table 7.5 summarises the AEs that were reported during solithromycin treatment (subjects 1, 4, 5 and 6). No AEs were reported with placebo. Treatment was terminated at day 23 for subject 1 due to significant hepatotoxicity. This was reported as a SUSAR. All other AEs related to mild gastrointestinal symptoms. These only occurred in those patients that experienced changes in liver function, i.e. subjects 1, 5 and 6 and not 4. No cardiac, renal or neurological AEs were reported. A peripheral eosinophilia was observed in subject 1.
Table 7.5. Adverse events reported with solithromycin. Subjects 1, 4, 5 and 6 received solithromycin as part of the CE01-204 protocol. Adverse events (AE) were reported, including Serious Adverse Events (SAE) and Suspected Unexpected Serious Adverse Reactions (SUSAR).

### 7.5.2.4. Pharmacokinetics of solithromycin in COPD

PK was assessed in CE01-204. Concentrations of solithromycin and two of its metabolites (N-acetyl CEM-101 and CEM-214) were measured at 4 hours following 400 mg of oral solithromycin at days 7, 14 and 28 (Figure 7.8). The upper limit for expected solithromycin concentrations (1.15 μg/ml) was based on previous studies, which had administered solithromycin over 7 days to healthy adult subjects (Still et al., 2011). Metabolite concentrations were expected to remain below 10% of the solithromycin concentration.
(information supplied by Cempra Inc.). Subjects 1, 4, 5 and 6 received solithromycin. Subject 1 discontinued treatment at day 23 due to hepatotoxicity. The concentrations of plasma solithromycin remained within the expected range for all subjects. The only exception was a mildly raised concentration (1.2 μg/ml) at day 7 for subject 5. For subjects 5 and 6, plasma concentrations of N-acetyl CEM-101 and CEM-214 were within the expected range and remained unchanged between day 7 and 28. For subject 1 however, N-acetyl CEM-101 rose and exceeded 10% of solithromycin by day 23 (solithromycin was discontinued on this day). This was not associated with any change in CEM-214. In subject 4, N-acetyl CEM-101 modestly exceeded 10% of solithromycin at day 28, while CEM-214 rose at day 7, but returned to < 10% when next measured (day 28).
Figure 7.8. Pharmacokinetics of solithromycin in COPD: (A) Solithromycin (B) N-acetyl CEM-101 (C) CEM-214. Subject 1 (●), subject 4 (○), subject 5 (●) and subject 6 (○) received 400 mg of oral solithromycin (once daily) as part of the CE01-204 protocol. Blood samples were taken 4h after the dose on days 7 (± 2), 14 (± 2) and 28. They were not measured at baseline (B). Unscheduled samples were also taken for subjects 1 and 6 (day 23). Samples were kept on ice, centrifuged to obtain plasma and shipped to an independent bioanalytical laboratory for mass spectrometry. Subject 1 was withdrawn at day 23 (solithromycin discontinued. Expected concentrations are represented by grey shading.
7.5.3. Primary outcome measure

7.5.3.1. Sputum neutrophils

The primary outcome measure was the relative effects of solithromycin and placebo on the number of neutrophils per ml of sputum. These data are only shown for the patients who completed 28 days of treatment with solithromycin or placebo (Figure 7.9). A trend towards a reduction in sputum neutrophils was observed with solithromycin but not placebo. All patients responded similarly. Due to the small sample size, no statistical comparisons have been made.

![Figure 7.9. Effect of placebo and solithromycin on sputum neutrophils: (A) Placebo (B) Solithromycin. Subject 4 (○), subject 5 (■) and subject 6 (□) received placebo and solithromycin as part of the CE01-204 protocol. Both treatment periods were 28 days. Subject 2 (⊗) withdrew from CE01-204 and only received 28 days of placebo. Induced sputum was collected at baseline (B) and day 28 (D 28). Plugs were homogenised and cells, fixed and stained. Differential cell counts were then performed. Data presented are individual subjects.](image-url)
7.5.4. Secondary outcome measures

7.5.4.1. Measures of sputum CXCL8, IL-6, MPO, MMP-9, CCL2 and TNF-α

One of the secondary outcome measures was the relative effects of solithromycin and placebo on concentrations of CXCL8, IL-6, MPO, MMP-9, CCL2 and TNF-α in induced sputum supernatants. CE01-204 was terminated early. Therefore, the effects of placebo and solithromycin on inflammatory mediators have only been analysed for CXCL8. This is shown in Figure 7.10, for the patients who completed 28 days of either treatment. No trends were identified and due to the small sample size, no statistical comparisons were made.

![Figure 7.10. Effect of placebo and solithromycin on concentrations of sputum CXCL8: (A) Placebo (B) Solithromycin. Subject 4 (○), subject 5 (●) and subject 6 (●) received placebo and solithromycin as part of the CE01-204 protocol. Both treatment periods were 28 days. Subject 2 (⊗) withdrew from CE01-204 and only received 28 days of placebo. Induced sputum was collected at baseline (B) and day 28 (D 28). Sputum plugs were homogenised before centrifugation to obtain supernatants. Concentrations of CXCL8 were then measured using ELISA. Data presented are individual subjects.](image-url)
7.5.4.2. Measures of nasal CXCL8

Another secondary outcome measure was the relative effects of solithromycin and placebo on concentrations of CXCL8 in the nasal ELF. This is shown in Figure 7.11, for the patients who completed 28 days of either treatment. The median baseline concentrations of nasal CXCL8 (± IQR) were 3.94 (± 4.64) ng/ml and 3.48 (± 2.60) ng/ml prior to starting treatment with placebo and solithromycin respectively. These concentrations were similar to those measured in Chapter 6 for 13 COPD patients (3.81 ± 2.55 ng/ml, p = 0.69) (see Section 6.5.3). Although there was not a clear trend for placebo, treatment with solithromycin appeared to reduce nasal CXCL8 for 2 out of 3 patients. No statistical comparisons were made.

Figure 7.11. Effect of placebo and solithromycin on concentrations of CXCL8 in the nasal ELF: (A) Placebo (B) Solithromycin. Subject 4 (○), subject 5 (●) and subject 6 (∇) received placebo and oral solithromycin as part of the CE01-204 protocol. Both treatment periods were 28 days. Subject 2 (∇) withdrew from CE01-204 and only received 28 days of placebo. Nasosorption was performed at baseline (B) and day 28 (D 28). Concentrations of CXCL8 within the eluates were measured by ELISA. Data presented are individual subjects.
7.5.4.3. Measures of FEV₁

The relative effects of solithromycin and placebo on FEV₁ was another secondary outcome. This is shown in Figure 7.12, for the patients who received a full 28 days of either treatment. There were no clear trends suggesting that either solithromycin or placebo affected FEV₁ (L or % predicted). No statistical comparisons were made.

![Figure 7.12](image-url)

**Figure 7.12. Effect of placebo and solithromycin on FEV₁:** (A - B) FEV₁ (L) (C - D) FEV₁ (% predicted).

Subject 4 (○), subject 5 (○) and subject 6 (⊗) received placebo and solithromycin as part of the CE01-204 protocol. Both treatment periods were 28 days. Subject 2 (⊗) withdrew from CE01-204 and only received 28 days of placebo. Spirometry was performed at baseline (B) and day 28 (D 28) to determine FEV₁. Data presented are individual subjects.
7.5.4.4. Measures of $R_{5-20}$ (Impulse Oscillometry)

IOS is capable of directly measuring small airway calibre (i.e. $R_{5-20}$). In COPD, these airways are chronically inflamed and remodelled. The relative effects of solithromycin and placebo on $R_{5-20}$ was therefore a secondary outcome. This is shown in Figure 7.13, for the patients who completed 28 days of either treatment. There were no clear trends suggesting that either solithromycin or placebo influenced small airway calibre. As before, no statistical comparisons were made.

