UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Academic Unit of Clinical and Experimental Sciences

Biomarkers of inflammation and infection in Chronic Obstructive Pulmonary Disease: utility of disease stratification and management

by

Dr Viktoriya Kim

Thesis for the degree of Doctor of Philosophy

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This thesis is embargoed from public release for 36 months after submission of the final version to the University. All analyses for publications resulting from the work produced in collaboration with GSK will be ratified in advance by the AERIS publication steering committee. All relevant manuscripts will be reviewed and approved prior to submission.

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ABSTRACT

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Exacerbations landmark the natural course of COPD and are associated with worsening airway obstruction, impact patients health status and have a considerable socio-economic impact. There is a gap in our understanding of different phenotypes of COPD exacerbations and therefore the strategies for treatment of exacerbations have remained unaltered for many years. Diagnosing an exacerbation event is largely subjective and depends on the assessment of presenting symptoms. Treatment of an exacerbation event is often generic and does not take into account different inflammatory mechanisms and underlying aetiological factors. The aim of this work was to determine whether clinically available airway and blood biomarkers can be used, separately or jointly, to identify exacerbations of COPD and in particular bacterial infection at exacerbations

The analyses were conducted on data collected from the Acute Exacerbation and Respiratory InfectionS in COPD cohort (AERIS) - a longitudinal observational study conducted from 2011-2014. The cohort consisted of patients with moderate to very severe COPD with a previous history of at least one moderate exacerbation. Patients were seen during clinical stability on a monthly basis and at exacerbations. Exacerbation events were monitored prospectively and patients were seen within 72 hours of an onset of the exacerbation event. Pulmonary function was assessed, and sputum samples were collected on a monthly basis and at exacerbations. Sputum samples were processed for microbiology and biomarkers. Blood samples were collected quarterly and at exacerbations and processed for biomarkers. The AERIS study was delivered at the standard of a randomised controlled trial with the rigorous monitoring and data cleaning, therefore, the data quality and the study conclusion are sufficiently robust to set up the vaccine study.

This work for the first time describes an association between the persistence of eosinophilic inflammation in COPD during clinical stability with an increased likelihood of eosinophilic exacerbations, and an association between eosinophilic inflammation, seasonality and bacterial presence. Namely, bacterial infection at exacerbation was higher in the Winter than Summer in patients with predominantly blood eosinophils≥2% at stable visits. A larger proportion of the

exacerbations with raised blood eosinophil ≥2% were observed during the Summer compared to Winter season. The findings confirm that sputum purulence can be examined by the five-graded Southampton sputum colour chart and the sputum colour grade was associated with airway neutrophils and airway bacterial presence at exacerbations. The numeric change in sputum colour was associated with airway bacterial presence; the higher the numeric change the greater was the frequency of airway bacterial detection. Higher sputum colour, higher CRP, higher fibrinogen, higher blood neutrophils and lower blood eosinophils were useful in identifying an exacerbation event with airway bacteria.

Therefore, these observations support the view that there are markers that are readily accessible in routine clinical care. These biomarkers reflect airway and systemic inflammation, they can be used in the diagnosis of airway bacterial infection at exacerbations and so may aid the management of COPD exacerbations. Further work to confirm the impact of these findings in improving clinical outcomes in intervention studies is required.

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Academic Thesis: Declaration Of Authorship

I, Dr Viktoriya Kim, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

"Biomarkers on inflammation and infection in Chronic Obstructive Pulmonary Disease: utility of disease stratification and management"

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as (listed below):

Signed: .....

Date: .....

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Firstly, I would like to thank my supervisor Professor Tom Wilkinson who provided me with his inspiration, guidance and support throughout my years in research. I wish to thank my supervisor Dr Steve Wootton for his support , encouragement and continual enthusiasm. I am grateful to my supervisor Dr Karl Staples for his patience, guidance and considerable support he provided to complete this thesis. My thanks go to Dr Ngaire Coombs for her guidance in statistical approach for my publications and thesis. I appreciate the help with the machine learning classifiers that Dr Ashley Heinson provided for my analysis. I am grateful to Dr Guy Roberts, my clinical supervisor, for his understanding and support to complete my thesis.

I am grateful to GSK Belgium for funding this study and, in collaboration with the University of Southampton, giving me the opportunity to deliver this study. I wish to thank the team's laboratory technicians for help with processing all the samples and Public Health England for their microbiology assessment of sputum.

My special thanks go to my colleagues Kris, Nick, Malwina, Anna, Des and Peter for helping me to run this study. I thank a marvellous team of research nurses and administrators who helped to conduct this highly intense single centred study smoothly. I am grateful to the colleagues in the SCBR office that provided me with much needed encouragement, particularly, in my final stretch of research. And of course I am grateful to all study participants who persevered with the study participation and without whom to deliver this study would have been impossible.

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Statement of the work performed

The present work is based on the AERIS study - a large collaboration between the GlaxoSmithKline, University of Southampton and University Hospital Southampton NHS Foundation Trust. The AERIS was a single-centre prospective epidemiological study of 127 with moderate to very severe COPD patients conducted between 2011 and 2014. The primary aim of this study was to assess the occurrence of all cause exacerbations of COPD and the contribution of airway infection to exacerbations of COPD. The results of the analyses reported in this thesis represent tertiary objectives of the AERIS programme.

I was involved in the design of the AERIS study. I was also involved in obtaining Research Ethics Committee and local Research and Development approvals for this study. I actively participated in the study set up. I personally screened, recruited and consented patients for the study. I was a clinical research fellow in the AERIS cohort thus involved in 1:2 and 1:3 study on call rota to capture exacerbations prospectively within 72 hours. With the team of nurses I was involved in screening exacerbation alerts and performed telephone or face-to-face consultations. I run multiple study stable and exacerbation visits when I personally collected the history, clinical examination, performed spirometry with bronchodilator testing, conducted blood and sputum sampling, body composition, questionnaires data collection. As one of the clinical research fellows I dealt with the radiological findings, particularly, when incidental findings of lung malignancy and other abnormal findings were reported. Furthermore, I was part of the team actively involved in rigorous data cleaning. Under the guidance of the team statistician Dr Ngaire Coombes I was responsible for all the statistical data analysis for the present work. Part of this work I presented in the form of abstracts and publication. Machine learning classifiers approach was performed by Dr Ashley Heinson.

Definitions and Abbreviations

6MWT	6 Minute Walk Test
AAT	Alpha 1 antitrypsin
AERIS	Acute Exacerbation and Respiratory InfectionS in COPD
ATS	American Thoracic Society
AUC	Area Under the Curve
BNP	B-type natriuretic peptide
CAT	COPD Assessment Tool
CCL2	C-C motif Ligand 2
CCL3	C-C motif Ligand 3
COPD	Chronic Obstructive Pulmonary Disease
CRP	C- Reactive Protein
CT	Computed Tomography
CXCL11	C-X-C motif Ligand 11
CXCL9	C-X-C motif Ligand 9
CXR	Chest X-Ray
ECLIPSE	Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points
EI	Eosinophilic inflammation
ERS	European Respiratory Society
EXACT-PRO	EXAcerbations of Chronic Pulmonary Disease Tool Patient-Reported Outcome
FEV1	Force Expiratory Volume in 1 second
FGF	Fibroblast Growth Factors
FLAME	EFfect of Indacaterol Glycopyronium Vs Fluticasone Salmeterol on COPD Exacerbations study
FVC	Forced Vital Capacity

GOLD	Global initiative for chronic Obstructive Lung Disease
HRQoL	Health Related Quality of Life
IAE	Intermittent Airway Eosinophils>3%
IBE	Intermittent Blood Eosinophils≥2%
IFN-γ	Interferon Gamma
IL	Interleukin
ILC2	type 2 innate lymphoid cells
IP-10	Interferon (IFN) inducible protein of 10 kDa
KCO	Transfer coefficient for carbon monoxide
LTB4	Leukotriene B4
mMRC	Modified Medical Research Council
MPO	Myeloperoxidase
NE	Neutrophil Elastase
NEADL	Nottingham Extended Activities of Daily Living
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
PAE	Predominantly Airway Eosinophils>3%
PBE	Predominantly Blood Eosinophils ≥2%
PCT	Procalcitonin
PPM	Potential Pathogenic Microorganism
RAE	Rarely Airway Eosinophils>3%
RBE	Rarely Blood Eosinophils ≥2%
RV	Residual Volume
SSC	Southampton Sputum Colour (SSC) Chart
Sp02	Peripheral Oxygen Saturation
SPD	Surfactant Protein D
SVC	Slow Vital Capacity

TGF-β	Transforming Growth Factor beta
Th1	Type 1 helper T cell
Th2	Type 2 helper T cell
TLC	Total Lung Capacity
TLCO	transfer factor for carbon monoxide
TNFα	Tumour Necrosis Factor alpha
TNT	Troponin-T
TORCH	TOwards a Revolution in COPD Health
WBC	White Blood Cells
WHO	World Health Organisation

Prologue

I first met Pete, a 70 year old ex-mechanic, during his admission to the acute medical ward with an “exacerbation of COPD”. His only presented symptoms on that occasion were an increase in breathlessness, that he frequently experienced, and some cold symptoms. However, as he was a “frequent attender” and a known COPD patient he was commenced on Prednisolone, Doxycycline, nebulised Ipratropium and Salbutamol on admission. Pete was well enough to be discharged a few hours after admission although it was felt he should complete a week’s worth of antibiotics and steroids after discharge. He frequently attended A&E with the acute onset of COPD symptoms and was always treated for an exacerbation of COPD. I then followed him up in our chest clinic. He described symptoms of breathlessness on exertion with occasional wheeze and very scarce daily sputum production. Pete had his post bronchodilator spirometry and was classed as severe COPD. He was commenced on a LABA/ICS and LAMA combination a few years ago.

I saw Jim, a 65 year old ex-sailor, in the chest clinic. He presented with symptoms of progressive breathlessness, daily sputum production and frequent episodes of acute worsening of these symptoms. Jim usually goes to his GP who commences him on repeated courses of the standard package “oral steroids and antibiotics” for these acute episodes. In fact, when I first met him in clinic he presented with symptoms of increased breathlessness and increased volume of purulent sputum. Jim has been on a prolonged course of antibiotics and steroids by his GP. He was initially diagnosed with COPD a few years ago which, based on post-bronchodilator FEV1, was classed as severe. Over time Jim was commenced by his GP on a LABA/ICS and LAMA combination but despite of that was still having frequent exacerbations.

Both patients report that COPD has significantly impacted their lives; with Pete now wheelchair-bound for mobilizing outdoors and Jim feeling that he can not do as much as he used to when worked in the Navy and always fearful of another exacerbation. Jim and Pete had their COPD medications fine tuned to their condition, with small airways disease and patients’ dexterity taken into account. In addition, they had lifestyle change advice and were referred for pulmonary rehabilitation. They subsequently had a trial course of prophylactic antibiotics commenced. Following these changes Jim has improved but Pete continued to have a high number of exacerbations.

In relation to these seemingly similar clinical COPD cases, albeit with different clinical responses, I ask myself the following questions:

- Are all episodes of acute worsening of symptoms, that Jim and Pete are receiving the treatment for, true exacerbations of COPD?
- How to objectively differentiate infectious exacerbations of COPD that benefit from antibiotics?
- How to predict the group of patients that will respond to treatment (steroids and antibiotics)? Moreover, how to predict the group of patients that does not need antibiotics?
- Which clinical and biological measurements, alone or in combination, will best predict these responses?

These cases have informed my thinking and helped to shape the overall aim of the whole work described in this thesis.

Chapter 1 Introduction

1.1 COPD overview

Chronic obstructive pulmonary disease (COPD) is the favoured term for conditions such as chronic bronchitis and emphysema in the presence of confirmed airway obstruction. The American Thoracic Society (ATS) and European Respiratory Society (ERS) define COPD as

> "a preventable and treatable disease state characterised by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases, primarily caused by cigarette smoking. Although COPD affects the lungs, it also produces significant systemic consequences".[1]

1.1.1 Epidemiology and socio-economic burden

COPD is an established major cause for mortality and morbidity in the Western world with a significant socioeconomic impact. According to the World Health Organization (WHO) report there are sixty four million people diagnosed with COPD and three million people died of COPD. In 2004 COPD was listed as the fourth leading cause of death.[2] The WHO prediction is that of COPD being the third leading cause of death worldwide by 2030, corresponding to 5% of all deaths globally.[3] In addition the WHO predicts that by 2030 COPD will rank fifth in worldwide disease burden and account for 3.8% of total DALYs (Disability adjusted life years).[2] Furthermore, COPD is widely underdiagnosed. The British Lung Foundation estimates that there are two million undiagnosed COPD patients in the UK and this report has led the drive to identify this population.[4]

COPD is a debilitating condition and it has a substantial impact on the quality of life. Health related quality of life significantly deteriorates with the disease severity in COPD patients.[5] It was estimated that the cost of the severe COPD in Spain is seven-times higher than mild COPD and three-times higher than moderate COPD. [6] In Sweden the cost of severe COPD was more than ten times higher than mild COPD and three times higher than moderate COPD patients.[7] According to the European White Lung Book the total cost for COPD in Europe in 2001 was €38.7 billion (€4.7 billion for ambulatory care, €2.7billion for drugs, €2.9billion for inpatient care and €28.4billion for lost work days).[8] COPD costs also increase with an increase in exacerbation frequency.[9] Most economic burden is rising from the hospitalisation associated with these exacerbation episodes.[9]

Severe exacerbations of COPD also have an independent negative impact on the patient's prognosis and frequent severe exacerbations of COPD are associated with high risk of mortality.[10]

1.1.2 History of COPD

Some early descriptions of COPD can be found in the conditions of emphysema and bronchitis. Attempts to describe emphysema can be traced back to the 12th century when cadaverous lungs were described as "voluminous" and "turgid" where as a first clinical description of patients with chronic bronchitis was made only in the early 19th century.[11] In the 1960s, Dorhorst offered a landmark description of two extreme clinical phenotypes of patients with respiratory insufficiency.[12] The classic "Blue Bloater" was described as a younger patient with chronic bronchitis who often presented with right heart failure. The classic "Pink Puffer" was an older patient with muscle wasting suffering with disabling dyspnoea and clear evidence of emphysema. In 1962 the ATS diagnostic standards defined chronic bronchitis using clinical terms such as productive cough lasting for at least three months for at least two years whereas emphysema was defined in anatomical terms as enlarged alveolar space and a loss of alveolar walls.[13] Although spirometry has been in use since the late 19th century it was not until later in the 20th century when the physiological changes of airway obstruction were taken into consideration when the diagnosis of COPD was made.[14]

1.2 Diagnostic criteria of COPD

A diagnosis of COPD is suspected in patients presenting with symptoms such as dyspnoea, chronic cough, chronic sputum production, exposure to noxious particles and family history of COPD. A formal diagnosis is made when the presence of airway obstruction is confirmed based on post-bronchodilator spirometry. Airflow obstruction is defined as a reduction of forced expiratory volume in one second (FEV1) to forced vital capacity (FVC) ratio of less than 0.7 (FEV1/FVC < 0.7). Classification of the severity of COPD is based on post bronchodilator FEV1, as the percentage of predicted value.