Figure 7.13. Effect of placebo and solithromycin on $R_{5-20}$ (small airway function). Subject 4 (○), subject 5 (○) and subject 6 (○) received placebo and solithromycin as part of the CE01-204 protocol. Both treatment periods were 28 days. Subject 2 (⊗) withdrew from CE01-204 and only received 28 days of placebo. IOS was performed at baseline (B) and day 28 (D 28) to determine $R_5$ and $R_{20}$. Data presented are individual subjects.
7.5.4.5. COPD assessment test scores

The CAT is validated to provide a measure of health status in COPD (Kon et al., 2014). As such, the relative effects of solithromycin and placebo on CAT scores was chosen as a secondary outcome. These data are shown in Figure 7.14, for the patients who completed 28 days of either treatment. There were no clear trends suggesting an effect on CAT scores. No statistical comparisons were made.

![Figure 7.14. Effect of placebo and solithromycin on COPD assessment test scores.](image)

Subject 4 (○), subject 5 (○) and subject 6 (●) received placebo and solithromycin as part of the CE01-204 protocol. Both treatment periods were 28 days. Subject 2 (●) withdrew from CE01-204 and only received 28 days of placebo. COPD assessment tests were undertaken at baseline (B) and day 28 (D 28). Data presented are individual scores.

7.5.4.6. Measures of serum biomarkers (C-reactive protein and fibrinogen)

COPD is associated with systemic inflammation and upregulated levels of serum inflammatory markers, such as C-reactive protein (CRP) and fibrinogen (Garcia-Rio et al., 2010). As such, the relative effects of solithromycin and placebo on CRP and fibrinogen were chosen as secondary
outcomes. These data are shown in Figure 7.15, for the patients who completed 28 days of either treatment. There were no clear trends suggesting an effect on CRP or fibrinogen. No statistical comparisons were made.

Figure 7.15. Effect of placebo and solithromycin on serum biomarkers: (A - B) C-reactive protein (C - D) Fibrinogen. Subject 4 (○), subject 5 (○) and subject 6 (Θ) received placebo and solithromycin as part of the CE01-204 protocol. Both treatment periods were 28 days. Subject 2 (⊗) withdrew from CE01-204 and only received 28 days of placebo. Blood samples were taken at baseline (B) and day 28 (D 28). Concentrations of C-reactive protein and fibrinogen were reported by the RBHT clinical biochemistry and haematology laboratories respectively. Data presented are individual subjects. Upper and lower limits of normal are represented by the grey shading.
7.6. Combined summary of all outcome measures

The primary and secondary outcome measures are summarised in Figure 7.16. These data are presented as mean changes in each parameter following 28 days of treatment with either placebo or solithromycin (i.e. results at baseline minus results from day 28). Due to the small sample size, no statistical comparisons have been made. However, there are trends suggesting that solithromycin (and not placebo) reduces sputum neutrophils and concentrations of CXCL8 in both sputum and nasal ELF. This might also be associated with small improvements in small airway function ($R_{5-20}$). There were no trends suggesting an effect on FEV$_1$ (L or % predicted), CRP, fibrinogen or CAT scores.
Figure 7.16. Effect of placebo and solithromycin on the primary and secondary outcomes: (A) Change in sputum neutrophils (Nφ) / ml (B) Change in concentrations of sputum CXCL8 (C) Change in concentrations of nasal CXCL8 (D) Change in FEV₁ (L) (E) Change in FEV₁ (% predicted) (F) Change in R₅₋₂₀ (G) Change in levels of CRP (H) Change in levels of fibrinogen (I) Change in CAT scores. Subjects received placebo and solithromycin as part of the CE01-204 protocol. Both treatment periods were 28 days. Primary and secondary outcome measures were subsequently analysed. Data presented are mean ± SEM for placebo (□) and solithromycin (■). n = 3-4.
7.7. Discussion

As a drug class, macrolide antibiotics are associated with hepatotoxicity, but the risk is considered to be low. Typically, this manifests as a transient, asymptomatic elevation in aminotransferases and this is observed in as few as 1 - 2% of patients (Hopkins, 1991). Oral solithromycin however, at a dose of 400 mg for 28 days, was associated with an unacceptable risk of hepatotoxicity in COPD patients. Three out of the four patients who received solithromycin in CE01-204 developed deranged liver function tests. These data are concerning, as they potentially predict a greater risk of drug-induced liver injury than treatment with telithromycin, a structurally similar ketolide. It was also the primary reason why the FDA recently refused market approval for solithromycin (Cempra, 2016, FDA, 2016). Data from CE01-204 significantly exceeds the hepatotoxic signal observed in earlier phase 1 and 2 studies, which had used shorter treatment periods (≤ 7 days). Elevated concentrations of ALT were only identified in 6.8% of these subjects (data pooled and provided by Cempra Inc.). Larger phase 3 clinical trials have also demonstrated a similarly low risk of hepatotoxicity. When solithromycin was used to treat pneumonia for 5-7 days, only 7.2% of the 856 subjects developed an ALT that was three times greater than normal (Oldach et al., 2013, Barrera et al., 2016).

Three distinct patterns of hepatotoxicity were observed in CE01-204. Subject 1 developed a cholestatic hepatitis with elevated concentrations of ALT, AST and ALP, followed by hyperbilirubinaemia, icterus and pruritus a week later. This has been reported with macrolides before, albeit very rarely (Chandrupatla et al., 2002, Maggioli et al., 2011), indicating that this event was idiosyncratic. The associated eosinophilia suggests hypersensitivity as a possible basis for this reaction and raises the possibility of prior sensitisation to structurally-related macrolides (unknown in this case). Moreover, improvements in biochemistry were only observed once solithromycin was discontinued. In contrast, subject 6 exhibited much milder
elevations in ALT, AST and ALP. Although these had also developed by day 14, there was no hyperbilirubinaemia or eosinophilia, and the biochemistry resolved despite continued dosing. This suggests hepatic adaptation, a phenomenon that is commonly described with many other drugs (Devarbhavi, 2012). Subject 5 also developed a transaminitis with elevated concentrations of ALT and AST. However, this was evident much later during dosing (at 28 days) and there were no associated rises in ALP, serum bilirubin or eosinophils. Furthermore, this transaminitis persisted for 3 days after discontinuing solithromycin, indicating that unlike in subject 6, hepatic adaptation had not occurred.

It remains unclear why the incidence of liver injury exceeded that of prior studies. One explanation might be the fundamental differences in populations. In CE01-204, the patients were considerably older with a mean age of 69.3 years, compared to 56.0 years in a phase 2 trial (Oldach et al., 2013). Furthermore, the current study solely recruited patients with COPD, a condition that is associated with significant medical comorbidity, which might increase the likelihood of hepatotoxicity. Indeed, in CE01-204, almost all of the patients had a comorbidity (83%) compared to only 60% in one phase 2 clinical trial (Oldach et al., 2013). That said, prior trials have identified as many as 62 COPD patients, who upon receiving solithromycin did not have an increased risk of liver injury, relative to other study participants (Oldach et al., 2013, Barrera et al., 2016). A low BMI, as is often the case with emphysema (Guerra et al., 2002), was also unable to explain the high incidence of hepatotoxicity in CE01-204.

Solithromycin is a potent inhibitor of CYP3A4 (Jamieson et al., 2015) raising the distinct possibility that drug interactions could have contributed to the hepatotoxicity. Subject 5 was taking 20 mg of atorvastatin. This is a HMG-CoA reductase inhibitor (statin), commonly prescribed for hypercholesterolaemia. It is metabolised by CYP3A4 and co-administration with a macrolide could therefore have resulted in accumulation (Neuvonen et al., 2006) and liver
injury (Liu et al., 2010). As such, it is possible that atorvastatin contributed to the transaminitis experienced by subject 5. Subject 1 was taking 5 mg of finasteride for benign prostatic hypertrophy. It too, is metabolised by CYP3A4 (Huskey et al., 1995). Although serum concentrations of finasteride were found to be elevated in this subject, it is equally possible that the liver injury itself (rather than solithromycin) impaired drug clearance. Furthermore, there is no evidence that finasteride induces cholestatic hepatitis and there have been no case reports of interactions with macrolides. Moreover, four other individuals received concomitant finasteride in other solithromycin studies, without such adverse effects. It therefore, seems unlikely that finasteride contributed on this occasion. Nevertheless, the potential for such a wide range of drug interactions is still concerning. This would include many medications that are used to treat COPD (e.g. xanthines) as well as associated comorbidities. Ultimately, this would significantly restrict patient suitability if solithromycin were ever marketed.

Hepatotoxicity was only observed after 7 days of treatment. It is therefore possible that inhibition of CYP3A4 by solithromycin led to the auto-inhibition of its own metabolism and a gradual accumulation of both drug and metabolites with delayed hepatotoxic consequences. Indeed, CE01-204 is the first human study to extend solithromycin treatment past 7 days. When analysing the PK data however, concentrations of solithromycin, N-acetyl CEM-101 and CEM-214 were, for the most part, within the expected range. There was a modest rise in N-acetyl CEM-101 for subject 1 but similar small increases in both N-acetyl CEM-101 and CEM-214 were also observed for subject 4, who did not experience a transaminitis. Furthermore, these data are serum concentrations and may not correlate with levels within hepatocytes, the principle site of drug accumulation in animal studies (data held by Cempra Inc.). A second, and more recent ongoing study, exposing patients with non-alcoholic steatohepatitis to 400 mg of
oral solithromycin over 13 weeks, also found an unacceptable risk of liver injury (FDA, 2016). When taken together, these data suggest that the duration of treatment (i.e. > 7 days) is the primary determinant of hepatotoxicity at this dose of solithromycin.

It is accepted that daily dosing of 400 mg solithromycin is unsafe in COPD. Nevertheless, available outcome measures were still analysed to inform any possible future studies. There were too few data for statistical comparison and no conclusions could be drawn. However, possible trends did suggest an anti-inflammatory effect. Potential reductions in both sputum neutrophils and CXCL8 were observed. These were mirrored by the concentrations of CXCL8 in the nasal ELF and not demonstrated with placebo. This might suggest that solithromycin downregulates the expression of this chemokine in vivo, with a reduction in neutrophil recruitment. This could result from an inhibition of NF-κB (Kobayashi et al., 2013b) and/or the restoration of corticosteroid sensitivity (Kobayashi et al., 2013a), as all subjects were taking an ICS. These potential anti-inflammatory actions were observed in the absence of bacterial colonisation, thereby excluding any potential confounding effect from reductions in bacterial load (i.e. airway sterilisation with an antibiotic). Furthermore, although solithromycin did not influence FEV₁, there was a trend towards an improvement in small airway calibre (i.e. R5-20), reflecting the sensitive nature of IOS.

It may be worth repeating a clinical trial of solithromycin in COPD, using a lower dose (e.g. 200 mg) and/or a less frequent dosing (e.g. three times a week). Although such a study would require more regular liver function testing and the strict avoidance of CYP3A4 substrates and inhibitors, it might demonstrate an anti-inflammatory effect without an unacceptable risk of hepatotoxicity. If CE01-204 were to be repeated, then several limitations in the current design should also be addressed. Although a crossover design facilitates a smaller study population, there is the potential for ‘carry over’ of treatment effects in patients who received
solithromycin first. This could be limited by increasing the washout duration. Furthermore, the anti-inflammatory mechanisms could be explored by isolating sputum macrophages and nasal epithelial cells to examine the levels and activity of NF-kB and HDAC2. Ideally, a stronger study design would also involve a larger patient population, treated over a longer duration. This would allow subgroup analyses to predict those patients who are most likely to respond to solithromycin. Obviously, this would not be justifiable ethically, given the current safety concerns. An alternative might be to develop and trial the solithromycin analogues that were found to be anti-inflammatory in Chapter 5. Some of these molecules had the added advantage of not being antibiotics (e.g. CEM-153, CEM-154 or CEM-232).

The hypothesis for this chapter was: ‘solithromycin is immunomodulatory in vivo and capable of downregulating airway inflammation in stable COPD patients’. This hypothesis cannot be accepted. At a daily dose of 400 mg, oral solithromycin was associated with an unacceptable risk of hepatotoxicity in COPD, resulting in the early termination of CE01-204. As a result, no conclusions could be drawn and the potential anti-inflammatory actions that were demonstrated in Chapter 5, could not be confirmed in vitro.
CHAPTER 8

General Discussion
8.1. Summary of key findings

The hypothesis for this thesis was: ‘Bacterial phagocytosis by alveolar macrophages is defective in COPD, results from unstable microtubule polymers and can be augmented by solithromycin, a novel macrolide with immunomodulatory properties’. After considering all of the data, this hypothesis can only be partially accepted. The following summarises the key findings:

- Using MDM, bacterial phagocytosis was defective in COPD (H. influenzae and S. pneumoniae).
- Defective bacterial phagocytosis in COPD was associated with a predominance of unstable microtubules; polymers within macrophages were more susceptible to disassembly and contained less acetylated tubulin.
- Solithromycin did not augment defective bacterial phagocytosis. It was immunomodulatory however, and inhibited the release of TNF-α from LPS-stimulated MDM, derived from non-smokers.
- The above finding supported previously published data (Kobayashi et al., 2013a, Kobayashi et al., 2013b) and formed the rationale for a clinical trial. Solithromycin however, was associated with an unacceptable risk of hepatotoxicity and the trial was terminated early, before the outcome measures could be assessed.
- A novel sampling technique was developed to facilitate the in vivo study of inflammation in COPD. Nasosorption was used to sample the nasal ELF as a surrogate for the bronchi. This demonstrated that nasal CXCL8 is not only upregulated in COPD but also correlates with bronchial concentrations.

These data, and other important findings, will now be closely examined.
8.2. **MDM are required to study phagocytosis in COPD**

Several studies have demonstrated defective bacterial phagocytosis in COPD (Berenson et al., 2006, Marti-Lliteras et al., 2009, Taylor et al., 2010, Hodge and Reynolds, 2012, Berenson et al., 2013). It was not possible however, to confirm these findings within this thesis when using AM isolated from BALF. Although trends did favour a defect, power calculations suggested that significantly more samples would be required for statistical significance. Given the challenges in recruiting volunteers and obtaining sufficient cells from BALF, it was judged impractical to undertake this number of bronchoscopies. Indeed, Chapter 3 highlighted that there is a poor recovery of BALF and/or AM from both COPD patients with advanced disease and non-smoking controls, with over half of potentially-suitable subjects reluctant to consent, fearing the invasive nature of bronchoscopy and potential for harm. Furthermore, there is a sampling bias associated with using AM, as bronchoscopies are infrequently undertaken in patients with severe disease ($\text{FEV}_1 < 50\%$). This may have partly explained why defective bacterial phagocytosis was not demonstrated with AM, as the magnitude of this defect may correlate with airflow obstruction (Berenson et al., 2013).