- Mild – FEV1 ≥80% predicted
- Moderate – FEV1 ≤50% to <80% predicted
- Severe – FEV1 ≤30% to <50% predicted
- Very severe – FEV1 <30% predicted

Spirometric classification has been useful in predicting health status, utilisation of healthcare resources, and prognosticating the rate of exacerbations and mortality in COPD.[15-17,18,1] However, there is no association between FEV1 and clinical symptoms.[19] Furthermore, the simple spirometric grading system is not without limitations, specifically, there is a tendency to underestimate airflow obstruction in younger individuals but to overestimate airway obstruction in the normal aging lung.[20] These previous classifications also did not account for patients' symptoms, particularly the significance of exacerbations. Therefore, in 2011 Global Initiative for Chronic Obstructive Lung Disease (GOLD) proposed to stratify patients by measuring the impact of the COPD by means of the modified Medical Research Council (mMRC) dyspnoea score and COPD assessment test (CAT). However, GOLD 2011 ABCD classification of COPD did not offer better prognostic value for mortality than a simple spirometry based classification, moreover, it was confusing as the risk stratification was based on the exacerbation frequency and/or severity of the airflow obstruction.[19, 21, 22] Therefore, a refinement of the ABCD GOLD classification was proposed in 2017.[23] This classification offers a separate spirometric assessment of the airflow obstruction severity from the symptoms and exacerbation history based ABCD classification.[23] (**Figure 1.1**) Furthermore, spirometry and symptoms based classification does not reflect complex underlying pathophysiological mechanisms and thus does not account for patients with different COPD phenotypes and does not offer phenotype tailored management.

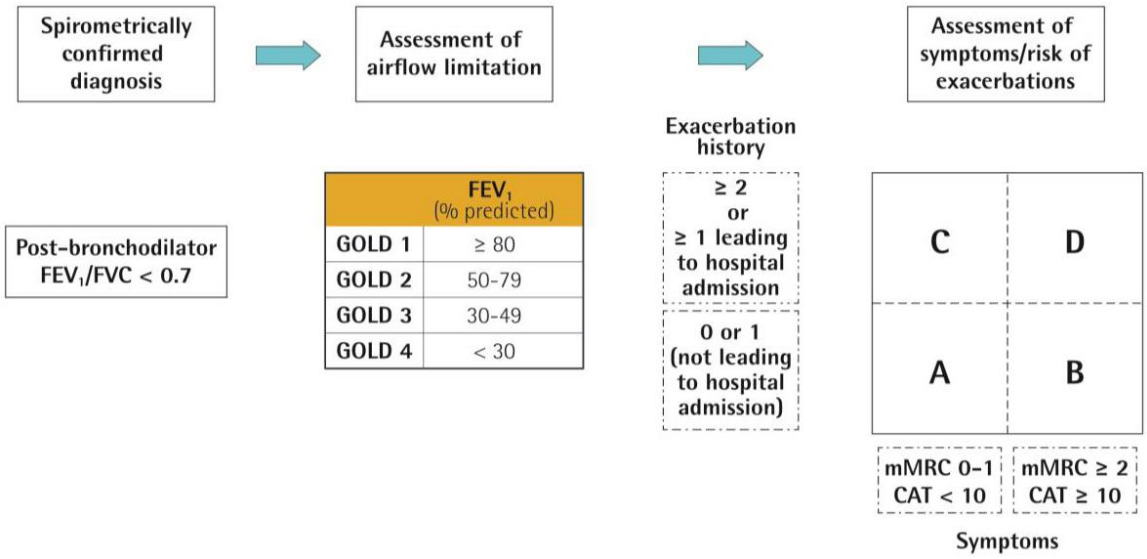


Figure 1.1 The refined ABCD assessment tool. Adapted from Vogelmeier et al 2017.

1.3 Aetiology of COPD

COPD is a multifactorial disorder caused by the exposure to an environmental factor and genetic risk determinants. The primary cause of COPD in high- and middle-income countries is tobacco smoke, however, in low-income countries the biggest risk factor is thought to be an exposure to indoor air pollution.[2, 24-26] Studies on women in developing countries exposed to various levels of indoor pollutants including biomass and charcoal suggested that persistent exposure was associated with chronic airflow obstruction. Reactive oxidant substances that are produced by the persistent tobacco smoking and noxious gases result in an infiltration of the airways with inflammatory cells.[27] However, only 25% of smokers develop COPD in their lifetime[28] thus leading to the hypothesis that this condition is a result of complex host-environmental interactions. COPD was observed to aggregate in families with stronger associations between parents and children and between siblings.[29] The one proven genetic risk factor for the development of COPD is alpha 1 antitrypsin (AAT) deficiency. However, AAT deficiency is responsible for only a small proportion of COPD.[30] As COPD is such a heterogeneous disease, the gene combinations responsible for the COPD will likely be different for different phenotypes.[29, 31] Although there has been some progress in understanding COPD genetics since the discovery of the AAT gene, much of the genetic basis for the disease remains undiscovered.

1.3.1 Airway inflammation

Airway inflammation is thought to be a major component in the pathogenesis of COPD. Cigarette smoke and other noxious particles activate resident lung macrophages and epithelial cells to release cytokines that attract further inflammatory cells such as neutrophils and CD8+ T cells to the lungs and in the long term lead to remodelling events such as airway wall destruction, fibrosis of the small airways and mucus hypersecretion.[32] Activated epithelial cells and macrophages stimulate fibroblast proliferation via growth factors such as Transforming Growth Factor beta (TGF-β) and Fibroblast Growth Factors (FGFs) resulting in peribronchial fibrosis. Fibrosis is thought to be the main contributing mechanism towards the irreversible airway obstruction associated with COPD.[33] Activated epithelial cells and macrophages also release the pro-inflammatory cytokines Interleukin-6 (IL-6), Tumour Necrosis Factor alpha (TNFα) and chemoattractants such as Interleukin-8 (IL-8), C-C motif Ligand 2 (CCL2) and C-C motif Ligand 3 (CCL3) that lead to further recruitment of neutrophils and monocytes, the latter differentiate into lung macrophages.[33] The chemokines C-X-C motif Ligand 9 (CXCL9), Interferon (IFN) inducible protein of 10 kDa (IP-10), C-X-C motif Ligand 11 (CXCL11) attract Th1 cells (Type 1 helper T cell) that stimulate further release of IFN-γ.

Activated neutrophils and CD8+ T cells are involved in antimicrobial activity and have been shown to contribute to airway damage via release of inflammatory mediators (IL-8, TNFα, IFNγ), serine proteinases (neutrophil elastase, cathepsin K, matrix metalloproteinase), other cytotoxic granules (granzyme and perforin) [34-36] and reactive oxygen species. The lungs are given structure by the meshlike pulmonary extracellular matrix that consists of proteins such as collagen, elastin, proteoglycans, fibronectin and laminin.[37] The effect of cytokines along with the protease-mediated degradation of elastin and apoptosis of alveolar endothelial cells result in destruction of the key components of extracellular matrix and cause emphysema. Persistent airway inflammation and goblet cell hyperplasia resulting in mucus hypersecretion leads to airway remodelling.[38] In addition, an increased metaplasia of the epithelium is also characteristic in COPD. TGF-β and FGFs have the potential to contribute towards airway remodelling by the increase in thickness of airway smooth muscle layer, mostly due to hypertrophy of smooth muscle cells, and the increase in extracellular matrix protein deposition around airway smooth muscle.[37, 39] Airway thickening in COPD leads to the worsening of airflow obstruction.[40]

An infiltration and increased number of alveolar macrophages, neutrophils, cytotoxic T-lymphocytes in the airways that stimulate the secretion of inflammatory mediators (chemokines, cytokines, growth factors, and pro-inflammatory cytokines) along with the high level of oxidative stress (via activation of NFκB)[41] lead to airway remodelling and destruction of airway.[42, 43]

COPD is characterised by predominance of CD4+ T cells typically with a Th1-cell subtype response (IL-8, IL-12, IL-17, TNFα and IFNγ production).[34, 44] Neutrophilic inflammation is thought to be central in COPD pathogenesis is associated with underlying airway bacterial presence.[45-48] Some authors reported a correlation between IL-8 concentration and bacterial presence indicating[49] that airway bacteria may trigger neutrophilic inflammation via release of IL-8 in the airway.[48] Sethi et al proposed a vicious cycle hypothesis where bacterial pathogens established in the lower airways, they persist by furthering impairment of mucociliary clearance, mucociliary hypersecretion and airway epithelial injury. Bacterial products trigger recruitment of neutrophils into the airways. Influx of neutrophils and their degranulation in the airways and lung parenchyma may contribute to the airway inflammation and progression of lung damage.[50]

Although COPD is characterised by predominantly neutrophilic inflammation there is a mounting evidence for the significance of eosinophilic inflammation in a subgroup of COPD patients. As opposed to the Th1 pathway characteristic of neutrophilic inflammation, eosinophil-mediated inflammation is thought to be orchestrated via Th2 cells through release of IL-13 and IL-5.[32, 51]

1.3.2 Systemic effects

There is also evidence for inflammatory sequelae outside of the lung in COPD. Gan et al analysed 14 original studies to identify the association between FEV1, FVC and systemic inflammatory markers.[52] They showed that, compared to healthy controls, those with airflow limitation have significantly raised levels of systemic markers such as C-reactive protein (CRP), fibrinogen, leukocytes and TNFα.[52] Donaldson et al showed the association of raised airway and systemic inflammation and faster FEV1% decline.[53] Although COPD is associated with raised systemic markers, the origin of the systemic inflammation in COPD remains unclear. There are few proposed theories of the systemic inflammation in COPD: a "spillover" theory, tobacco smoking, hypoxia, dynamic hyperinflation, aging process and other part of body such as bone marrow producing inflammatory mediators.[54-58] There is no general consensus on which theory explains the mechanism that drives systemic inflammation. It is most likely that a combination of these mechanisms contributes towards the raised systemic inflammation in a subpopulation of COPD patients.

The most plausible explanation for raised systemic inflammation in COPD is the "spillover" hypothesis where the inflammation from the airways and lung parenchyma "spills over" into the systemic circulation. Hurst et al demonstrated the association between lower airway and systemic inflammation at exacerbations.[59] However, this association was not present during clinical stability.[60] Similarly Singh et al showed no correlation between sputum and serum neutrophils during clinical stability.[61] Another proposed theory is that tobacco smoke drives systemic inflammation. It is well known that tobacco is a cause of systemic inflammation in atherosclerosis and coronary artery disease.[62] This theory is challenged by the fact that ex-smokers with COPD also have persistent systemic inflammation despite smoking cessation.[63] This might imply that tobacco smoking triggers off inflammation but perhaps a different mechanism maintains it. Hypoxia is also a common finding in COPD patients and could be driving the inflammation. Mild COPD patients who underwent a hypoxia challenge had increased serum IL-6[64] and the degree of hypoxia was also associated with the serum TNFα level.[65] Hypoxia could evolve from dynamic hyperventilation, which is another common feature of COPD and one that does not depend on disease severity. Gatta et al demonstrated that decreased inspiratory capacity was associated with the higher level of CRP.[66] It has been suggested that perhaps bone marrow could be initiating a persistent low grade systemic inflammation in COPD. This is an attractive theory as the bone marrow is the primary matrix for development and maturation of inflammatory cells. It is known that bone marrow is affected by the persistent smoking and air pollution.[67, 68] Palange et al studied the role of the bone marrow in systemic inflammation on an example of a haemopoetic progenitor (CD34+ cells) in a small cohort of COPD patients (n=18) and controls (n=12).[69] They

reported that the CD34+ level was decreased in COPD patients and CD34+ was also associated with the airflow obstruction.[69] However, the role of the bone marrow in COPD has not been sufficiently studied to draw definitive conclusions.

Systemic inflammation in COPD is also associated with the development of other conditions.[70, 71, 72] For example, COPD is commonly associated with cardiovascular disease, lung cancer, weight loss and osteoporosis. Cardiovascular disease shares smoking as a risk factor with COPD and it is not therefore surprising that COPD was reported to be an independent risk factor for cardiovascular morbidity and mortality.[73] Mannino et al reported a significant association between respiratory impairment, cardiovascular disease, diabetes and hypertension.[74] They also reported that COPD patients were likely to have at least two other co-morbidities and those COPD patients with comorbidities were at higher risk of hospitalization and mortality. [74]

1.3.3 The lung microbiome in stable COPD

The previous belief that the lungs of a healthy person are sterile has been refuted using modern molecular detection techniques. There is a mounting evidence that the lower respiratory tract is inhabited by a community of microorganisms that may contribute to health and disease, specifically in COPD. [75, 76] The microbiome in COPD patients undergoes dynamic changes during clinical stability and at exacerbation.

In healthy individuals, the respiratory epithelium plays a vital role in host defence by producing mucus, chemokines, cytokines, antimicrobial peptides, surfactant proteins and proteinase inhibitors. Despite the large number of viral and bacterial antigens encountered throughout life, these innate immune mechanisms and the mucociliary clearance activity of the epithelium prevents colonisation of the lower airways by pathogenic organisms.[77] As mentioned above, in COPD the innate lung defence mechanisms are impaired, predisposing patients to bacterial establishment in the lower respiratory tract.

The bronchial secretion from the distal airways was reported to be positive to the potentially pathogenic microorganisms (PPM) in approximately one third of stable COPD patients when standard microbiological methods were applied.[78, 79] _ENREF_76 Chronic bacterial colonisation perpetuates inflammation and contributes to the progression of COPD severity.[53] For example, bacterial presence in the lower airway during clinical stability is associated with higher airway inflammation and decline in FEV1 in COPD patients.[80] An in-depth understanding of the mechanisms modulating the natural course of COPD, specifically the impact on the exacerbation

frequency, by bacterial colonisation is still largely unknown. However, there is evidence to show that bacterial colonisation is associated with increased exacerbation frequency[49], baseline airway inflammation in frequent exacerbators as evidenced by an increased level of systemic inflammatory cytokines such as IL-6, IL-8[81] and IL-1β[82]. Bacterial colonisation with the organism, *Haemophilus influenzae* (HI), in particular is associated with increased sputum purulence and symptom count at exacerbations[49] as well as higher level of local inflammatory response and dose response relationship between HI load and IL-8[82]. Persistent colonisation with HI during clinical stability also showed a relationship with airway obstruction, namely, patients colonised with HI had lower FEV1 compared to those not colonised. [49]

Another factor that might lead to chronic bacterial colonisation is biofilm formation. Bacterial biofilms are an aggregate of microorganisms where cells adhere to each other on a surface and are enclosed by a self-produced matrix.[83] There is evidence that a range of different microorganisms exist in biofilms that promote microbial resistance to pharmaceutical or immune resistance and the majority of persistent infections develop biofilms.[84, 85] These biofilms can also have a significant host component that includes neutrophil extracellular traps (NETs).[86] Non-typeable HI (NTHI) was shown to initiate NET formation by means of multiple molecular patterns and the bacteria demonstrated to be highly resistant to killing by newly recruited neutrophils within NET structures.[87] Bacteria growing in biofilms appear to be highly resistant/tolerant to a wide variety of clinically relevant antibiotics and immune effectors.[88-90]

1.3.4 Treatments of COPD: Stable Disease

The aim of COPD treatments is to minimise the frequency and severity of exacerbations, reduce COPD symptoms and improve exercise tolerance and health status.[23] The GOLD guidelines (2017) suggest a treatment algorithm for patients with stable COPD based on the severity of airflow obstruction, clinical symptoms and exacerbation rate.[91] (**Figure 1.2**) The current algorithm, however, is largely generic and does not take into account individual underlying inflammatory process and the presence of airway infection.