MDM are a validated model of AM (Burgess and Metcalf, 1980, Akagawa et al., 2006, Winkler et al., 2008) and were therefore adopted by this thesis. Of particular relevance, they express similar cell surface receptors, demonstrate an equivalent capacity for phagocytosis (Akagawa et al., 2006, Winkler et al., 2008) and internalise both inert particles and bacteria, using the phagocytic pathways exhibited by AM (Taylor et al., 2010). Furthermore, defective phagocytosis is replicated in COPD when using MDM (Taylor et al., 2010), including the dichotomous results observed with beads and bacteria. MDM are differentiated from peripheral blood monocytes. As such, sampling is minimally invasive, relatively risk free, potentially favourable to research volunteers and could be undertaken in severe COPD. In
summary, MDM avoid many of the previously-identified limitations associated with the use of AM.

Until now, no study has directly compared phagocytosis by individually-paired AM and MDM. Correlations presented in Chapter 3 (Section 3.5.3) are the first to demonstrate a potential relationship between the phagocytic capacity of these two macrophage populations. This was regardless of prey, with positive correlations observed for inert particles, Gram-negative and Gram-positive bacteria. Although these findings were limited by sample size and therefore unable to demonstrate independent correlations for each of the subject groups, they still lend further support to the use of MDM to study phagocytosis. Furthermore, assays with AM revealed positive correlations between the phagocytosis of different prey (Sections 3.5.2.5 and 3.5.2.6), including the internalisation of *H. influenzae* and *S. pneumoniae*. This suggests that phagocytic responses are not specific to bacterial strain; another characteristic that was shared by MDM (Chapter 4; Section 4.5.2). Clearly MDM are a suitable surrogate. However, findings were still validated in AM whenever possible, to provide conclusive evidence.

### 8.3. Bacterial phagocytosis is defective in COPD

Using MDM allowed a greater number of assays to be undertaken and provided adequate statistical power. It was subsequently confirmed that macrophages from COPD patients are indeed defective in bacterial phagocytosis with an approximate 50% reduction in the uptake of heat-killed bacteria, relative to non-smokers. This was observed for both *H. influenzae* and *S. pneumoniae*, and could not be explained by any demographic variable or the use of medication. Given that MDM are cultured from circulating monocytes that have never been directly exposed to noxious stimuli within the lung, defective bacterial phagocytosis is therefore most likely to be inherent to the monocyte-macrophage lineage, rather than being
acquired by AM. Alternatively, the monocytes could have been influenced by systemic inflammation or oxidative stress (Agusti et al., 2012, Zeng et al., 2013) with subsequent differentiation unmasking the defect (Taylor et al., 2010).

Defective bacterial phagocytosis is reproducible in COPD. It has been replicated with MDM (Taylor et al., 2010, Hodge and Reynolds, 2012), validated in AM (Berenson et al., 2006, Marti-Lliteras et al., 2009, Taylor et al., 2010, Berenson et al., 2013) and demonstrated for a multitude of bacterial species and non-bacterial prey, such as A. fumigatus (Belchamber et al., 2016) and C. albicans (Ferrara et al., 1996, Vecchiarelli et al., 1991). This defect is also unlikely to be an artefact of the experimental methodology employed by this thesis, as similar findings have been reported with live bacteria (Taylor et al., 2010, Berenson et al., 2006, Berenson et al., 2013), with or without complement opsonisation (Berenson et al., 2013) or when using different laboratory techniques, such as flow cytometry (Hodge and Reynolds, 2012).

8.4. Less stable microtubules predominate in COPD

MDM were also used to study the underpinning mechanism(s) of defective bacterial phagocytosis. Until now, this has remained unclear. Microtubules were chosen as the focus of this study, as they are of particular importance to SR and the phagocytosis of non-opsonised prey (Newman et al., 1991, Allen and Aderem, 1996, Sulahian et al., 2008). Due to the relative lack of serum in the lung, such microtubule-dependent pathways are likely to predominate (Donnelly and Barnes, 2012). Furthermore, any significant contributions from a dysfunctional actin cytoskeleton and/or differential receptor expression have been excluded previously (Taylor et al., 2010). Indeed, fully functional actin microfilaments are essential for the internalisation of all prey (Aderem and Underhill, 1999) and any defect might be expected to result in a complete failure of both bacterial and bead uptake, rather than a selective 50%
reduction in bacterial phagocytosis. This has been observed by previous PhD students when examining the effects of an actin microfilament inhibitor, cytochalasin D, on phagocytosis (Taylor, 2009, Thomas, 2012).

Two markers of microtubule stability were used to demonstrate for the first time that less stable polymers predominate in COPD macrophages. These cells contained less acetylated tubulin and bacterial phagocytosis was more sensitive to the effects of nocodazole-induced depolymerisation (relative to non-smokers). There were obvious limitations associated with these data, including the small sample size and a lack of acetylated tubulin levels from AM. Nonetheless, such complementary findings and their partial validation in AM, still provides evidence that unstable polymers do predominate in COPD. Furthermore, these data correspond to the phagocytic responses of non-smokers, smokers and COPD patients. Specifically, differences in bacterial phagocytosis, associated nocodazole sensitivity and levels of acetylated tubulin were only found between COPD patients and non-smokers (but not smokers). This thesis therefore confidently proposes that the microtubule population within AM is skewed towards less stable polymers in COPD, which rapidly depolymerise, less frequently associate with MAPs, and are unable to provide the necessary cytoskeleton for effective bacterial phagocytosis.

8.5. Possible mechanism for microtubule instability in COPD

In the developed world, tobacco smoking is the commonest risk factor for COPD. It directly affects leukocytes, promotes inflammation and contributes to oxidative stress, both locally and systemically (Agusti, 2007, Zeng et al., 2013, Barnes, 2014). As such, smoking might be important in the aetiology of defective bacterial phagocytosis. This thesis however, did not demonstrate any association between the smoking habits of individual COPD patients (i.e.
smoking status or tobacco exposure (pack years)) and the magnitude of defective phagocytosis. Furthermore, no associations were identified between smoking and microtubule stability, when levels of acetylated tubulin were correlated with pack years. Although, one might conclude that smoking is therefore not important in the aetiology of microtubule instability and defective bacterial phagocytosis, these data could equally reflect the underlying self-perpetuating nature of inflammation and/or oxidative stress in COPD, both of which persist despite smoking cessation (Gamble et al., 2007, Louhelainen et al., 2009). In other words, tobacco smoke may initiate a less stable microtubule population, but continued exposure to inflammation/oxidative stress maintains this polymer instability, allowing defective phagocytosis to endure smoking cessation.

To clarify whether cigarette smoke plays a role in either microtubule instability or defective bacterial phagocytosis, further work is required to compare results from smokers without airflow obstruction to those from non-smoking controls and COPD patients. This was not possible, due a wide variability in data from smoking subjects, which led to statistical underpowering. This most likely reflected the heterogenous nature of this group and/or an over-reliance on one-off measures of spirometry to differentiate smokers from COPD patients with mild airflow obstruction (Aaron et al., 2017).

Oxidative stress is not only implicated in the pathogenesis of COPD but is also a feature of several neurodegenerative diseases (Wilson and Gonzalez-Billault, 2015, Penazzi et al., 2016), myocardial infarction (Drum et al., 2016) and acute respiratory distress syndrome (Kratzer et al., 2012). Research into microtubule structure and function within these conditions has repeatedly identified a role for oxidative stress in the generation of polymer instability with important consequences for neurones (Gardiner et al., 2013), myocytes (Drum et al., 2016) and epithelial cells alike (Kratzer et al., 2012). This is thought to be mediated through aberrant
post-translational modification of tubulin (Iqbal et al., 2009). Indeed, akin to COPD, tubulin acetylation is reduced in Alzheimer’s disease (Zhang et al., 2015) and Huntington’s chorea (Dompierre et al., 2007). It is therefore possible that mitochondrial dysfunction, ROS overproduction and/or an imbalance in pro- and antioxidants might influence microtubules within circulating monocytes and AM from COPD patients. This warrants further investigation (Section 8.9.3).