The GOLD guidelines recommend that patients with confirmed COPD are commenced on bronchodilators either as monotherapy or dual therapy depending on the degree of symptoms and history of exacerbations. Bronchodilators are central in management of stable COPD[23]. They are shown to increase FEV1, reduce dynamic hyperinflation and improve exercise performance.[92, 93] Furthermore, the treatment with long acting muscarinic receptor antagonist (LAMA) shown to improve symptoms and health status, reduces exacerbation rate.[94] LAMA and long acting beta-2

receptor agonist (LABA) are recommended for use in treatment naïve patients whose symptoms aren't controlled with short acting equivalents (SABA and SAMA). Both LABA and LAMA work via different complementary pathways. Namely, bronchodilation by β2 agonists is achieved via activation of β2 receptors on the airway smooth muscles resulting in airway smooth muscle relaxation, whereas muscarinic antagonist work through the antagonism at muscarinic receptors thus preventing airway smooth muscle contraction. Therefore, combined therapy has the potential to improve symptoms when used in patients not responding to monotherapy. Previous studies of LAMA/LABA combination showed superiority in improving airflow obstruction versus monotherapy.[95, 96] Furthermore, LABA/LAMA demonstrated a greater improvement in patient's symptoms and quality of life compared to monotherapy.[95-97] However, LABA and LAMA therapy does not appear to be sufficient to control symptoms and exacerbations frequency, thus, those patients with uncontrolled symptoms and frequent exacerbations are recommended to be commenced on anti-inflammatory agents such as inhaled corticosteroids (ICS) or more rarely long-term oral corticosteroids.[91]

The mechanism of action of ICS is thought to be via suppressing inflammation. This is achieved mainly by switching off multiple activated inflammatory genes through effects at gene promoters such as reversing histone acetylation resulting in a reduction in the number of inflammatory cells (including eosinophils, T lymphocytes, mast cells and dendritic cells). [98] Although, GOLD advocates the use of ICS in patients with poorly controlled symptoms and frequent exacerbations, the role of ICS in general COPD populations remains controversial.[99] Some authors found reported inhaled corticosteroids and inhaled long acting bronchodilators to be beneficial by the maintained control of COPD and reduced the rate of exacerbations.[100-102] A Cochrane review, to determine the efficacy of regular use of ICS in stable COPD that included 13139 patients, confirmed the finding that long term use of ICS reduced the exacerbation rate.[103] However, the Cochrane authors also reported no benefit of long term ICS on FEV1 decline and no statistically significant effect on mortality.[103] Furthermore, the TORCH (TOwards a Revolution in COPD Health) study in COPD reported a concern that ICS/LABA treatment was associated with higher risk of pneumonia especially in those with more severe disease.[104] Other authors also confirmed an increased incidence of pneumonia in patients on ICS.[105]

Further studies demonstrated that LABA/LAMA treatment significantly improved lung function compared to LABA/ICS.[106, 107] Moreover, the FLAME study (EFfect of Indacaterol Glycopyronium Vs Fluticasone Salmeterol on COPD Exacerbations) specifically looked into patients with moderate to very severe COPD and history of at least one exacerbation LABA/LAMA had greater prevention in exacerbations than LABA/ICS.[105] Thus there is a need for an individually tailored treatment for

stable COPD. Therefore, it is perhaps prudent to consider integrating the nature of an underlying inflammatory profile into the treatment algorithm.

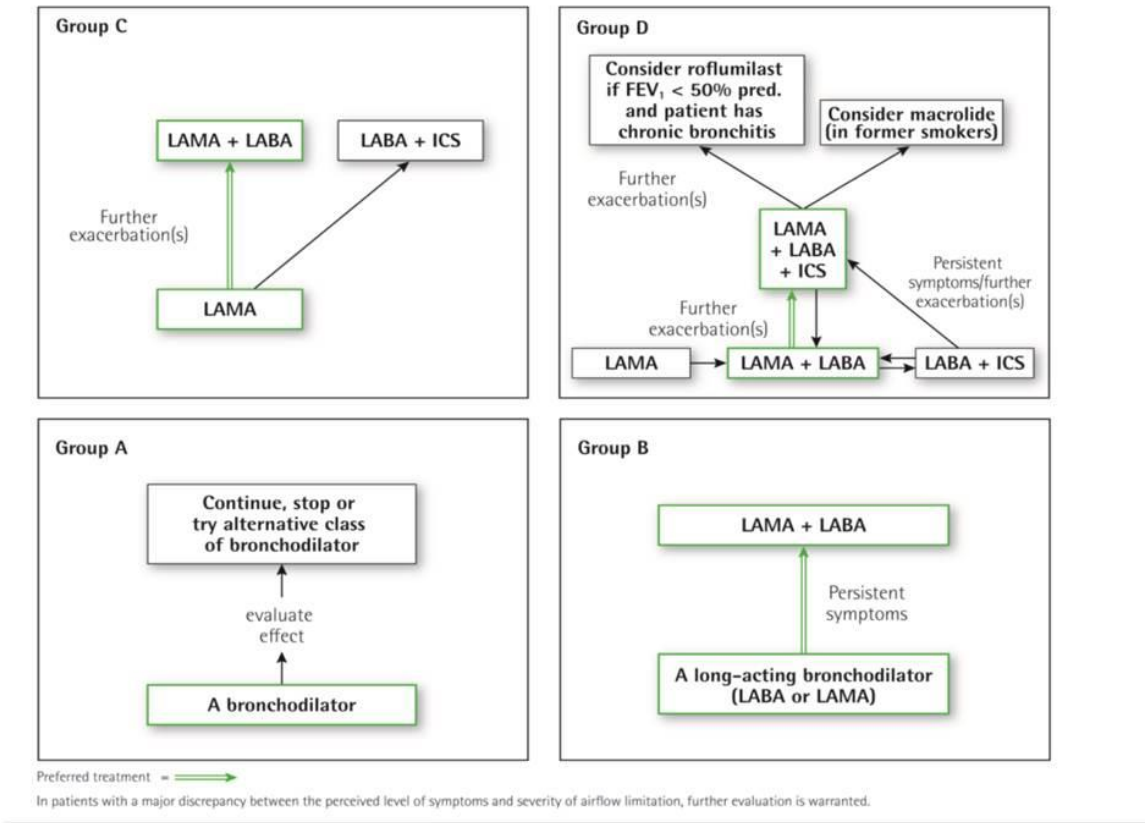


Figure 1.2 Pharmacologic treatment algorithms by GOLD Grade. Adapted from Vogelmeir et al 2017

There have been a series of trials aimed at reducing exacerbation rates by means of bacterial eradication[108] using long-term prophylactic antibiotics such as quinolones[109] or macrolides[110-112]. One study investigated the eradication of bacterial colonisation of the lung with a five-day course of Moxifloxacin demonstrated a significant reduction in airway bacteria but the frequency of re-colonisation of new Potentially pathogenic microorganism (PPM) in eight weeks' time was high in both Moxifloxacin and placebo treatment groups.[108] The PULSE study, which assessed the impact of the long-term intermittent pulsed treatment with Moxifloxacin on the frequency of exacerbations, demonstrated that the group pulsed therapy with Moxifloxacin has significantly reduced the odds of exacerbations, specifically in the group with purulent/mucopurulent sputum at baseline.[109] Other authors investigated the use of Azithromycin in COPD and showed that it could prevent future exacerbations of COPD in an older group of ex-smokers with milder

disease.[113] Long-term prophylactic therapy with macrolides also demonstrated a reduction in exacerbation rates but therapy increased the risk of hearing decrement and more importantly microbial resistance to the antibiotics.[110] Thus, although there is some evidence to support a long term antimicrobial treatment the antimicrobial agent should be carefully selected and monitored for adverse effects. We should also remember the significance of antimicrobial resistance associated with long-term antibiotic administration.

1.4 Exacerbations of COPD

The natural course of COPD is punctuated by acute changes in patients' respiratory symptoms beyond daily variations, and sufficient to make changes in management. These events are known as acute exacerbations of COPD (AECOPD),[1] these events have a major impact on quality of life. The significance of frequent exacerbations was studied by Donaldson et al, who demonstrated that frequent exacerbations of COPD are associated with more rapid decline in lung function.[114] Mackay et al studied the impact of COPD by using the COPD Assessment Tool (CAT) score and demonstrated worsening in health status at exacerbations as evidenced by an increase in the CAT score. The CAT score also reflected exacerbation severity (as measured by exacerbation length and reduction in lung function) and was higher at baseline in a subgroup of frequent exacerbators.[115] Other groups examined patient's health related quality of life, using St George's Questionnaire, in the context of exacerbations and reported that the quality of life was significantly worse in patients with higher number of exacerbations.[116, 117] Furthermore, exacerbation events were associated with further risk of exacerbations and an increased risk of death.[118-120]

1.4.1 Aetiology of exacerbations of COPD

Exacerbations of COPD are thought to be caused by the complex interactions between the host, airway infection and environmental pollution.[47, 121-126] Seasonality appears to be an important factor associated with COPD exacerbations.[127, 128] The multicentre, international TORCH study demonstrated a higher rate of exacerbations during the winter months in the northern and southern hemispheres.[127]

1.4.1.1 The role of bacteria in exacerbations of COPD

Bacteria are frequently found in patients' airways during exacerbations and assumed to play a major part in exacerbations of COPD.[122, 124, 129-131] For instance, bacterial exacerbations are associated with an increased evidence of neutrophilic inflammation (IL-8, TNF-α, neutrophil elastase, leukotriene B4, myeloperoxidase) than non-bacterial episodes at exacerbations.[121-123] Sethi et al demonstrated a strong association between HI and *Moraxella catarrhalis* (MC) in sputum and neutrophilic airway inflammation at exacerbations.[122]

An increase from the baseline airway inflammation corresponds with the clinical manifestation of classic symptoms of exacerbation of COPD.[132-134] Various infectious and non-infectious agents can cause an increase in airway inflammation or exacerbation of COPD. Approximately two-thirds of all exacerbations of COPD are of bacterial and/or viral origin [124, 135] HI 20-30%, *Moraxella catarrhalis* 10-15% (MC), *Streptococcus pneumoniae* 10-15% (SP) and *Pseudomonas aeruginosa* 5-10% (PA) are most common pathogens associated with COPD exacerbations.

Significant genetic diversity in individual strains of bacterial species and alterations in the surface antigenic structure allows bacterial strains to evade pre-existing host immunity thus causing recurrent exacerbations.[134] In their prospective study, Sethi et al examined the role of a new strain acquisition using molecular typing of bacteria at exacerbations. Sethi et al found that the new strain acquisition was significantly higher at exacerbations compared with stable visits.[136] They also reported that the new strain acquisition was associated with a two-fold increase in COPD exacerbations.[136] However, not every new strain acquisition leads to an exacerbation. Three out of four major pathogens examined were associated with an increased risk of acute exacerbations (HI, MC, SP), but PA showed no such association.[134, 136] It is also proposed that the onset of a new exacerbation could be due to the complex host-pathogen interaction i.e. the balance between bacterial virulence and host defence system determine the outcome of every new bacterial strain.[137]

1.4.1.2 The role of viruses in exacerbations of COPD

In addition to bacteria, respiratory viruses are also detected in 20 - 56% of COPD patients during exacerbations.[124, 138, 139] Respiratory viruses were reported to be associated with longer time to exacerbation recovery[140]. Human rhinovirus (HRV) in particular was associated with lower airway inflammation[141], and co-infection of HRV and HI was associated with higher HI bacterial load[139]. Other respiratory viruses detected at exacerbation include Parainfluenza virus 5-10%, Influenza virus 5-10% Respiratory Syncytial virus 5-10%, coronavirus 5-10% and adenovirus 3-5%.[135] Mallia

et al in conducted an experimental model of virus induced exacerbations of COPD.[142] They demonstrated that all 4 COPD patients when inoculated the lowest dose of rhinovirus developed symptomatic colds, associated with the reduction in FEV1, an increase of cytokine (IL-6 and IL-8) and positive serological response to rhinovirus in nasal lavage.[142] Some authors proposed that eosinophilic inflammation of the tracheobronchial tree to be a good predictor of viral infection.[124] However, Bafadhel et al in their study of clinical phenotypes of acute exacerbations reported that IP-10 was the best predictor of airway viral presence at exacerbations.[47]

1.4.2 Diagnostic criteria for exacerbation of COPD

There is no universally accepted definition of AECOPD and no generally agreed classification. Anthonisen et al were first to delineate exacerbation phenotypes and suggested three types of exacerbations.[143] Type I – the increase of dyspnoea, sputum volume and sputum purulence; Type II – when two of these symptoms were present; Type III – when one of these symptoms was present in association with at least one of the following symptom: sore throat, nasal discharge, fever, wheeze, cough and increase in respiratory or heart rate by 20%. Based on this work the East London group defined a COPD exacerbation as the new onset of at least two "major" symptoms (dyspnoea, sputum amount and sputum purulence) or a combination of one "major" and one "minor" (wheeze, sore throat, cold and cough) persisting for at least two consecutive days.[140, 144] Whereas the GOLD definition of an acute exacerbation is less specific and defined as "an acute event characterised by a worsening of patient's respiratory symptoms that is beyond normal day-to-day variations and leads to a change in medication".[125] Furthermore, classification of the severity of an exacerbation event differs widely from previous recommendations to rank exacerbations (Level I – outpatient management, Level II – required hospitalisation, Level III – leads to respiratory failure) to more recent classifications based on the requirement of treatment for an exacerbation event (mild – no treatment required, moderate – outpatient treatment, severe - hospitalised).[1, 91] Despite numerous attempts to define COPD exacerbations, there is no universal consensus and clinical diagnosis of exacerbations remains largely subjective. Severity of COPD and psychosocial factors are likely to impact on the patients' perception of symptoms and lead to higher healthcare resources use.[145] Therefore, currently there is no additional objective tool to confirm a diagnosis of COPD exacerbation. There is clearly a need for validated biomarkers of exacerbation to enable more objective, standardised and accurate diagnosis of an exacerbation.

1.5 Biomarkers

There are several proposed definitions of biological markers, more frequently known as biomarkers. The WHO in coordination with the United Nations defined biomarkers as "*any substance, structure, or process that can be measured in the body, its products and influence or predict the incidence of outcome or disease*".[146] According to the National Institutes of Health a biomarker is "*a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*".[147] Strimbu et all referred to biomarkers as a "*subcategory of medical signs – that is, objective indications of medical state observed from outside the patient – which can be measured accurately and reproducibly*".[148] Conventionally, the term "biomarker" has been used when the markers were measured in biological samples such as cells, tissue, exhaled breath, sputum, serum or plasma. The term is occasionally used to refer to clinical measurements such as exacerbation rate etc.

Biomarker applications range from disease diagnostic abilities, to use as prognostic indicators and the monitoring of therapeutic intervention efficacy. COPD is a heterogeneous condition encompassing different clinical, pathophysiological and inflammatory characteristics. Specifically, exacerbations of COPD vary in their inflammatory profile and aetiological factors but currently management is indifferent to different phenotypes. There were a number of studies proposing the significance of inflammatory markers and their association to the disease stage. Currently the only accepted marker of COPD is FEV1; both for the diagnosis of COPD and measuring its severity. However useful this measure is, it is accepted that this marker does not reflect the underlying pathological processes, it is not modified by the interventions and, therefore, other markers should be considered. However, to date no marker is ready for a clinical translation as has not fulfilled all the required criteria of a true biomarker. Sin and Vestbo suggested that a true COPD biomarker should fulfil the following criteria of biological plausibility, strong, consistent and independent association with disease, strong, independent association with hard clinical outcomes, modification by interventions, with changes in clinical outcome to be reflected by changes in biomarker status.[149] Furthermore, there is a gap in understanding the role of biomarkers to predict exacerbations and identify bacterial presence at exacerbation. The identification such biomarkers may lead to improved scientific and clinical outcomes.

1.5.1 Airway biomarkers

Sputum production is a hallmark of COPD and it is largely a result of the altered mucin overproduction and mucociliary clearance.[150] Sputum induced by inhalation of hypertonic saline is

derived from the lumen of the central airways[151] and is considerably less invasive than obtaining a bronchial biopsy or bronchoalveolar lavage.[152, 153] Sputum analysis is widely analysed both in research and clinical practice. Sputum can be assessed for the presence of airway infection and visually for sputum purulence.[123, 154] Sputum is also examined for underlying airway inflammation by examining the infiltrating leukocytes as well as for specific cytokines and chemokines, although this is more common in the research setting.[152, 153, 155]

1.5.1.1 Sputum microbiology and virology

Sputum samples are universally used in clinical practice for the assessment of bacterial and/or viral presence.[47, 78, 123] The most widely used method in clinical practice is a conventional sputum analysis for bacterial culture and antibiotic sensitivity analysis. Most UK hospitals have access to Polymerase Chain Reaction (PCR) analysis for detection and identification of viruses, but this is not performed routinely. There are other available such as PCR analysis and Fluorescence In-Situ Hybridisation (FISH) for bacterial presence but these are not routinely available for clinical use.

1.5.1.2 Sputum colour

Sputum can be assessed visually for purulence that in turn represents an underlying airway neutrophilic inflammation. Airway neutrophils play a significant part in the airway inflammation in COPD patients.[153] The product of degradation of neutrophils, myeloperoxidase, is an indicator of a degree of inflammation. The amount of myeloperoxidase in sputum determines the colour of the sputum, which in turn defines the degree of the purulence.[156] Miravittles et al assessed a group of patients with COPD during clinical stability using five graded sputum colour tool and reported that more than 80% of patients with dark yellow or greening colour were positive to bacterial presence.[157] Stockley et al assessed a group of patients presented with moderate exacerbation of COPD and then reassessed them in two months later during clinical stability.[123] They demonstrated a clear relationship between the airway neutrophil count and sputum colour number at exacerbations. Furthermore, they reported that the green (purulent) sputum was associated with higher prevalence of airway bacterial presence at exacerbations but, opposite to the Miravittles et al report, not during clinical stability.[123, 157] Stockley et al concluded that sputum colour is a useful tool in management of exacerbations in an outpatient setting.[123] Although, Stockley et al used a 9-grade sputum colour assessment they reported their findings as green (purulent) and white (mucoid). Soler et al examined sputum purulence in a cohort of patients hospitalised with an exacerbation of COPD event.[158] The group also demonstrated that purulent sputum samples yielded higher airway bacterial presence. However, Soler at al divided the cohort into purulent and non-purulent based purely on patient's sole report and did not supplement it

with staff visual sputum assessment. One might argue that this is closer to the "real world" scenario, however, the objectivity of the purulence assessment remains unanswered to a degree.

Although there is evidence for using sputum colour as a surrogate for airway neutrophilia and airway bacterial presence it is important to note that the methods used for the assessment of the degree of sputum purulence had been performed using various assessment tools. These ranged from using a straightforward "purulent-yes/no" description approach to multi-coloured colour charts.[156, 158, 159] In an attempt to examine sputum colour with underlying airway inflammation and bacterial presence Stockley et al applied a nine-point sputum chart on patients with only chronic bronchitis and bronchiectasis not including patients with emphysema in an outpatient setting.[123, 156] Stockley et al summarised their findings into purulent and mucoid exacerbations groups. Soler et al used sputum colour to randomise patients with severe exacerbations of COPD to guide antibiotic treatment and assigned them to sputum purulent and non-purulent groups based on patients reported change in sputum colour.[158] Allegra et al used 10-colour Pantone catalogue chart when assessing the purulence from patients with severe exacerbation of COPD.[159] Interestingly, the group reported a high yield of bacterial isolates in both mucoid (colour 0-1) and purulent sputum (colour 2-9), opposite to the report by Stockley et al.[123, 158] More recently a Bronkotest, a five-point sputum colour chart, was introduced for an assessment of sputum purulence and association with airway bacterial presence. Daniels et al used a five-point sputum colour on patients with exacerbations of COPD and demonstrated an association between the purulent sputum (sputum colour 3-5) and bacterial presence.[160] In contrast, Brusse-Keiser et al in his study of severe exacerbations using five-point sputum colour chart reported a weak correlation thus advocating against assessing sputum colour as a potential marker of bacterial infection.[161]

Therefore, the assessment of the sputum purulence remains challenging with no universally accepted sputum colour chart and contradicting evidence on airway bacterial presence.

1.5.1.3 Sputum cellular markers

Sputum analysis, particularly, sputum cell differential, gives an insight into the airway inflammatory processes associated with respiratory diseases. Neutrophils and eosinophils represent markers of underlying airway inflammation in COPD.

1.5.1.4 Neutrophils

Recruitment of neutrophils to the airways is regulated by various mediators. CXCL1 and CXCL2 via CXCR2 attract neutrophils to the airways. TNFα and LTB4 play a role in stimulating activation of neutrophils. In particular, activated neutrophils may initiate chronic airway inflammation directly by producing neutrophil elastase, leukotriene B4, IL-8 and indirectly by triggering a cascade of chemokines and cytokines that maintain perpetual inflammation and lead to alveolar destruction and emphysema.[162] It has been suggested that neutrophil elastase is a major mediator of bronchial inflammation by means of damaging epithelial cells, causing goblet cells hyperplasia and mucus hypersecretion. Neutrophil elastase has previously demonstrated that it might also inhibit lung defences through reducing immunoglobulin function, delayed mucociliary clearance and proteinase inhibitors.[163, 164]

Previous studies have demonstrated that sputum neutrophils are increased in smokers and patients with COPD compared to healthy individuals.[153, 165] However, despite the increased airway neutrophils, these cells in COPD population appear to have different chemoattractant properties, higher speed but less migratory accuracy, compared to healthy subjects.[166] The immediate assumption is that the higher the airway inflammation the worse/lower the degree of airflow limitation. Some authors demonstrated such an association between the airway neutrophils and FEV1 at exacerbations.[124, 167] However, Singh et al reported only a weak association between airway neutrophils and FEV1% during clinical stability.[61] They also found no relationship with the rate of exacerbations and emphysema.

Neutrophils play an essential role in the antimicrobial response of the lungs that is enhanced even further during an exacerbation event.[168] Sethi et al found that the presence of bacteria during clinical stability was associated with neutrophilic airway inflammation.[45] Furthermore, as described earlier, airway neutrophils at exacerbation were found to be significantly associated with airway bacterial presence by some authors.[169] Papi et al also reported that the influx of neutrophils in the airway lumen is not limited to bacterial presence but also proposed an association with viral infection.[124]

TNFα plays an important role in pathogenesis of COPD and previously appeared to be a plausible cytokine to target in the treatment of COPD. Rennard et al reported a study with Infliximab in stable moderate-severe COPD patients and concluded that these patients did not benefit from the Infliximab treatment. Furthermore, the concern over the more cases of malignancy and pneumonia in the group treated with Infliximab.[170] Aaron et al examined the role of anti-TNF α treatment at exacerbations using a different agent from Rennard et al (Etanercept).[171] Aaron et al compared if Etanercept was more efficient to the systemic corticosteroids treatment in

management of exacerbations of COPD. Aaron et al found that Etanercept was not more effective than Prednisolone in management of exacerbations. To date there is no accepted neutrophil-targeted treatment available on the market for management of COPD thus further research into biomarkers of neutrophil associated inflammation in COPD is required.

1.5.1.5 Eosinophils

Eosinophilic inflammation was historically thought to be a feature of asthma with neutrophilic inflammation being a classical hallmark of COPD.[121, 165, 172-174] However, previous studies have demonstrated that eosinophilic inflammation is present in a subset of COPD patients both during clinical stability and at exacerbation.[47, 175] Th2 cells stimulate eosinophilic inflammation via secretion of IL-5 cytokine. Bafadhel et al assessed sputum IL-5 for patients with stable COPD and response to treatment with corticosteroids.[176] They reported an association between sputum IL-5 and sputum eosinophilia (≥3%) , and reduction in IL-5 level in response to oral steroids. Sputum eosinophil >3% is an accepted marker of airway eosinophilic airway inflammation and derived from the reported enhanced response in this group of patients to corticosteroids at a stable state.[175, 177] Furthermore, COPD subjects during clinical stability with sputum eosinophils>3% demonstrated a significant improvement in reduction of sputum eosinophils, greater improvement in FEV1 and health status after a short course of oral steroids. [175, 177] Fujimoto et al compared patients at stable state and during exacerbations and found a significant increase in all airway cellular markers, including sputum eosinophils, during exacerbations of COPD, thus suggesting that not only sputum neutrophils but also sputum eosinophils play important role in the pathogenesis of exacerbations of COPD.[178] In the light of this finding, Bathoorn et al examined the hypothesis that the beneficial response to steroids during an exacerbation is due to suppression of airway eosinophils. After a run-in period, at exacerbation they randomised patients to different treatment arms: LABA/ICS and placebo tablets, oral steroids and placebo inhalers and placebo tablets and inhaler. They found that high dose of LABA/ICS compared to placebo significantly suppressed airway eosinophils and improved symptoms. Papi et al proposed that the rise in airway eosinophils was related to viral infection although other authors found no such association.[47, 124] Furthermore, there is emerging evidence of an association between airway infection and eosinophils. Kolsum et al in their study demonstrated that at exacerbations lower airway eosinophils were associated with higher airway bacterial presence.[179]

There is also evidence to suggest that patients with sputum eosinophilia had a greater improvement in FEV1[175, 177], reported symptoms and reduction in sputum eosinophil count following oral corticosteroids.[175] Another trial of inhaled corticosteroids in an EI group showed

small but significant improvements in FEV1 however, no striking change in their sputum eosinophilia.[180]

Previous asthma studies have demonstrated that a management strategy aimed at minimising airway eosinophilia improved the rate of exacerbations.[181] In an attempt to test this hypothesis in COPD cohort Siva et al randomised a cohort into two treatment groups: first followed the traditional guidelines and second with a strategy targeted at reduction of eosinophilic inflammation (maintaining airway eosinophilia <3%). They demonstrated that the strategy of targeting airway eosinophilia was associated with a significant reduction of severe exacerbations but no difference was seen in mild or moderate exacerbations.[182] In another study high eosinophilia was reported to be one of the significant hazards associated with increased exacerbations after the withdrawal of ICS.[183] Furthermore, Contoli et al in their randomised proof of concept study of LABA/ICS versus LABA demonstrated that only patients with lower sputum and blood eosinophils that were treated with LABA/ICS had higher bacterial load compared to LABA/ICS arm with raised sputum and blood eosinophils.[184] Thus there is a mounting evidence to suggest that patients with eosinophilic inflammation should be treated and be maintained on corticosteroid treatment whereas for those patients with no eosinophilic inflammation corticosteroid treatment does not appear to be beneficial and may perhaps even be harmful.

1.5.1.6 Limitations to sputum analysis

Although sputum analysis is an important tool for an assessment of airway inflammatory process there are several disadvantages to sputum collection. Firstly, the success of satisfactory sputum sample collection varies and some patients are generally unable to produce any sputum.[123, 152, 158, 185] Secondly, although it is considered to be a safe procedure in patients with airways disease, there are rare side effects that are associated with the procedure.[186, 187] From the experience of sputum sampling in AERIS cohort some patients did not like the salty taste during sputum induction. Thirdly, sputum sampling and sputum processing require trained staff.[185] A further limitation of sputum analysis is that it represents a whole central airway but not distal airways and it is not specific to a particular location of the bronchial tree.[151] Furthermore, sputum sampling and sputum processing are not widely used in clinical practice thus it is not possible to translate these findings into clinical practice. Therefore attempts are made to identify sensitive and easily accessible systemic biomarkers that accurately identify the type of airway inflammation and bacterial presence.

1.5.2 Systemic biomarkers

Although venepuncture is an invasive procedure it allows an easy and fast access to the assessment of systemic inflammation in a patient to guide the management.

1.5.2.1 White blood cells and neutrophils

White blood cells (WBC) or leukocytes are derived from the bone marrow stem cells and consist of neutrophils, eosinophils, monocytes, lymphocytes and basophils.[188] Neutrophils are the most common type of leukocyte and represents 50-60% of all granulocytes. Neutrophils are the first line defence cells activated in response to the invasion of a microbial pathogen. After the initial local response triggered by the resident airway macrophages to the airway bacterial infection, a cascade of pro-inflammatory cytokine and chemokines are produced that in turn recruit activated blood neutrophils into the airways.[189] Pneumonia is a common example of a systemic neutrophil response to airway bacterial invasion. In the study of patients admitted with severe and non-severe community acquired pneumonia plasma concentration of cytokines (IL-6, IL-8 and TNFα) and blood neutrophil functional state were reported to be enhanced in patients with pneumonia compared to the healthy controls.[190] Moreover, patients with severe compared to non-severe pneumonia demonstrated a suboptimal lung inflammatory response and exaggerated systemic inflammatory response.[190] Namely, a moderately but significantly reduced phagocytosis-stimulated respiratory burst neutrophils activity (as measured by the production of H2O2 stimulated by the phagocytosis of S. aureus), sputum cytokines (IL-1β, IL-6, and TNFα and IL-17) and enhanced systemic response (IL-6, TNFα, and IL-8) when compared to the non-severe exacerbations.[190] In contrast, in COPD increased numbers of WBC were reported to be mildly elevated in patients during exacerbations of COPD.[191] Furthermore, Thomsen et al reported that simultaneously raised leukocytes along with CRP and fibrinogen were associated with an increased risk of future exacerbations of COPD.[192] Neutrophils are known for their antibacterial role which is increased during AECOPD.[168, 188] Neutrophils are recruited to the site of infection with the primary goal of phagocytosis and destruction of invading microorganisms.[193] Furthermore, exacerbations of COPD associated with airway bacterial infection evoke systemic responses and an increase in circulating neutrophils was also observed.[59, 123, 158, 194] Thus neutrophils play an important role and previously demonstrated an association with airway bacterial presence. However, it is not clear how accurately systemic neutrophils could be used to detect airway bacterial infection in COPD.