Microtubules bind and interact with a plethora of MAPs, which carry out a variety of different functions, including the stabilisation and destabilisation of the polymers (Itoh and Hotani, 1994). Moreover, there is evidence from several neurodegenerative conditions that oxidative stress influences MAPs via downregulation, proteolysis or phosphorylation (Kaindl et al., 2006, Grand et al., 2014). It is therefore plausible that defects in MAPs might arise in COPD from oxidative stress and contribute to microtubule instability. This theory has not been investigated in the literature before. The expression and function of MAPs should therefore be explored in COPD and under conditions of oxidative stress. αTAT1 represents a logical target for these studies (Section 8.9.3). It is a recently identified MAP that has been implicated in both the acetylation of α-tubulin and the stabilisation of microtubule polymers (Al-Bassam and Corbett, 2012, Shida et al., 2010).

8.6. The phagocytosis of beads is not defective in COPD

In contrast to bacterial phagocytosis, the uptake of inert beads was not defective in COPD. This has been reported before (Taylor et al., 2010) and suggests that their internalisation is mediated via alternative phagocytosis pathways, which are initiated by different cell surface receptors and/or less reliant on microtubule polymers. The latter is supported by data presented in Chapter 4, which demonstrated that the phagocytosis of beads and bacteria differ
following microtubule depolymerisation. Specifically, nocodazole appeared to have less of an inhibitory effect on the internalisation of beads, with no differences identified between subject groups. A stronger argument could have been made had lower concentrations of nocodazole been used to identify the linear portion of the dose-response curve, with similar EC$_{50}$ values subsequently calculated for COPD patients and controls.

8.7. Clinical implications

8.7.1. Defective phagocytosis might contribute to COPD progression

AM account for the vast majority of airway leukocytes (Harris et al., 1970) and are the primary phagocytes (Martin and Frevert, 2005). Moreover, the bacterial prey chosen for this thesis were specific respiratory pathogens (PPB) that commonly colonise COPD airways (Rosell et al., 2005) and frequently trigger acute exacerbations (Sethi and Murphy, 2008). The phagocytosis assays were also performed without opsonins, in order to resemble the AM-pathogen interaction within the ‘serum-free’ airways (Donnelly and Barnes, 2012). As such, the observed defective bacterial phagocytosis is very likely to have important clinical consequences. Specifically, the inability of AM to clear \textit{H. influenzae} and \textit{S. pneumoniae} might permit airway colonisation, promote bacterial dysbiosis and contribute to exacerbations, thereby perpetuating inflammation (Hill et al., 2000, Banerjee et al., 2004b, Sethi et al., 2006, Marin et al., 2010, Marin et al., 2012, Singh et al., 2014) and disease progression (Wilkinson et al., 2003).

To test the above hypothesis, associations were sought between the magnitude of defective phagocytosis and COPD severity. It can be challenging to quantify disease severity (Jones, 2009) and several different markers were therefore used, including FEV$_1$, symptom burden (mMRC and CAT scores), smoking status, frequent \textit{versus} infrequent exacerbations and the combined GOLD assessment (a combination of the aforementioned parameters). However, no
statistically significant associations were identified. This may be evidence that defective bacterial phagocytosis is not important in the pathogenesis of COPD, bacterial colonisation/dysbiosis or exacerbations. However, these data were limited by sample size and there was an uneven distribution of ‘severity’ within the cohort; all patients displayed moderate/severe airflow obstruction, most reported mMRC scores of ≥ 2 or CAT scores ≥ 10, and over half were assigned to GOLD group D. Furthermore, other innate and adaptive immune responses are defective and likely to be important in permitting bacterial colonisation (Shaykhiev and Crystal, 2013, Bhat et al., 2015). As such, a close linear relationship between phagocytic capacity and COPD severity is unlikely.

8.7.2. Solithromycin does not augment bacterial phagocytosis

AM from COPD patients are capable of an equivalent uptake of beads when compared to control subjects. They are therefore not completely dysfunctional as phagocytes, suggesting that defective bacterial phagocytosis can be augmented pharmacologically. Such an approach could prove to be disease-modifying, as potential reductions in bacterial load and associated inflammation are to be expected. The regular administration of macrolides to COPD patients reduces the frequency of exacerbations (Donath et al., 2013). Although this could directly result from antibiotic actions, not all exacerbations are bacterial in origin (Wedzicha and Seemungal, 2007) and macrolides are proven to be potent immunomodulators (Kanoh and Rubin, 2010) with possible effects on bacterial phagocytosis (Hodge et al., 2008). The possibility that macrolides might augment defective bacterial phagocytosis was therefore formally investigated by this thesis. There are concerns that the long-term administration of currently available macrolide antibiotics could promote microbial resistance (Serisier, 2013). Therefore, it was decided to simultaneously examine the effects of solithromycin, a novel
fluoroketolide with a lower potential for bacterial resistance (McGhee et al., 2010, Rodgers et al., 2013).

Despite demonstrating both antibiotic and anti-inflammatory actions in vitro, solithromycin did not augment phagocytosis, including the uptake of bacteria by MDM from COPD patients. This was despite using maximal concentrations (greater concentrations significantly impaired MDM viability), which were physiological, i.e. similar to those achieved within the pulmonary ELF after oral administration (Rodvold et al., 2012). Moreover, no effect was observed with similar concentrations of azithromycin. The sample size was very small however, and only a single incubation timepoint was adopted. The latter is relevant as macrolides are immunomodulators and therefore capable of inducing multiphase effects on leukocytes at different time points in vitro (Culic et al., 2002, Shinkai et al., 2006) or during prolonged administration in vivo (Konno et al., 1992, Parnham et al., 2005). Nonetheless, the presented data are convincing for a lack of effect by this class of drugs and it seems very unlikely that macrolides, ketolides or fluoroketolides, would directly augment bacterial phagocytosis in vivo. Although related compounds, with significantly large macrocyclic lactone rings, are capable of stabilising microtubules (Mitsui-Saito et al., 2002, Field et al., 2009), there is no evidence that macrolide antibiotics per se, have similar effects. Furthermore, the in vivo augmentation of bacterial phagocytosis reported by Hodge et al. (2008) and the association between azithromycin use and an increased uptake of S. pneumoniae (Section 4.5.5.3), could both be explained by indirect mechanisms that had secondarily influenced AM function (e.g. airway sterilisation or via effects on other leukocytes).

8.7.3. Targeting microtubule instability to improve bacterial phagocytosis

Restoring polymer stability in AM may augment bacterial phagocytosis in COPD. It therefore represents a potentially novel approach to tackling airway colonisation and associated
inflammation. ‘Microtubule stabilisers’ are already commercially available as chemotherapy agents (Stanton et al., 2011). Their effect on AM phagocytosis could be trialled in COPD. However, microtubules are ubiquitous and involved in a variety of other important cellular functions. As such, any potential benefits are likely to be offset by the ‘indiscriminate’ stabilisation of microtubules and resultant harmful side-effects. Although, this might be avoided through directed drug administration (i.e. inhaled), further characterising the pathophysiology and/or localising the specific polymer defect(s) (e.g. dysfunctional MAPs), would identify a more focused target for pharmacotherapy. As previously suggested, oxidative stress could prove to be important in destabilising the microtubule population. Indeed, there is some evidence that anti-oxidants promote bacterial phagocytosis in COPD (Harvey et al., 2011) and this might account for the reduction in exacerbation frequency that is observed during their oral administration (Cazzola et al., 2015, Fowdar et al., 2017). This is preferable to toxic chemotherapy and could be investigated further in vitro, using MDM (Section 8.9.3).