1.5.2.2 Blood Eosinophils

Previous studies demonstrated a good relationship between airway and systemic eosinophil counts.[47, 195, 196] Different cut offs expressed both in terms of absolute cell numbers and percent of eosinophils in the blood have been explored in an attempt to describe a clinically relevant sputum eosinophilic phenotype. It was previously reported that a 2% blood eosinophil cut off had a high sensitivity in identifying >3% airway eosinophilia during AECOPD[47], suggesting that peripheral eosinophils are a clinically more accessible marker to predict airway eosinophilia. In different studies alternative cut offs have been applied with variable predictive results. [197, 198]

Singh et al examined a longitudinal eosinophilic phenotype in the ECLIPSE cohort using 2% of blood eosinophils as a cut off for sputum eosinophilia. He reported 37.4% of subjects had persistently elevated blood eosinophils, 46.5% intermittently and 13.6% persistently low (<2%) blood eosinophils.[195] However, the ECLIPSE study did not have a benefit of prospective assessment of exacerbation visits. There was some evidence to suggest that the group with persistently elevated serum eosinophils had better clinical characteristics such as higher FEV1% and lower mMRC and SGRQ scores but exacerbations were not sampled nor infection rates estimated.[195] Some authors report a negative relationship between eosinophils and FEV1[199, 200] but this finding was not supported by other studies.[33, 201] A higher pneumonia risk was previously reported to be associated with long term ICS use in COPD patients.[104, 105] However, in the meta-analysis of 10,861 patients from ten trials (ICS treatment versus controls) Pavord et al examined if the risk of pneumonia irrespective of ICS treatment could be identified using blood eosinophils (≥2%) as a biomarker.[202] The group reported that there was a slightly higher prevalence of pneumonia in patients with blood eosinophils of less than 2% compared to the group with the blood eosinophils of 2% or more irrespective of ICS treatment.[202] Although this meta-analysis was performed retrospectively on ten studies, these findings may be clinically relevant when stratifying patients at higher risk of pneumonia.[202] However, the magnitude of the increased pneumonia risk was small therefore this needs to be tested in further studies. Data from the Copenhagen study suggest peripheral eosinophilia was associated with an increase in both moderate and severe exacerbations in COPD patient but this is not a consistent finding.[195, 198] There is an evidence to suggest that severe exacerbations of COPD with eosinophilic inflammation are associated with a shorter length of stay in hospital but no difference in re-admission rate in a UK based study.[203] Conversely, a US based study reported a higher readmission rate in those with higher eosinophils and no difference in the hospital length of stay.[204] In another study of severe exacerbations Couillard et al demonstrated that raised blood eosinophils (≥200cells/uL or ≥2%) predicted a threefold increase in readmission rate with exacerbation of COPD event.[205]

As mentioned for sputum eosinophil measurements, blood eosinophils may also help predict treatment responses. Pascoe et al in a post-hoc analysis of two double blind trials of LABA versus LABA/ICS investigated the usefulness of blood eosinophils as a biomarker of response to ICS treatment.[206] Pascoe et al reported that patients with the blood eosinophilia (≥2%) benefited from the additional inhaled corticosteroid. The ICS/LABA arm of this study had a lower exacerbation rate compared to LABA alone. There seemed to be no effect on blood eosinophils in patients in ICS/LABA arm.[206] Furthermore, a post-hoc analysis of the WISDOM (Withdrawal of Inhaled Steroids during Optimized Bronchodilator Management) trial revealed that blood eosinophils at screening were associated with higher exacerbation rates following ICS withdrawal in subjects with severe to very severe COPD and history exacerbations.[207] Thus eosinophilic inflammation is an important COPD phenotype but little is known about the persistency of eosinophilia in COPD population, eosinophil associated exacerbations of COPD and the impact of infection and thus seasonality. Better understanding of these phenotypes might optimise a phenotype-targeted approach with steroid treatment in this group of COPD patients

1.5.2.3 CRP, fibrinogen and IL-6

The most commonly studied blood markers in COPD are C-reactive protein (CRP) and fibrinogen. CRP and fibrinogen are acute phase proteins.[208] CRP and fibrinogen are primarily synthesized in the liver. Fibrinogen is a glycoprotein that takes part in the blood coagulation pathway, converting thrombin into fibrin. Increased levels of CRP are seen in variety of inflammatory conditions, particularly of infectious aetiology. CRP binds to polysaccharides in pathogens and activates the classical complement pathway. CRP and fibrinogen production is triggered by IL-6 - a major inducer of hepatic protein synthesis.[209] IL-6 is an inflammatory cytokine secreted by activated macrophages and monocytes in response to the inflammatory stimuli, for example, bacterial pathogen.[210] IL-6 is produced in the airway or blood in response to a stimulus, such as infection.[210, 211] IL-6 acts as a signal in T-cell activation triggers T cell differentiation and induces antibody secretion by B cells. CRP, fibrinogen and IL-6 were studied in the COPD and found that a subpopulation of COPD patients has persistently raised CRP and fibrinogen during clinical stability.[212-214] Although some large studies reported an association of fibrinogen and FEV1[215]; no such relationship was confirmed by others when these findings were adjusted for age, sex, BMI and smoking history.[214] FEV1 decline as a surrogate marker of disease activity was not associated with fibrinogen in ECLIPSE study.[215] However other authors reported that raised CRP, fibrinogen and Il6 were found to be associated with reduced FEV1 in COPD.[214, 216] Broekhuizen et al reported that irrespective of FEV1 raised CRP was associated with reduced exercise capacity and health

status.[217] This finding was confirmed by Garcia-Rio et al, they reported that raised CRP and Il6 were inversely associated with exercise tolerance.[214] Reported data on correlations between airflow obstruction and CRP levels is inconsistent with reports of weak to moderate correlations between FEV1 and CRP.[214, 217] CRP level was found to be increased during exacerbations[218] and reduced in patients on inhaled corticosteroids providing a good evidence for a potential biomarker.[212]

CRP, fibrinogen and IL-6 were also studied in the context of COPD exacerbations and were found to be associated with increased exacerbations and mortality.[218, 219, 220] Specifically, CRP was found to be useful in confirming an exacerbation visit when associated with a major exacerbation symptom.[218] Furthermore, fibrinogen was studied in the context of predicting future exacerbation events and high fibrinogen was associated with an increased risk of severe exacerbations over the following twelve months.[221] In another study of severe COPD exacerbations, raised CRP levels along with body temperature were associated with viral and mixed viral/bacterial presence.[222] Some authors suggested that CRP was therefore useful in detecting airway infection at exacerbations.[47, 158, 223] Thus, there is mounting evidence that both CRP and fibrinogen have a potential role in identifying bacterial presence at exacerbations to aid antibiotic administration, however, further research is required.

1.5.2.4 Procalcitonin

Procalcitonin (PCT) is peptide precursor of the peptide Calcitonin.[224] It is produced by parafollicular cells of the thyroid gland and by the neuroendocrine cells of the lung.[224, 225] PCT is a marker of systemic inflammation and its sustained elevated release is observed during sepsis.[225] Particularly the use of PCT is advocated in sepsis of a bacterial aetiology.[224] PCT was previously studied and proposed to be a good guide for antibiotic treatment in patients with exacerbations of COPD.[226 227]

A study of a general population, admitted to the ICU setting with sepsis and a suspected diagnosis of infection, demonstrated that PCT was more discriminatory of sepsis compared to IL-6 and IL-8 in distinguishing sepsis from other severe systemic non-infectious inflammatory conditions.[228] These authors concluded that PCT is a promising biomarker of sepsis. In a different ICU based study of patients admitted with AECOPD PCT of >0.25 μg/L was a good marker of mortality.[229] PCT levels of less than 0.10 μg/L demonstrated a low likelihood of airway bacterial presence (40%) but there was no difference in PCT levels in groups with and without airway bacteria.[229] However, the sample size was small (n=39) and interestingly so was the bacterial yield which was remarkably

scarce (12.8%).[229] The authors reported that only HI and PA species were detected in sputum in five out of thirty one patients. Whether the authors tested sputum for other common COPD airway bacterial pathogens is unclear.[229] Bafadhel et al examined patients hospitalised with exacerbation of COPD, asthma and pneumonia and found that PCT and CRP in COPD were significantly lower than those with AECOPD.[223] Furthermore, Bafadhel et al demonstrated a strong association between CRP and PCT and proposed that CRP could be used as a guide to antibiotic administration in patients admitted with acute respiratory conditions illnesses. However, Soler et al in their study of severe exacerbations demonstrated no such association between CRP and PCT, moreover, they demonstrated no association between PCT and airway bacterial presence.[158]

There are no trials specifically investigating PCT in the context of bacterial colonisation in COPD. In their study Daubin et al reported that PCT level was less than 0.1 µg/L in patients with known bacterial colonisation (n=3) compared to the PCT of less than 0.25 µg/L in all patients with detected PA on admission to ICU (n=4).[229] A study on a paediatric patients, admitted with either suspected infection or severe burns, examined the usefulness of the PCT levels in different subgroups of patients: bacterial colonisation, bacterial, viral and local infections.[230] The group reported that the PCT level in patients with bacterial colonisation and no infection was increased in patients with bacterial, viral and local infections compared to patients with bacterial colonisation.[230]

A Cochrane review analysed the safety and efficacy of PCT guided treatment in patients with acute respiratory infection.[231] The review was based on 14 trials including a total of 4221 patients in different clinical setting (primary care, emergency department, hospitalised, including ICU). The authors concluded that PCT guided initiation/discontinuation of antibiotics was non-inferior to standard practice. Moreover, PCT guided treatment reduced the use of antibiotic prescription significantly. However, this review included patients from different clinical settings, with different underlying respiratory pathologies and with different severities of clinical symptoms. It is unsurprising that sepsis in the ICU will be associated with more enhanced inflammatory responses to acute respiratory infections treated in the community. Thus the recommendations of this Cochrane review for COPD population should be taken with caution.[232]

Stolz et al conducted a randomised double-blinded study of Procalcitonin guided antibiotic administration at severe exacerbations. Using a cut off of <0.10 µg/L suggestive of absence of bacterial infection, >0.25µg/L suggestive of bacterial presence and 0.10-0.25 µg/L of possible bacterial presence.[233] Stolz et al concluded that PCT use offered an advantage over the standard treatment regimen, reducing antibiotic use. However, this study included patients that had already been on antibiotics at the time of recruitment (22%) and the airway bacterial yield was

remarkably low in this cohort with severe exacerbations (37%). Furthermore, airway bacterial presence was not associated with raised PCT levels. In addition, there was no difference in PCT levels in patients with and without sputum purulence, and no difference in patients with different types of exacerbations as per Anthonisen et al.[143]

Therefore, the evidence for PCT as a biomarker of bacterial infection in COPD is inconsistent and further research is required to assess its role, particularly, in moderate exacerbations of COPD.

1.5.2.5 IP-10

Another biomarker of airway infection is IP-10. IP-10 is secreted by bronchial epithelial cells, monocytes, neutrophils and lymphocytes in response to TNF- α and interferon-γ and elevated in COPD patients in response to viral infection.[142, 234, 235] Quint et al examined COPD patients during clinical stability and at exacerbation. They demonstrated that serum IP-10 was increased at exacerbations of COPD and correlated with sputum rhinovirus load at exacerbations.[235] In their study of AECOPD phenotypes, Bafadhel et al also identified IP-10 as the best marker of exacerbations associated with respiratory viral infection.[47] There is therefore mounting evidence that IP-10 maybe a useful marker of respiratory viral infection, thus it is important to assess this biomarker in the AERIS cohort.

1.5.2.6 SPD

Surfactant protein D (SPD) is a large collagenous glycoprotein and a member of a family of innate immune molecules called collectins.[236, 237] SPD is secreted by type II epithelial and Clara cells in the lungs and is also detected in circulation. SPD plays a vital role in protecting lungs from developing emphysema and although the exact mechanism is not clear the most plausible mechanism is by reducing inflammation and oxidative stress in the lungs.[238] One of the ways of reducing inflammation is by accelerated clearance of microbial pathogens from the airways.[237] SPD mediates the host defence function, interact with leukocytes and modulate the function of phagocytic cells[236]. SPD acts as a pattern recognition receptor that binds to airway pathogens (including bacteria, viruses, and fungi), allowing recognition by alveolar macrophages thus facilitating phagocytosis and clearance.[236] SPD was evaluated in ECLIPSE cohort during stable visits. The authors reported that SPD level was significantly raised in COPD patients compared to controls (current and former smokers without airway obstruction).[239] These results are in line with previously reported findings.[240] Furthermore, in the ECLIPSE study, COPD patients with higher SPD levels during clinical stability were found to be at higher risk of future exacerbation events.[239] Furthermore, there is some evidence that SPD could be useful in diagnosing exacerbations of COPD, namely, the SPD levels were found to be increased at exacerbations compared to the

stable COPD visits in small observational studies.[240, 241] However, further research is required to confirm these findings.

1.5.2.7 TNT and BNP

Troponin-T (TNT) and B-type natriuretic peptide (BNP) are markers of myocardial injury and were previously examined in COPD population, particularly during acute exacerbations.[242 243, 244] Increased cardiac burden has been previously reported in patients during COPD exacerbations.[245] TNT and BNP were previously reported to be elevated in patients with AECOPD.[246, 247] In addition, during severe exacerbations of COPD elevated TNT and BNP were associated with higher morbidity and mortality.[243 248] Some studies also suggest that elevated cardiac Troponin in exacerbating COPD patients is associated with higher in-hospital mortality rate but others did not confirm this finding.[243, 249] Previous authors on the population based studies reported an association between acute respiratory infection events and cardiovascular morbidity.[250] Therefore, I tested if there is an association between exacerbation of COPD events, specifically of bacterial aetiology, and cardiac enzymes in the AERIS cohort.

In summary, there is no universally accepted objective measurement to aid the diagnosis of COPD exacerbation. Subsequently, inadequate diagnostic approach of an exacerbation event reflects on the approach to treating exacerbations that remains largely generic - “nebulisers, steroids, antibiotics”.

1.6 Treatment of COPD exacerbation

Large proportions of patients with an exacerbation are managed in an outpatient setting.[118, 251] Current guidelines advocate treatment of an exacerbation event with systemic corticosteroids and antibiotics.[91] However, there is no tailored approach to treatment of an exacerbation event, moreover, the response varies considerably and these corticosteroids in particular have a high side effect profile, whilst there is also the emerging spectre of antimicrobial resistance.

1.6.1 Corticosteroids in exacerbation of COPD

The subject of the benefit of using corticosteroids to treat exacerbations was addressed in a recent Cochrane review.[252] The Cochrane analysis of 13 studies that included 1620 patients reported that treatment of an exacerbation with systemic corticosteroids reduced the rate of treatment failure, had a lower rate of relapse but did not affect the mortality rate compared to placebo. After 3 days of treatment there was a significant improvement in symptoms, FEV1, increase in pO2 and decrease in pCO2 on arterial blood gases in those patients treated with

steroids. At the end of treatment, reports on dyspnoea were contradictory with some studies reporting a significant improvement whereas other authors a statistically non-significant improvement. Furthermore, the 72 h improvements in FEV1 and pCO2 were not maintained at end of treatment. Systemic corticosteroid treatment significantly reduced the duration of hospital stay for general inpatient COPD population. However, this was not reflected in a subgroup of COPD patients requiring ICU admission for assisted ventilation, where there was no difference in the length of ICU stay observed between the placebo and steroid-treated groups. The Cochrane review concluded that overall patients with an exacerbation of COPD benefitted from treatment with systemic corticosteroids. However, there was an increased risk of various adverse events associated with systemic corticosteroids (hyperglycaemia, osteoporosis, candidiasis, secondary adrenal insufficiency, Cushing's syndrome etc). Specifically, the likelihood of hyperglycaemia was reported by Cochrane to be three times higher in the treatment group compared to the placebo group. [252]

As discussed earlier, eosinophilic inflammation gained considerable attention due to its favourable response to steroid treatment during clinical stability. There is also a mounting evidence for the beneficial use of systemic corticosteroids in patients with eosinophilic inflammation at exacerbations.[171, 253, 254] The non-inferiority study of corticosteroid treatment demonstrated that biomarker directed (blood eosinophils cut off of >2%) exacerbation treatment was not associated with an increase in treatment failure or worsening of symptoms compared with standard exacerbation of COPD treatment.[254] Moreover, biomarker-negative patients who received systemic corticosteroids had greater rates of treatment failure than those biomarker-negative patients who received placebo.[254] In contrast, a pooled analysis of three randomised controlled trials of prednisolone versus placebo to treat exacerbations of COPD demonstrated that there was no difference in treatment failure in the group with lower blood eosinophils(<2%) but there was a striking difference in treatment failure rate in the groups with higher blood eosinophils. Namely, patients with higher blood eosinophil level at exacerbation had significantly reduced treatment failure rate when treated with Prednisolone compared to those treated with placebo.[253] Similarly, when the response to treatment was investigated in a cohort of patients with severe exacerbations with and without raised blood eosinophils, the authors reported that severe exacerbations were associated with raised blood eosinophils had shorter length of stay compared to those with lower blood eosinophil level however readmission rate was similar.[203] Therefore, the subgroup of exacerbating patients with eosinophilic inflammation appears to gain maximum benefit from the treatment of systemic corticosteroids.

1.6.2 Antibiotics for exacerbations of COPD

For patients with acute exacerbations of COPD and sputum purulence current guidelines advocate the use of antibiotics.[91] However the evidence for the efficacy of antibiotic administration in COPD exacerbations remains controversial.[123, 143, 255, 256]

Earlier randomised controlled placebo controlled trial by Anthonisen et al investigated if there was any benefit of antibiotic administration in patients with different types of COPD exacerbations. They defined Type I exacerbations as a combination of three major symptoms: sputum purulence, sputum volume and dyspnoea; type II – the presence of the two out of three major symptoms; type III – a combination of at least one major symptom and one minor symptom of upper respiratory tract infection within the last five days (sore throat, nasal discharge), fever without other causes, increased wheeze, increased cough, increase in respiratory or heart rate by 20% from baseline.[143] Patients were randomised to treatment with antibiotics or placebo upon exacerbation. The outcome was a treatment failure that comprised of exacerbations with no improvement in symptoms that did not require further intervention and exacerbations that required further intervention. Patients with type I exacerbations benefitted the most from antibiotic treatment with 20% greater success rate in the antibiotic treated group and 30% greater treatment failure (no resolution and deterioration) in the placebo group. The group with type II exacerbations demonstrated only 10% greater success rate in the antibiotic-treated arm and 11% treatment failure in the placebo group. Anthonisen et al also reported that type III exacerbations showed no benefit in antibiotic treatment and concluded that antibiotics should not be used in this group of patients[143] Subsequently, the Birmingham group conducted a randomised study using sputum purulence as a biomarker to guide antibiotic treatment in patients with moderate exacerbations of COPD. They demonstrated that patients with purulent sputum had higher bacterial load and patients with white sputum colour improved without antibiotic therapy (94% versus 6% deteriorated and required treatment). [123] More recent research was based on the Procalcitonin (PCT) as a marker of bacterial presence to guide antibiotic treatment.[223, 257] However, other authors concluded that PCT is not useful in differentiating between the bacterial and non-bacterial infections.[158, 258]

A recent Cochrane review that included sixteen trials and 2068 participants concluded that there was a significant benefit from antibiotic administration for patients with exacerbations of COPD requiring admission to ICU.[256] Although it was acknowledged that this review included only one trial based on patients admitted to ICU (n-93). In this review authors also concluded that there was a significant reduction in treatment failure in the antibiotic treated group compared to placebo in the analysis of all inpatient and outpatient studies conducted between 1957 and 2012.

However, when the analysis was limited to only currently used antibiotics (amoxicillin-clavulanic acid, trimethoprim/ sulphamethoxazole, doxycycline, penicillin) this difference in treatment failure did not persist.[256] Therefore, the effect of antibiotics as a treatment for both outpatient and inpatient exacerbations of COPD remains inconclusive.

Despite the advantages of antibiotics, concerns are being raised globally over the significant detrimental consequences of antibiotic use. Since the wide use of antibiotics, antibiotics' overuse and inappropriate prescribing led to an increase in bacterial resistance.[259] Furthermore, antibiotic-associated bacterial infections, eg. Methicillin resistant *Staphylococcus aureus* (MRSA) infections and *Clostridium difficile*-induced colitis that leads to high mortality, are getting more common. These antibiotic resistant infections lead to longer hospital stays, higher costs of treatment and increased mortality. The WHO responded to this global issue by setting a plan of action to reduce bacterial resistance by increasing awareness on antimicrobial resistance, strengthen surveillance and research, reduce incidence of infection and, more importantly, to optimise the use of antibiotics.[260] Thus the prescribing of antimicrobial agents in AECOPD should be tailored to the group that will benefit from it the most.

To summarise, whilst steroids and antibiotics have some utility they do not seem to work in all COPD exacerbations which is why targeted therapy is required.

1.7 Conclusion

In summary, there is evidence that exacerbation events are detrimental for COPD patients as these events worsen airway obstruction, impact health status and are associated with increased mortality. In addition, COPD exacerbations have an enormous socioeconomic impact. However, the management of exacerbation events remains generic and is not tailored to exacerbation phenotypes. A number of studies examined the role of biomarkers in identifying an exacerbation visit and specifically testing these biomarkers for their ability to detect bacterial presence. However, there is a gap in our understanding of the role of clinically available markers in identifying an exacerbation, specifically of a bacterial aetiology. Whilst other studies used a similar approach in assessing the value of biomarkers in identifying an exacerbation and the presence of airway bacteria there is no study that has followed these markers over time and in particular study of the dynamics of these markers before during and after exacerbations.

Thus, when I encounter a patient presenting symptoms suggestive of an exacerbation of COPD I firstly ask myself the question if this patient is genuinely exacerbating and secondly if I need to treat this patient with antibiotics or steroids. In order to make a decision on the antibiotic treatment a clinician needs to ascertain if an exacerbation event is triggered by the airway

bacteria. The most commonly method used is an investigation of a sputum sample for the presence of bacteria (direct marker). However, this test may take days for the result to inform us on the nature of the exacerbation. Another approach is to examine other surrogate markers (putative) to boost the confidence in the bacterial aetiology of an exacerbation. (**Figure 1.3**)

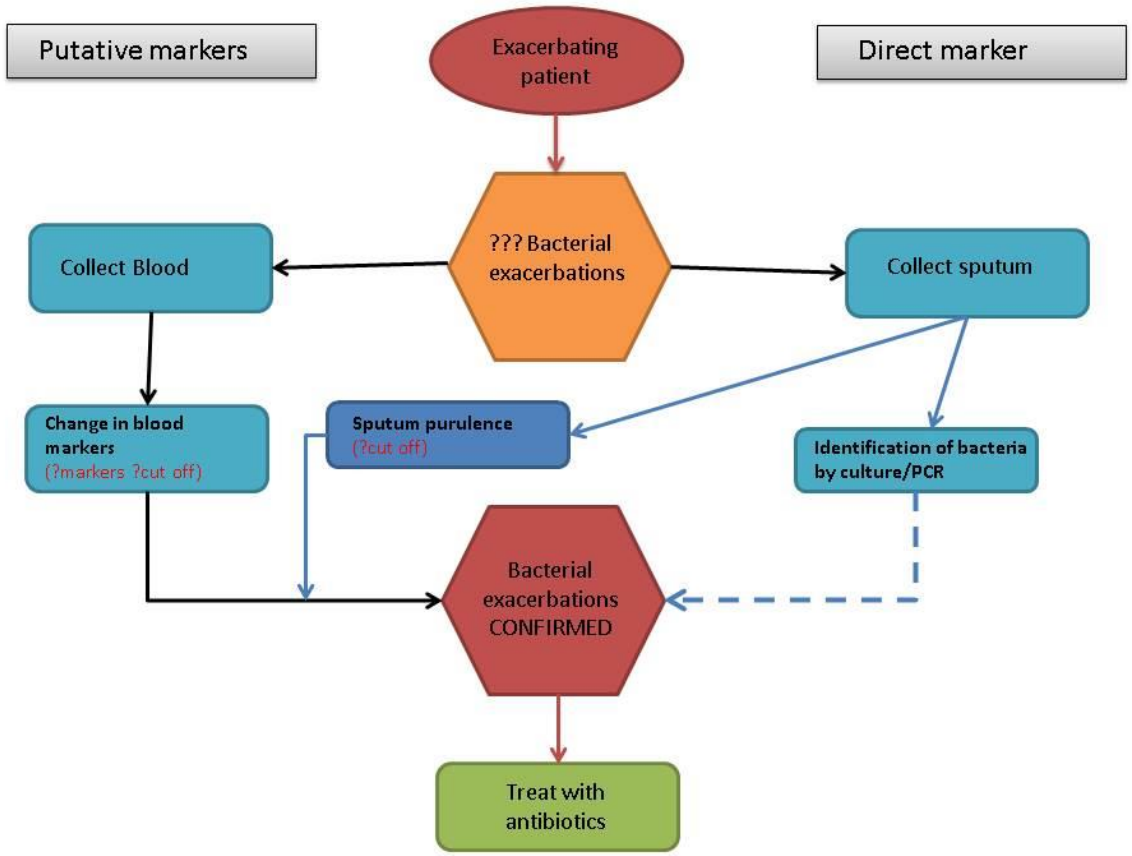


Figure 1.3 Flow diagram illustrating a "treat or not to treat" with antibiotics process.

Chapter 2 Hypothesis and Aims

As discussed there are currently no objective measurements routinely used in clinical practice to confirm an exacerbation event and specifically exacerbation phenotype (eg. bacterial aetiology).

My overall aim was to determine whether routinely measured biomarkers can be used, separately or jointly, to identify exacerbations of COPD and in particular bacterial infection at exacerbations. This would help guide appropriate antibiotic therapy and decrease reliance on steroids.

Therefore, my hypotheses were:

- Routine measures, separately or jointly, can identify exacerbations of COPD
- Routine measures, separately or jointly, can identify bacterial infection at exacerbations
- Biomarker defined endotypes of COPD are stable over time and hence may be used to stratify patient groups for future therapeutic trials

In order to test these hypotheses I determined whether:

Objective 1. differences in sputum colour can be used to identify a) exacerbations of COPD, b) bacterial infections at exacerbations (this is addressed in Chapter 4).

Objective 2. differences in airway and blood eosinophils measured over time can be used to predict a) exacerbations of COPD, b) bacterial infections at exacerbations (this is addressed in Chapter 5).

Objective 3. differences in blood biomarkers can be used to identify a) exacerbations of COPD, b) bacterial infections at exacerbations (this is addressed in Chapter 6).

Objective 4. these airways and blood biomarkers can be used in combination to identify bacterial presence (this is addressed in Chapter 6).

Chapter 3 Methods

3.1 AERIS study

I tested my hypothesis on the Acute Exacerbations and Respiratory InfectionS in COPD (AERIS) study. AERIS was a prospective epidemiological study of 127 subjects with moderate to very severe COPD patients with a history of at least one moderate exacerbation of COPD prior to recruitment. The primary aim of this study was to assess the occurrence of all cause exacerbations of COPD and the contribution of airway infection to exacerbations of COPD.[261] Therefore the study was powered to investigate the incidence of all-cause exacerbations of COPD and the occurrence of exacerbations with airway bacterial presence. The results of the analyses reported in this thesis represent tertiary objectives of the AERIS programme. Of the tertiary objectives the analyses of the present work focus on the sputum and blood measures as potential biomarkers of exacerbations and airway bacterial presence. The study was carried out from 2011 to 2014.

3.1.1 Ethics approval

The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice, and was approved by the Southampton and South West Hampshire Research Ethics Committee A (REC reference number 11/H0502/9). All participants provided written informed consent.

3.1.2 Study design and study participation

The AERIS[261] study is a single-centre, prospective, observational cohort study based at the University Hospital Southampton (ClinicalTrials.gov: NCT01360398). The recruitment phase was conducted over the 12 month period from June 2011 until July 2012. The study enrolled 127 subjects, male and female between the age of 45 and 85. All subjects were recruited with a confirmed diagnosis of COPD, post-bronchodilator FEV1 ≤80% FEV1/FVC ≤ 0.7 (GOLD stage II-IV), current or ex-smoker with smoking history ≥ 10 pack years. All subjects included had one or more exacerbations of COPD that were treated with antibiotics and/or steroids in the previous 12 months. Exclusion criteria included other known respiratory conditions such as asthma, as the only cause of the respiratory obstructive disorder, α-1 antitrypsin deficiency, cystic fibrosis, tuberculosis, lung cancer, previous history of lung surgery and other conditions imposing pneumonia risk. Subjects who were on a long-term antibiotic therapy at the moment of enrolment and those who had antibiotics and steroids in the previous 1 month prior to the

enrolment were also excluded. A full list of inclusion and exclusion criteria for AERIS study is presented below in **Table 3.1**.

Table 3.1. Inclusion and Exclusion Criteria for enrolment in AERIS cohort

Inclusion criteria
1. Subjects who the investigator believes can and will comply with the requirements of the protocol.
2. Written informed consent obtained from the subject.
3. Male or female subjects between, and including, 40 and 85 years of age, at the time of consent.
4. Subjects with confirmed diagnosis of COPD (based on post-bronchodilator spirometry). [262] with FEV1 of ≤80% of predicted normal and FEV1/FVC<0.7
5. Subjects have moderate, severe, or very severe COPD, according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) staging [262].
6. Subjects have a current or prior history of 10 pack-years of cigarette smoking. Former smokers are defined as those who have stopped smoking for at least 6 months. Number of pack years = (number of cigarettes per day/20) x number of years smoked.
7. Subjects present a documented history of ≥1 exacerbation requiring antibiotics and/or oral corticosteroids or hospitalization in the previous 12 months*.
**Subjects with recent COPD exacerbations, in stable condition, and having stopped antibiotics, can be enrolled one month post exacerbation.*
Exclusion criteria
1. Subject has a confirmed diagnosis of asthma (as only cause of obstructive respiratory disorder), cystic fibrosis, pneumonia risk factors or other respiratory disorders (e.g., tuberculosis, lung cancer).
2. Subjects having undergone lung surgery
3. Subject has a α-1 antitrypsin deficiency as underlying cause of COPD.
4. Subject who experienced a moderate or severe COPD exacerbation not resolved at least 1 month prior to enrolment visit and at least 30 days following the last dose of oral corticosteroids (subjects can be enrolled when their AECOPD or pneumonia has resolved).
5. Subject using any antibacterial, antiviral or respiratory investigational drug or vaccine up to 30 days prior to the enrolment visit.
6. Subject has other conditions that the principal investigator judges may interfere with the study findings, such as:
7. Subject at risk of non-compliance, or unable to comply with the study procedures.
8. Evidence of alcohol or drug abuse.

3.1.3 Subject participation

Subjects were seen for one enrolment visit and 24 scheduled monthly visits over the two-year period. Additionally, patients were seen at exacerbations.

3.1.3.1 Routine visits

Subjects were seen on a monthly basis (+/- 7 days) for routine follow up visits. Exacerbation visits replaced monthly visits if the former took place 2 week before or after the scheduled visit.

3.1.3.2 Exacerbation visits

In addition to routine monthly visits, research team conducted visits for exacerbating subjects within 72 h from the onset of symptoms. Exacerbation of COPD was defined as a combination of at least two major symptoms (dyspnoea, sputum amount and sputum purulence) or at least one major and one minor symptom (wheeze, sore throat, cold and cough) for two consecutive days. These criteria were published previously and subsequently validated on the East London cohort.[140, 143, 144] Exacerbations of COPD were captured via an e-diary. Each subject was provided with an e-diary and a peak-flow meter. Subjects were instructed to complete e-diaries on a twice daily basis, morning and evening. The morning questionnaire consisted of a list of questions aimed at registering worsening of symptoms within the last 24 h i.e. “in the last 24 hours did you experience a worsening of *breathlessness*?” When a combination of worsening symptoms persisted for at least 48 h, a potential exacerbation of COPD alert was transmitted to all members of the research group. A responsible clinician contacted the patient to confirm an exacerbation and arrange a visit within 72 h of the onset of symptoms. Along with the exacerbation alert sent to research team, the e-diary was programmed to instruct the subject to contact the research team as a safety net mechanism. In a few cases when the research team was unable to see subjects for the exacerbation visit, but exacerbation of COPD was confirmed by another clinician, those events were recorded as missed exacerbations.

All data from the e-diaries was transmitted centrally to the PHT website which was checked by the research team on daily basis. Subjects also completed a evening EXACT-PRO (Exacerbation of Chronic Pulmonary Disease Tool Patient Reported Outcome) questionnaire prior to sleeping. EXACT-PRO is a set of 14 questions designed to evaluate frequency, severity and duration of exacerbations of COPD.[263]

3.1.3.3 Exacerbation severity and treatment

All exacerbating patients were reviewed by a clinician on the day of the visit. It was a clinical decision if a treatment for exacerbation of COPD was required. Exacerbations were considered as

- **mild** if the attending clinician deemed that no treatment with antibiotics and/or oral steroids was required
- **moderate** – if the attending clinician deemed that treatment was required but that the patient was considered to be safe to be managed as an outpatient
- **severe** – if admission to the hospital was necessary.