It remains to be seen how effective microtubule stabilisation could be, both in vitro (AM phagocytosis) and ultimately clinically. Nocodazole activates intrinsic GTPase activity (Lin and Hamel, 1981, Mejillano et al., 1996) and was expected to induce microtubule ‘catastrophe’ at the micromolar concentrations used in this thesis (De Brabander et al., 1976, Cannon and Swanson, 1992). However, it only partially inhibited bacterial phagocytosis under these experimental conditions, in both control subjects and COPD patients. This either indicates that a significant proportion of microtubules remain stable in COPD and therefore resistant to disassembly, or suggests equally important roles for microtubule-independent pathways in the uptake of bacteria.
8.7.4. Solithromycin as an anti-inflammatory in COPD

Solithromycin shares many of the immunomodulatory properties demonstrated by other macrolide antibiotics (Kobayashi et al., 2013a, Kobayashi et al., 2013b) but with a limited potential for microbial resistance (McGhee et al., 2010, Rodgers et al., 2013). It was therefore considered as a possible novel anti-inflammatory treatment for COPD. Immunomodulation was firstly confirmed in vitro, when physiologically-relevant concentrations were shown to inhibit the release of TNF-α from LPS-stimulated MDM. Although these experiments were limited to MDM from non-smokers and only a single mediator was analysed, they do still complement previously published data, which strongly suggest that solithromycin would benefit COPD patients in vivo, by directly downregulating inflammatory pathways and restoring corticosteroid sensitivity (Kobayashi et al., 2013b, Kobayashi et al., 2013a).

Unfortunately, when oral solithromycin (400 mg daily for 28 days) was administered to a small group of COPD patients, it was associated with an unacceptable risk of severe hepatotoxicity. It is therefore an unsuitable treatment at this dose/dosing frequency. This could not have been predicted from earlier phase 1 and 2 studies (Oldach et al., 2012) or from two recent trials in community-acquired pneumonia (Oldach et al., 2013, Barrera et al., 2016), which had safely administered 400 mg daily, albeit for shorter durations. The hepatic signal observed in CE01-204 most likely resulted from an accumulation of solithromycin and/or metabolites within hepatocytes, which could have been mediated by auto-inhibition of its own metabolism (Jamieson et al., 2015) and prolonged drug exposure. Even if a lower dose (e.g. 200 mg) and/or less frequent dosing interval (e.g. 3 times a week) were both safe and effective, solithromycin is still hindered by a number of potential drug interactions, including commonly prescribed medication, such as statins. Outside of a clinical trial, this would frequently preclude its use in a multimorbid COPD population, where concomitant prescribing is commonplace.
8.7.5. **CEM-153, CEM-154 and CEM-232 as anti-inflammatories in COPD**

Concerns over the long-term use of oral solithromycin should not be confined to its potential for hepatotoxicity and/or drug interactions. There is also a theoretical risk of promoting bacterial resistance to earlier generations of macrolide (*i.e.* those that share common ribosomal binding sites). Furthermore, data from within this thesis has suggested that solithromycin might inhibit, rather than augment, bacterial phagocytosis. *In vitro*, this could offset any anti-inflammatory effects by promoting bacterial colonisation/dysbiosis. Therefore, it may be preferential to develop and trial CEM-153, CEM-154 and CEM-232. These solithromycin analogues do not appear to have significant antibacterial actions and may not even bind to the bacterial ribosome. Yet they remain capable of immunomodulation. Indeed, all three compounds inhibited the release of TNF-α from LPS-stimulated MDM, without any effects on the growth of *S. pneumoniae* or *M. catarrhalis*. Results were most promising for CEM-232, which had a similar efficacy to solithromycin and potentially greater potency than any of the other analogues. These data can only be considered preliminary however, and further work is certainly required (*Section 8.9.4*). This should be prioritised.

8.7.6. **Sinonasal inflammation and symptoms in COPD**

An intimate link between upper and lower airway inflammation is established for asthma and often referred to as the ‘one airway, one disease’ or ‘united airways’ concept (Grossman, 1997). Sinonasal inflammation is common in asthmatics, who have a high incidence of allergic or nonallergic chronic rhinitis, rhinosinusitis and nasal polyposis (Montnemery *et al.*, 2001). Asthma guidelines acknowledge the importance of this co-existing sinonasal disease and recommend its concomitant treatment (Bateman *et al.*, 2008, Brozek *et al.*, 2010, Reddel *et al.*, 2015). Indeed, the use of intranasal steroids not only leads to fewer nasal symptoms but is also associated with improvements in asthma control (Lohia *et al.*, 2013), including a reduction
in asthma-related emergency attendances and hospitalisation (Adams et al., 2002). It is less well appreciated, but COPD is also associated with a high incidence of chronic sinonasal symptoms (Montnemery et al., 2001, van Manen et al., 2001, Chen et al., 2003) and this was confirmed in a small non-atopic cohort within this thesis. Such symptoms correlate with pulmonary symptoms (Roberts et al., 2003), deteriorate during acute exacerbations (Huerta et al., 2015) and are a source of significant morbidity (Hurst et al., 2004).

It seems most likely that exposure of the sinonasal epithelium to noxious substances within tobacco smoke activates a chronic inflammatory process, that may be analogous to pulmonary inflammation in COPD (Hurst, 2010, Hurst et al., 2006b, Hurst et al., 2005, Hox et al., 2015). Indeed, this thesis did identify an increased concentration of CXCL8 in the nasal ELF of COPD patients. Potential underlying mechanisms were not explored further however, and a detailed clinical assessment of the upper respiratory tract was not performed (Section 8.9.5). Regardless, these data still highlight the importance of screening for nasal symptoms in COPD. Treating any underlying inflammation may also prove to downregulate sinonasal CXCL8 in COPD, with improvements in nasal blockage, discharge and postnasal drip (Callebaut et al., 2013), or possibly even cough and other respiratory symptoms (Callebaut et al., 2013, Calabrese et al., 2016).

### 8.7.7. Potential clinical uses for nasosorption

Nasosorption was used for the first time to sample the nasal ELF in stable COPD. CXCL8 was detected in the resulting supernatants and found to be upregulated, confirming data from previous studies that had used nasal lavage to demonstrate increased concentrations of this important chemokine (Hurst et al., 2005). Importantly, these upregulated concentrations were also reproducible in COPD, when comparing results from different patient cohorts across both Chapter 6 and 7. Moreover, concentrations of nasal CXCL8 were representative of bronchial
CXCL8 and levels were stable over time, providing a consistent baseline against which any acute changes (e.g. exacerbation) or clinical interventions could be assessed.

These data are preliminary however. They are limited by sample size, the lack of a mechanistic study and only a single inflammatory mediator was explored. Further work is therefore required, including a detailed characterisation of the nasal inflammatory profile in COPD (Section 8.9.5). Nonetheless, nasosorption is cheap, simple to perform, minimally invasive and safe. Unlike nasal lavage, it is not reliant on the adequate recovery of fluid, there are no potential issues with unknown or excessive dilution factors and it is practical to perform in a consultation room. As such, it is a promising research tool that could also be used clinically to personalise COPD management. It could have roles in diagnosis (including the identification of ‘at risk’ smokers), phenotyping, predicting or assessing treatment responses (e.g. steroid responsiveness), the earlier detection of exacerbations and differentiating bacterial from viral infections. Indeed, data presented herein has already demonstrated that nasal CXCL8, as measured by nasosorption, might be capable of differentiating frequent and infrequent exacerbators.