Treatment of exacerbation of COPD was at the discretion of attending clinician and as per routine practice consisted of oral steroids and/or antibiotics of choice.

3.1.3.4 Study procedures and measurements:

A range of measurements was taken during the study period. A full list of all study procedures for the routine visits and exacerbations is presented in **Table 3.2**.

In addition to daily diaries, subjects were asked to complete CAT.

Biological specimens such as blood, sputum, urine, nasopharyngeal swabs and breath were collected. Blood samples were processed for biomarkers, biochemistry and cell-mediated immune response. Sputum was processed for biomarkers, culture and multiplex PCR analysis.

Study-specific Standard Operating Procedures (SOP) for all procedures in AERIS study were written in accordance with the Good Clinical Practice (GCP) requirements for all the AERIS procedures.

Table 3.2. List of study procedures

Type of contact		**Time points**
Data Collection		
Physical examination*		every visit
Anthropometrics[1] and nutritional screening		Quarterly
Tests		
Lung function testing	Lung volumes	M0, M24
	Gas transfer	Every 6 months and exacerbation
	Spirometry	Each visit
6-min walk test		Every 6 months
Questionnaires		
mMRC		Every 6 months
CAT questionnaire		Quarterly and exacerbations
Sample collection		
Blood sampling		Quarterly and exacerbations
Sputum sampling		Monthly and exacerbations
Diary card use		
Daily monitoring (e-diary card)		
-exacerbation symptoms (morning)		Daily
-EXACT-PRO (bedtime)		

**M0 – Month 0/enrolment*

3.1.3.5 Smoking history

Smoking history was measured in pack years. Smoking history = n(cigarettes a day)/20(number of cigarettes in a pack)*n(years smoked). Ex-smokers were considered those who quit smoking for at least 6 months.

3.1.4 Questionnaires

3.1.4.1 E-diary: Morning and evening questionnaires.

The use of these questionnaires is described in Exacerbations section.

3.1.4.2 COPD Assessment Test (CAT)

All subjects completed CAT questionnaire on quarterly basis and during exacerbation visit. CAT questionnaire is a 8-items tool designed to assess the degree of COPD burden on an individual, it is sensitive to changes in COPD status, hence, allows monitoring of COPD health status.[264]

3.1.4.3 mMRC (modified Medical Research Council) dyspnoea scale

All subjects completed mMRC during routine visits six-monthly. mMRC dyspnoea scale is a 0-4 response scale. It is based on the original MRC dyspnoea scale where dyspnoea was previously graded 1-5[265] and designed to assess the degree of disability due to breathlessness in COPD.

3.1.5 Sputum sampling and processing

3.1.5.1 Sputum sampling: spontaneous versus induction.

Sputum samples were obtained spontaneously only if subject was unable to perform sputum induction. A 3-step cleansing procedure was always undertaken prior to each sputum sampling attempt to ensure as little contamination from upper airways as possible. The 3 step procedure instructions are as follows: “rinse and gargle mouth with water; scrape and clear the back of the throat; blow nose to reduce any contribution from the nasal and sinus cavities.”

3.1.5.2 Sputum induction

All subjects had Salbutamol 200 µg prior to sputum induction. During sputum induction post-bronchodilator FEV1 was closely monitored using spirometry. Based on FEV1 (L) subjects were divided into two groups: those with FEV1 ≥1L and those with FEV1<1L. . Subjects with FEV1≥1L were administered 3 cycles of nebulized 3% Sodium Chloride solution at a rate of 2-4ml/min each lasting 7 min using an Ultrasonic Nebulizer (Devilbiss Ultraneb U3000). After each 7 min cycle, subjects attempted to expectorate sputum. All subjected were instructed to perform the 3-steps cleansing technique prior to the official cough attempts. Spirometry was then performed to ensure FEV1 did not drop more than 20% from the baseline measurement. A further cycle of 7 min inhalation was then commenced. The total inhalation period did not exceed 21 min.

A modified protocol was applied for the “at risk” group with FEV1 <1L. For this group, 0.9% Sodium Chloride was administered at a low output nebulisation (≤1ml/min) using an Ultrasonic Nebulizer (Devilbiss Ultraneb U3000). Unlike the group with FEV1≥1L a cut off of 10% drop in FEV1 was applied at post-inhalation spirometry checks. Again the total inhalation period did not exceed 21 min. In both groups, sputum induction was terminated if subjects developed troublesome

symptoms, FEV1 dropped beyond the safety cut-off or when a sufficient sputum sample was obtained.

3.1.5.3 Sputum processing

Sputum processing was performed by the AERIS laboratory technicians. Sputum was collected in a Petri dish and processed within 2 hours of expectoration. Sputum plugs were selected from saliva in the Petri dish and mixed together. Sputum colour was then determined against the Southampton sputum colour chart (design of the chart and detailed sputum colour assessment is discussed below in relevant chapter). Selected sputum plugs were weighed in polypropylene tubes and divided for biomarkers and microbiology according based on weight with up to 0.2 g used for biomarker analysis with the remainder being sent to the microbiology lab for culture and bacterial identification. In cases when less than 0. 1g of sputum was obtained microbiological analysis was prioritized. A detailed flow chart for sputum sample prioritisation is below in **Figure 3.1**

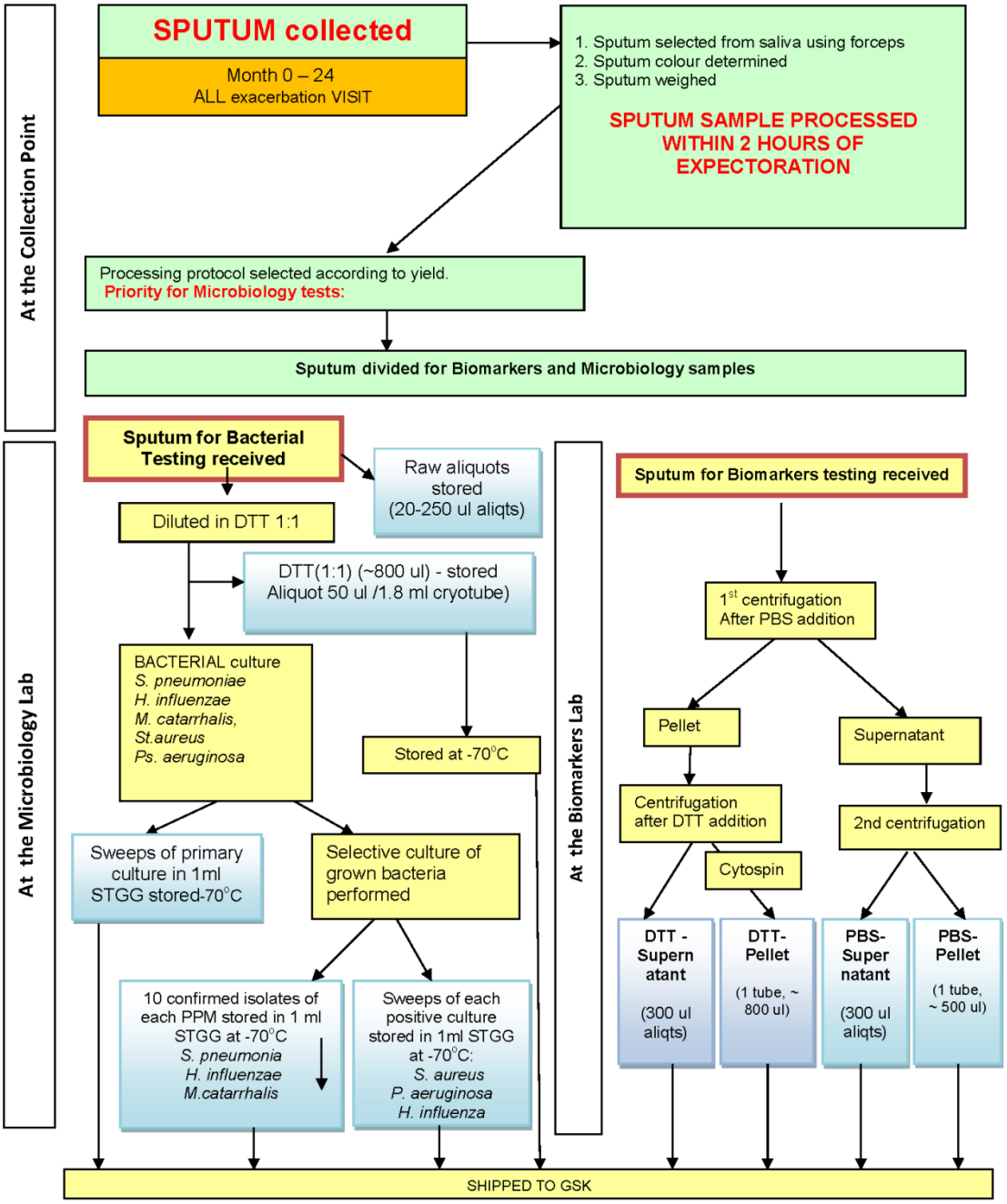


Figure 3.1 Sputum sample flow chart for biomarkers and microbiology samples

3.1.5.4 Sputum processing for biomarkers

The following two-step procedure was used to maximise the detection of inflammatory cytokines in sputum as the detection of many such analytes can be affected by the presence of dithiothreitol (DTT). 8 volumes of phosphate-buffered saline (PBS) was added to the sputum, vortexed for 15 sec and then incubated on ice on a bench roller for 30 min. Sampled then was centrifuged at 790g 4˚C for 10 min. This resulted in formation of a pellet and a supernatant. At this point sample was divided into two types of sample: a PBS sample and a DTT sample.

PBS sample

Six volumes of the supernatant was removed and centrifuged further at 1500 g 4˚C for 10 min. The supernatant was aliquoted and stored at -70˚C.

DTT sample

2 volumes of 0.2% DTT (Sigma, Poole, UK) were added to the remaining 2 volumes of supernatant. Samples vortexed for 15 sec and incubated on ice on a bench roller for 30 min. The sample was vortexed again, filtered using a 100µM cell strainer and the resulting filtrate was centrifuged at 790 g 4˚C for 10 min. The DTT supernatant was aliquoted and stored at -70˚C. The DTT pellet was resuspended in 1 ml of PBS and cell count performed using the tryptan blue exclusion method using a haemocytometer. The cell suspension concentration was then adjusted to 0.5 x 10^6 cells per ml with PBS and cytospin slides prepared using poly-L-lysine coated glass slides loaded onto a Shandon Cytospin3 centrifuge. Slides were centrifuged at 450 rpm for 6 min.

3.1.5.5 Cell differential count

After drying overnight, the slides were stained with haematoxylin and eosin using the Diff-Quick Rapid Romanowsky stain according to the manufacturer's instructions. A differential count was then performed on a maximum of 500 cells per slide at 40x magnification. This count included eosinophils, neutrophils, macrophages/monocytes, lymphocytes, bronchial epithelial cells and squamous cells. Squamous cells were regarded as a measure of a sputum quality. Sputum quality was considered acceptable if squamous cells were < 30%; fair if 30-60%; inadequate if >61%.[61]

3.1.5.6 Sputum processing for microbiology

A detailed sputum sample flow for microbiology and biomarkers presented in **Figure 3.1**.

Sputum was analysed to identify potentially pathogenic microorganisms common in COPD patients: *H.influenzae* (HI), *M.catarrhais* (MC), *S.pneumoniae* (SP), *S.aureus* (SA) and *P.aeruginosa*. Microbiology sputum samples were processed by a dedicated team of biomedical scientists, at Public Health England, within 6 hours of expectoration. Sputum sample was diluted with 0.2% (w/v) DTT in 1:1 ratio. The DTT sample was then inoculated onto a selective agar plates and incubated in 5% CO2 @37.0 for 24-48hrs. Selective plates included: Blood agar (horse blood) - a general plate that will grow all bacteria except HI; Chocolate agar (lysed horse blood) - nutritionally rich and grows all bacteria including Haemophilus; Bac – a chocolate agar plate

containing Bacitracin, this antibacterial agent eliminates all gram positive bacteria leaving only gram negative (HI and MC); CAN – blood agar plate containing antibiotics against gram negative bacteria, leaving only gram positive organisms to thrive (SP and SA); CFC – a plate containing various antibacterial agents and where PA is the only organism that grows on it.

Morphologically-identified colonies of each organism were selected then off of the plate and subjected to a further series of tests to confirm the identity of isolates.

- **HI** isolates were confirmed by supplementing the agar with X+Vfactors. HI requires X+V factors to grow after 24 hours of incubation.
- **MC** isolates confirmed via gram film (negative cocci); oxidase test (chemical colour change), Tributyrin test (colour change) and DNase (zone of clearing around colony) test are positive.
- **SP** isolates are confirmed by optochin test positive (zone of growth inhibition of more than 14mm).
- **PA** isolates confirmed by growth on CFC plate (bright green colonies and gram negative rods)
- **SA** isolates were confirmed by Gram positive cocci and morphologically distinct white colonies. The coagulase/Staphylococcus latex test was performed to confirm the isolate.

3.1.5.7 Viral detection in the sputum

Sputum samples were also analysed for the viral presence (HRV, RSV, influenza virus, parainfluenza virus, human metapneumovirus, adenovirus, human bocavirus and coronavirus).

Nucleic acids were extracted from sputum using the Viral NA Small Volume Kit (Roche Diagnostics) as per the manufacturer's instructions. The xTAG® Respiratory Viral Panel (RVP) Fast v2 (Luminex) is a qualitative nucleic acid multiplex test intended for the simultaneous detection and identification of multiple respiratory virus nucleic acids in respiratory specimens.[266] It detects influenza A, including subtypes of influenza A (H1 and H3), and distinguishes between 2009 H1N1 and other H1N1 (seasonal) strains, influenza B, respiratory syncytial virus, human metapneumovirus, parainfluenza virus 1–4, coronavirus (OC43, 229E, NL63, HKU1),rhinovirus/enterovirus, adenovirus, and bocavirus.

A quantitative real-time PCR (RT-PCR) assay was used for the detection and quantification of a fragment of a conserved region of the 5' noncoding region of rhinovirus[267] in samples displaying a positive signal for rhinovirus/enterovirus by xTAG® RVP Fast v2. The concentration of rhinovirus RNA in each sample, expressed in copies per mL, was inferred from the calibration curve (made of serial dilutions of an in vitro transcript containing the sequence targeted by the RT-PCR assay)

present in each RT-PCR plate and corrected against the dilution factors at each step of the process(nucleic acid extraction and RT-PCR reaction).

3.1.6 Sputum colour chart

The degree of sputum purulence was defined by the intensity of the sputum colour i.e. the darker the sputum the more purulent it was. Sputum colour description varies from study to study, with some studies using four grades whilst other use eight grades.[154, 156] We designed a sputum colour chart with one-to-five grades, according to a suspected degree of sputum purulence. Sputum colour 1 defined as white/clear, 2 – light yellow, 3 – dark yellow, 4 – light green, 5 – greenish. The sputum colour chart was developed using Pantone formula guide. Grade-1 coded as Pantone Cool Grey 1 grade tinted to 10%. Grade-2 coded as Pantone 607, grade tinted to 30%. Sputum colour grade-3 coded as Pantone 461, grade tinted to 100%. Sputum colour grade-4 coded Pantone 615, grade tinted to 100%. Sputum colour grade-5 coded as Pantone 617, grade tinted to 100%. Sputum colour chart was printed using the University Print Centre services.