8.8. Conclusion

This thesis was a bench to bedside investigation into defective innate immunity in COPD. Time spent at the laboratory bench has provided data to support the use of MDM to study AM phagocytosis and demonstrated that this important macrophage function is defective in COPD; most likely as a result of a microtubule population that is skewed towards instability. Furthermore, it has shown that macrolide antibiotics do not directly augment defective bacterial phagocytosis, but might still potentially act as anti-inflammatories through other immunomodulatory pathways. This property was shared by solithromycin and its analogues
CEM-153, CEM-154 and CEM-232, which were seemingly less active as antibiotics and could therefore have a lower potential for microbial resistance. Time spent with research volunteers has identified an increased prevalence of nasal symptoms in COPD and permitted the development of a novel sampling technique, with the potential to provide a surrogate measure of pulmonary inflammation (i.e. nasosorption). Nasosorption was utilised in a clinical trial assessing the anti-inflammatory potential of oral solithromycin. However, this novel fluoroketolide was found to be hepatotoxic in COPD at a dose of 400 mg daily.

These findings have important clinical implications that should be investigated further. They could also inform the methodology and/or design of future research projects.

8.9. Future Work

A number of important questions remain unanswered and further work could strengthen the arguments proposed by this thesis. In particular, there was a high degree of variability in data obtained from smokers. Augmenting the sample size could overcome this limitation and allow statistical comparisons to better understand the role of cigarette smoking in defective bacterial phagocytosis, microtubule stability and sino-nasal inflammation. Summarised below are several other investigations that could supplement the data presented in Chapters 3 - 7.

8.9.1. Future work to supplement Chapter 3

- Increase the sample size to: (i) determine whether the responses of paired AM and MDM correlate within each individual subject group (ii) determine whether the phagocytosis of different prey (e.g. *H. influenzae* vs *S. pneumoniae*) correlate for both AM and MDM (iii) confirm that bacterial phagocytosis by AM is defective in COPD.
8.9.2. Future work to supplement Chapter 4

- Confirm defective bacterial phagocytosis in COPD using live bacteria and further characterise this defect by: (i) using a wider range of bacterial and non-bacterial prey (ii) using pHrodo™-labelled bacteria to identify any differences in phagolysosomes formation (iii) using killing assays to identify any differences in bacterial elimination.

- Examine whether defective bacterial phagocytosis might contribute to obstructive bronchiolitis by correlating RFU with more sensitive markers of small airway function (i.e. Rs 5-20).

- Determine whether defective bacterial phagocytosis is important in regulating airway colonisation/dysbiosis by comparing phagocytosis assays from COPD patients with and without bacteria in induced sputum or BAL. The internalisation of specific bacterial species (i.e. RFU) could then be correlated with the quantity of bacteria within these respiratory samples using qPCR.

- Compare levels of acetylated tubulin in AM from non-smokers, smokers and COPD patients.

- Pre-treat macrophages with microtubule-stabilising agents (e.g. taxol or epothilone B) to determine whether bacterial phagocytosis can be augmented. Results from non-smokers, smokers and COPD could be compared (i.e. EC50 values).

- Undertake phagocytosis assays with lower concentrations of nocodazole to potentially identify the linear portion of concentration-response curves for bead phagocytosis, comparing EC50 values from non-smokers, smokers and COPD patients.

- Use Tubulin Tracker™ and confocal microscopy to: (i) confirm microtubule depolymerisation in the presence of nocodazole (ii) identify the relative presence of...
polymerised microtubules in macrophages from COPD patients and controls (unstimulated and after the addition of prey)

- Further characterise microtubule instability in COPD and identify any potential underlying mechanisms by: (i) examining the expression and function of αTAT1 (ii) examining the effects of oxidative stress on microtubule polymers and phagocytosis (e.g. by pre-treating MDM with hydrogen peroxide) (iii) examining the effects of anti-oxidants (e.g. N-acetylcysteine) on microtubule stability and phagocytosis.

8.9.3. Future work to supplement Chapter 5

- Perform time-courses with all the macrolide compounds to ensure that any immunomodulatory effects were not missed by selecting single time points (i.e. for phagocytosis and TNF-α release).
- Improve the sample size and adopt a lower range of drug concentrations to potentially identify the linear portions of TNF-α inhibition and enable statistical comparisons of potency (i.e. EC50 values).
- Screen CEM-153, CEM-154 and CEM-232 for effects on bacterial phagocytosis and identify MICs against a wider range of bacteria.
- Further investigate the effects of macrolides, solithromycin and associated analogues on macrophage function including: (i) TNF-α release by LPS-stimulated cells from COPD patients (ii) effects on other cellular functions, including the release of other mediators that are regulated by NF-κB (e.g. CXCL8).

8.9.4. Future work to supplement Chapter 6

- Identify the mechanisms for upregulated expression of sinonasal CXCL8 by performing concurrent nasosorption and nasal epithelial microcurettage, and analysing the samples
for CXCL8 and NF-kB activation respectively. Results could be compared between COPD patients and controls.

- Further characterise sinonasal inflammation and disease in COPD by: (i) using multiplex ELISA to analyse supernatants for a wider range of inflammatory and anti-inflammatory mediators; comparing results between COPD patients and controls, and correlating levels from nasal and bronchial samples (ii) comparing results from stable and exacerbated patients (iii) comparing results from different COPD phenotypes (iv) correlating levels of mediators with sensitive markers of small airway obstruction (i.e. Rs-20) (v) using a larger COPD cohort and identifying sinonasal disease and specific pathology with rhinomanometry, acoustic rhinometry, sinus CT and/or nasal endoscopy.

- Examine the effects of topical treatment on sinonasal symptoms and inflammation in COPD (e.g. intranasal steroids).

- Use nasosorption as a surrogate for bronchial inflammation in future studies.

### 8.9.5. Future work to supplement Chapter 7

- Consider undertaking a further clinical trial of solithromycin, but using a lower dose (200 mg) and less frequent dosing interval (three times a week) with regular liver function monitoring, the strict avoidance of any CYP3A4 substrates/inducers/inhibitors, and the exploration of any potential anti-inflammatory mechanisms (e.g. NF-kB and/or HDAC activity).

- Alternatively, develop the solithromycin analogues CEM-153, CEM-154 and CEM-232 by urgently undertaking pre-clinical studies to determine their bioavailability, toxicity and metabolism. This would be preferable to another trial with solithromycin.
REFERENCES


References


Matera, M. G., Calzetta, L. & Cazzola, M. 2010. TNF-alpha inhibitors in asthma and COPD: we must not throw the baby out with the bath water. Pulm Pharmacol Ther, 23, 121-8.


References


APPENDICES

Appendix A, B, C, D and E
Appendix A: Modified Medical Research Council (mMRC) Dyspnoea Scale

<table>
<thead>
<tr>
<th>Grade</th>
<th>Severity of Dyspnoea</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Breathless with strenuous exercise</td>
</tr>
<tr>
<td>1</td>
<td>Short of breath when hurrying on level ground or walking up a slight hill</td>
</tr>
<tr>
<td>2</td>
<td>Walk slower than people of the same age on level ground because of breathlessness; or stop for breath when walking at own pace on the level</td>
</tr>
<tr>
<td>3</td>
<td>Stop for breath after walking about 100 metres (or few minutes) on level ground</td>
</tr>
<tr>
<td>4</td>
<td>Too breathless to leave the house or breathless when dressing or undressing</td>
</tr>
</tbody>
</table>

The modified Medical Research Council (mMRC) Dyspnoea Scale. Breathlessness can be graded according the mMRC scale. Adapted from Fletcher (1952).
Appendix B: The COPD Assessment Test™ (CAT)

This questionnaire will help you and your healthcare professional measure the impact COPD (Chronic Obstructive Pulmonary Disease) is having on your wellbeing and daily life. Your answers and test score, can be used by you and your healthcare professional to help improve the management of your COPD and get the greatest benefit from treatment.

For each item below, place a mark (X) in the box that best describes you currently. Be sure to only select one response for each question.

Example: I am very happy [X] 2 3 4 5 I am very sad

I never cough [ ] 0 1 2 3 4 5 I cough all the time

I have no phlegm (mucus) in my chest at all [ ] 0 1 2 3 4 5 My chest is completely full of phlegm (mucus)

My chest does not feel tight at all [ ] 0 1 2 3 4 5 My chest feels very tight

When I walk up a hill or one flight of stairs I am not breathless [ ] 0 1 2 3 4 5 When I walk up a hill or one flight of stairs I am very breathless

I am not limited doing any activities at home [ ] 0 1 2 3 4 5 I am very limited doing activities at home

I am confident leaving my home despite my lung condition [ ] 0 1 2 3 4 5 I am not at all confident leaving my home because of my lung condition

I sleep soundly [ ] 0 1 2 3 4 5 I don’t sleep soundly because of my lung condition

I have lots of energy [ ] 0 1 2 3 4 5 I have no energy at all

COPD Assessment Test and the CAT logo is a trade mark of the GlaxoSmithKline group of companies. © 2009 GlaxoSmithKline group of companies. All rights reserved. Last Updated: February 24, 2012
Appendix C: Impulse Oscillometry in Non-Smokers and COPD Patients

Impulse Oscillometry was validated in COPD. It was performed in 10 COPD patients, as described in Section 2.2.2.4, and results were compared to those obtained from 8 age-matched non-smokers (Figure I). Measures of $R_{5r}$, $R_{20}$, $R_{5-20}$, $X_{5r}$, AX and RF were statistically different.

$R_{5-20}$ (a surrogate of small airway obstruction) was also correlated with spirometric measures of airflow obstruction in 18 COPD patients, including FEV$_1$ (% predicted) and MEF$_{25-75}$ (mid-expiratory flow; a marker of smaller airway narrowing) (Figure II). Correlations were observed between $R_{5-20}$ and both FEV$_1$ % predicted and MEF$_{25-75}$. 
Figure I. Comparison of impulse oscillometry results from non-smokers and COPD patients: (A) $R_5$ (B) $R_{20}$ (C) $R_{5-20}$ (D) $X_5$ (E) AX (F) RF. IOS was performed with COPD patients ($n = 10$) and age-matched non-smokers ($n = 8$). Measures of $R_5$, $R_{20}$, $R_{5-20}$, $X_5$, AX and RF were recorded. Data presented are median ± IQR. *** represents $p < 0.001$, ** represents $p < 0.01$, * represents $p < 0.05$ relative to non-smokers.
Figure II. Correlations between $R_{5-20}$ and spirometry (airflow obstruction): (A) $FEV_1$ (% predicted) (B) $MEF_{25-75}$. IOS was performed with COPD patients ($n = 18$) and correlated with paired spirometric measures of $FEV_1$ (% predicted) and $MEF_{25-75}$. Data presented are Spearman rank correlation coefficients.
Appendix D: Concomitant Prescribing in CE01-204

Guidance was provided from Cempra Pharmaceuticals, regarding concomitant prescribing in CE01-204. Patients who were prescribed drugs known to prolong the QT interval and/or drugs, foods or herbal products known to be moderate to potent inhibitors/inducers of CYP3A4 isozymes were not enrolled. Patients were also excluded if they were prescribed medications with narrow therapeutic indices that are principally metabolized by CYP3A4 or transported by P-glycoprotein (i.e. a drug interaction with solithromycin might result in higher and possibly unsafe exposures to these drugs). The following lists were used as a guideline but are not exhaustive:

Drugs known to prolong the QT interval:

- Class Ia (quinidine, procainamide)
- Class III (amiodarone, sotalol)

Drugs, foods or herbal products known to be moderate to potent inhibitors of CYP3A4:

- Oral antifungal agents (e.g. ketoconazole, itraconazole, posaconazole, fluconazole and voriconazole)
- HIV protease inhibitors (e.g. ritonavir and saquinavir)
- HCV protease inhibitors (e.g. boceprevir and telaprevir)
- Nefazodone
- Fluvoxamine
- Conivaptan
- Diltiazem
- Verapamil
Appendices

- Aprepitant
- Ticlopidine
- Crizotinib
- Imatinib
- Grapefruit or grapefruit juice

**Drugs or herbal products known to be moderate to potent inducers of CYP3A4:**

- St. John’s Wort
- Rifampin
- Rifabutin
- Anti-convulsants (e.g. phenobarbital, carbamazepine, phenytoin, rufinamide)
- Modafinil
- Armodafinil
- Etraverine
- Efavirenz
- Bosentan

**Drugs with narrow therapeutic indices that are principally metabolized by CYP3A4 or transported by P-glycoprotein (P-gp):**

- *CYP3A4 substrates:* alfentanil, astemizole, cisapride, cyclosporine, dihydroergotamine, ergotamine, fentanyl, midazolam, pimozide, quinidine, sirolimus, tacrolimus, everolimus and terfenadine.
- *P-gp substrates:* digoxin and colchicine.
Appendix E: Study Drug Discontinuation Criteria for CE01-204

The following study drug discontinuation criteria were applied for the CE01-204 clinical trial (Chapter 7):

**Hepatotoxicity:**

a) Study drug should be held immediately until repeat testing can be performed (within 72 hours) for the occurrence of the following abnormalities in the absence of an alternative explanation (e.g. viral hepatitis, alcoholic or autoimmune hepatitis, or exposure to known hepatotoxic drugs, evidence of obstruction, such as elevated alkaline phosphatase typical of gall bladder or bile duct disease, or malignancy, or impaired glucuronidation capacity caused by genetic (e.g. Gilbert’s syndrome) or pharmacologic (e.g. treatment with atazanavir or other drugs) factors):

   i. ALT or AST > 8x ULN.
   ii. ALT or AST > 3x ULN and total bilirubin level > 2x ULN or INR > 1.5.
   iii. ALT or AST > 3x ULN with fatigue, nausea, vomiting, RUQ pain or tenderness, fever, rash and/or eosinophilia (>5%).

   If confirmed on repeat testing, study drug should be stopped and not restarted. If repeat testing does not confirm any of the above criteria, the investigator and the sponsor Medical Monitor will discuss whether the subject’s clinical condition allows for restarting study drug.

b) Study drug should be interrupted for an ALT or AST > 5x ULN for more than 2 weeks.

**QT prolongation:**

a) Study drug will be interrupted for a QTc (F) > 500 ms which is confirmed on the same or successive days.
b) Study drug will be interrupted for any increase in QTc (F) > 60 ms relative to baseline, provided this QTc(F) value is > 480 ms.

**Increases in heart rate:**

a) Study drug will be interrupted for the occurrence of the following changes in HR which are not attributable to other causes:

i. New onset (i.e. not present at baseline) HR > 120 bpm.

ii. HR which is > 100 bpm and a 30 bpm increase from baseline, which is confirmed on the same or successive days.

iii. Any increase from baseline which is accompanied by symptoms consistent with angina or heart failure.

iv. Any new onset atrial fibrillation or flutter, or any ventricular tachyarrhythmia (not included isolated PVCs or couplets).

**AEs potentially associated with inhibition of nicotinic acetylcholine (nACh) receptors:**

a) New onset of the following symptoms which cannot be explained by other causes:

i. Blurred vision.

ii. Loss of consciousness.

iii. Symptoms consistent with myasthenia gravis.