Specially trained laboratory staff assessed sputum colour. Sputum colour was evaluated against the sputum colour chart (**Figure 3.2**) by choosing the highest number that matched most of the sputum sample. Sputum samples were recorded in patients' CRFs and laboratory worksheets.

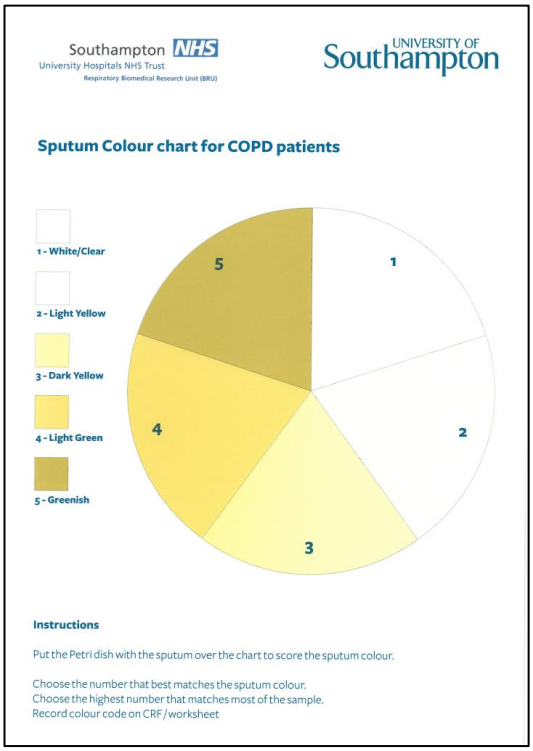


Figure 3.2 Southampton COPD group Sputum Colour chart

3.1.7 Blood sampling and processing

Venous blood was collected by venepuncture at enrolment, quarterly and exacerbation visits using standard techniques. Samples were processed for specific biomarkers including full blood counts, derived fibrinogen, C-reactive protein (CRP) and procalcitonin (PCT).

FBC, CRP and fibrinogen analyses were performed by the University Hospital Southampton laboratory. Procalcitonin was sent quarterly to Queen Alexandra Hospital, Portsmouth and processed on a Kryptor analyser. Surfactant protein (SP)-D, high sensitivity cardiac Troponin-T (hs-cTNT), Interleukin (IL)-6, Interferon-γ induced protein-10 (IP-10) and N-terminal pro-brain β-natriuretic peptide (NT-proBNP) were processed in the laboratory by GSK, Belgium. Immunoassay and ranges for SPD, IP10, IL6, BNP and TNT are summarised in **Table 3.3**. The range of IP-10, NT-proBNP, SPD and hs-cTNT was significantly wide, thus, to alleviate the interpretation of the odd ratios following rescaling methods were applied: IP10 and SPD were downscaled by 10 (interpretation of OR results changed to "per 10 Units"); BNP was downscaled by 100 ("per 100 Units"); and TNT upscaled by 100 ("per 0.01 Unit"). If samples for SPD, IL-6, IP10, BNP, TNT were not available or the values were below the limit of detection then the results were not reported and handled as missing data. For CRP when the reported values were <1.0 mg/L then the results was entered as 0.9mg/L.

Table 3.3 Blood Biomarker Immunoassay and ranges

Analyte	Vendor	Matrix Tested	Quantitation Range	Units
SP-D	Biovendor	Serum	7.8 – 2000	ng/mL
IP-10 (CXCL10)	R&D Systems	Serum	15.6 – 16,000	pg/mL
IL-6	Siemens Laboratory Diagnostics	Serum	2 – 1000	pg/mL
NT-proBNP	Siemens Laboratory Diagnostics	Serum	20 – 35,000	pg/mL
hs-cTNT	Roche Diagnostics	Serum	0.003 - 10	μg/L

For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

For routine biochemistry, blood was collected in 1x5ml BD Serum-separator Vacutainer® tubes (SST) and transferred directly to the pathology department at Southampton University Hospitals NHS Trust (SUHT) and processed by automated analysers. For the enrolment visit only, CRP was also analysed using this sample.

For biomarker analysis, 26.8 ml of whole blood was collected into 1x2ml BD EDTA Vacutainer® tube, 1x1.8ml BD citrate Vacutainer® tube, 1x6ml BD EDTA Vacutainer® tube and 2x8.5ml BD SST. Blood collected in the SST was allowed to clot for at least 30 minutes at ambient temperature. The SST tubes and the 6ml EDTA blood sample were then placed into a centrifuge at 1500-2000 g for 10-15 minutes at 20ºC with the brake on. Following centrifugation, aliquots of at least 0.5ml were aliquoted using plastic transfer pipettes into cryovials and stored at -70/80ºC in the SUHT Wellcome Trust Clinical Research Facility (WTCRF) until required for future processing. For all time points except enrolment, 0.5ml of serum was aliquoted from the SST 'biomarker' tube and transferred directly to the pathology department at SUHT for CRP analysis, described later in this chapter.

Blood collected for full blood count analysis, (1x2ml BD Vacutainer® tube) was transferred directly to the pathology department at SUHT and processed by automated cytometers.

Blood for derived fibrinogen levels (1x1.8ml sodium citrate Vacutainer® tube), based upon the prothrombin time (PT), was collected and processed in the pathology department at SUHT.

The values in table were taken as the normal reference ranges. (**Table 3.4**)

Table 3.4 Normal ranges for haematology blood samples

Blood count parameter	Unit	Reference range-Adult	Reference range-Male Adult	Reference range-Female Adult
Haemoglobin	g/L		130 – 170	120 – 150
White blood cell count (WBC)	*10^9/L	4.0 – 11.0		

Neutrophils	$*10^9$/L	2.0 – 7.5
Eosinophils	$*10^9$/L	0.04 – 0.5
Fibrinogen	g/L	2.0 – 5.0
C-reactive protein	mg/L	0 – 7.5

PCT

PCT measurement was carried out at the Department of Chemical Biochemistry, Portsmouth Hospitals NHS Trust, Queen Alexandra (QA) Hospital, Southwick Hill Road, Hampshire, UK using the B·R·A·H·M·S KRYPTOR analyser. This provides automated, immunofluorescent assays of PCT in human serum. 0.5ml aliquots of serum were stored at -70/80ºC in SUHT WTCRF. When required, samples were transferred in dry ice with a temperature monitor from SUHT to QA Hospital. For processing, samples were thawed at ambient temperature, centrifuged and analysed on a Kryptor analyser.

High sensitivity CRP

CRP measurement was carried out in the UHS NHS laboratory using the Beckman Unicel DXC system until May 2013, thereafter the analysis platform was changed to the Beckman Coulter AU500 system. Correlation between the two methods showed no significant differences. Briefly, both methods measure CRP concentration by a turbidimetric method. Following binding of an anti-CRP antibody-coated particle to CRP in serum, agglutination occurs with the formation of insoluble aggregates which can be measured by the rate of decrease in light intensity transmitted through the suspended particles. The linear range of the assay is from 0.2 to 480 mg/L, which represents high sensitivity, compared to routine CRP testing where the linear range is from 5 to 300mg/L.

3.1.10. Spirometry

Spirometry was performed with a Micro-Lab Carefusion spirometer in accordance with ATS guidelines by a trained individual.[268] Briefly, forced spirometry was selected on the spirometer and the flow volume graph screen appeared. The subject was seated and a nose clip was applied during the procedure. The patient was instructed to take a deep breath in filling their lungs to

total lung capacity, making a good seal around the mouthpiece and blowing out as hard and as fast as possible, and for as long as possible. This continued until the volunteers lungs were completely empty and they were encouraged throughout the process. The blows were repeated, or rejected as necessary and up to 8 blows were allowed to ensure repeatability or 3 blows with the best blow. Data was only used if it was deemed to be of sufficient quality i.e. the two highest FEV1 values and two highest FVC values were within 5% OR 150mls of each other respectively. Spirometry results recorded were FEV1, FEV1% predicted, FVC, FVC% predicted, FEV1/FVC ratio, FEF25%–75% and FEF25%–75% % predicted. For the purposes of using only good quality data in the study analyses the spirometry readings were categorised into A, B and C: quality A – acceptable spirometry with all three attempts FEV1 and FVC within 5% (150ml), quality B – acceptable with the FEV1 and FVC of at least two attempts were within 5%, quality C – unacceptable when only only one good quality attempt was produced.

3.1.11. Plethysmography

This was performed in the AERIS study using a Body Plethysmograph HDpft 4000 (Inspire Healthcare Ltd.) Briefly, a sterile mouthpiece was placed on the on the end of the disposable viral filter and it was adjusted so that it was the correct height for the patient. The plethysmograph door was closed and the patient was instructed to place the mouthpiece in their mouth (ensuring a tight seal and no leaks) and attach their nose clip and breathe normally. After 5-6 breaths a vertical line appeared on the computer software, after which on exhalation the space bar was pressed, causing the shutter to close on the subject's next inspiration. The subject was coached to pant against the shutter aiming for a frequency of 30-60 breaths/min (0.5-1Hz) for 3-4 breaths. Following the panting manoeuver the subject was asked to perform an SVC manoeuver by breathing into TLC and then slowly exhaling to RV and holding for 2-3 s before returning to tidal breathing. The space bar was pressed to end the test. The VTG graph and alveolar pressure versus plethysmograph pressure loops were viewed and assessed for technical acceptability. If there was more than one loop then the VTG values of those loops needed to be within 5% of each other. A minimum of three acceptable measurements of FRCpleth were obtained and two measurements of FRCpleth must have been within 5% (Highest-lowest/mean) of each other and the mean reported. The two best IC measurements must have been within 150 ml or 5% of each other. The two best VC measurements must have been within 150 ml or 5% of each other.

3.1.12. Gas transfer

Gas transfer was performed using a Collins CPL machine or HDpft4000 by a trained member of staff following the SOP. Briefly, tidal breathing was traced to ensure there are no irregularities.

The patient was then instructed to exhale slowly to residual volume and tap their leg when they had blown out all their air. At this point the space bar on the computer software was pressed and the patient instructed to immediately take a deep and full breath into TLC. When TLC was reached the V key was pressed which shut off the valve thus preventing air going back into the bell spirometer. The patient was then asked to hold their breath for 10 s until the red sample line reaches the vertical lines at which point they were asked to breathe out with medium force until RV (the red sample line touches/plateaus with the RV dotted blue line). The space bar was pressed at RV. Four minutes were given between tests, allowing adequate washout of the gases from the lungs. Tests were acceptable if SOT (complete inspiratory breath) were within 2 s, breath hold were between 8 and 12 s and complete exhalation was within 4 s. A maximum of 5 tests were performed and two DLCO results needed to be within 10% of each other.

3.1.113. 6-Minute Walk Test

The 6MWT was performed by a suitably qualified member of staff, on a 30 metre pre-prepared course. A finger-tip pulse oximeter was attached to the patient's finger and the oximeter checked to make sure that it is gave a good signal. The oximeter readings were observed for at least five minutes to ensure stability and to ensure the patient was rested. Baseline measurements at rest were recorded (heart rate, oxygen saturation). The Patient was instructed to walk round the pre-prepared course at their own pace for 6 min and were informed they could stop to rest if necessary. The number of laps walked in 6 min, end SpO_2, lowest saturations and end heart rate were noted. Measurements of SpO_2 and heart Rate were recorded at 1 and 2 min post exercise.

3.1.8 Statistical analysis

The distribution of continuous variables was assessed using histograms. Normally distributed data was presented as mean (SD). Most data presented was non-parametric and are presented as median (IQR). Correlations between non-parametrically distributed continuous variables were assessed using Spearman's rho. Bivariate analyses testing for differences between independent groups were conducted using Kruskal-Wallis, ANOVA, Chi-Square, or Fisher's Exact test, as appropriate. Bivariate analyses between paired groups were conducted using Wilcoxon Signed Rank test or paired t-test as appropriate, based on the distribution of the difference, or McNemar test for categorical data. Receiver Operator Curves (ROC) area under the curve was used to assess associations between biomarkers and binary outcomes of interest, and sensitivity and specificity was used to assess the predictive ability of different cut offs to identify the presence of a given outcome e.g. exacerbation, sputum eosinophilia. Intra-class correlations were used to assess the reliability of measures within individuals over time. Because some subjects were represented

multiple times descriptive analyses were limited to the enrolment visit or the first exacerbation visit occurring to each subject. Multivariate analyses with binary outcomes (eg. presence/absence of different conditions) were conducted using conditional logistic regression, including the subject number as a random effect to account for multiple observations per subject. Kaplan Meier survival curves were used to examine time to first exacerbation, with log rank test used to assess differences in time to first exacerbation between different groups. Cox proportional hazard regression was used to assess the association between different biomarkers/groups and the hazard of first exacerbation.

Multiple comparisons were made in the analyses, but no multiple comparison correction was applied. It is acknowledged that when multiple comparisons are made, there is an increasing likelihood that a Type I error will occur (e.g. false discovery phenomenon). Whilst some authors advocate to apply the multiple testing correction, such as Bonferroni correction, to counteract the problem with multiple comparisons.[269], this approach comes at a cost of potentially producing false negative results (Type II error).[270]. Therefore, on balance, following statistical advice it was decided that the data would be reported without multiple correction. The risk of a Type I error is acknowledged.

Machine learning classifiers were used to assess the value of combined biomarkers to predict the presence of bacteria at exacerbation. To perform these prediction a logistic regression classifier was trained and evaluated on the complete dataset using a leave tenth out cross validation (LTOCV) method to obtain an estimation of the classifiers performance. This was represented in a Receiver Operating Characteristic Curve (ROC), where the Area Under the Curve (AUC) is a measure of a classifiers performance. Machine learning classifiers were implemented in Python using the toolkit Scikit-learn.[271]

For pulmonary function test, data of quality C was excluded. For sputum differential only good quality sputum was included for analyses (squamous cells <30%). Missing data was not imputed, and was excluded on a casewise basis.

SPSS (version 21) was used for all analyses with the exception of intra-class correlation coefficients (ICC) and conditional logistic regression, which were conducted using STATA (version 11). Some graphs were produced using Microsoft Excel 2010. All of these analyses should be considered post hoc as they were not pre-specified in the AERIS statistical analysis plan.

The analyses for my doctoral thesis were performed on the data for year 1 only as these were available at the time of the completion of my thesis. Furthermore, although the cohort was

relatively stable over the first year, the dynamics was such that few patients dropped out after the first year (105 patients completed Year 1 and only 88 completed Year 2).

Chapter 4 General demographics and subject characteristics

4.1 Introduction

Chronic obstructive pulmonary disease is a condition that encompasses different clinical subgroups and is known for its chronic progressive course punctuated with acute episodes of clinical deteriorations known as exacerbations. The need for a longitudinal, observational in COPD is great as further understanding of the course and nature of COPD, particularly COPD phenotypes and endotypes are required in order to aid personalised management[272-274].

The AERIS study offers a deeply characterised cohort of moderate to very severe COPD patients during both stable disease and at exacerbation over time. The AERIS cohort was a prospective observational study of 127 subjects with moderate-very severe COPD. Detailed inclusion and exclusion criteria, outline of study visits are described in the Methods section (Chapter 3). Briefly, only subjects with FEV1 of ≤80% of predicted who were current or ex-smokers were enrolled into the study. These subjects had to have at least one moderate-severe exacerbation of COPD over the previous 12 months and were not on long term antibiotics/oral steroids at the time of enrolment.[261] Patients were recruited from the primary and secondary care. After the completion of AERIS study some patients expressed their willingness in participating in upcoming respiratory research studies.

In this chapter I present the general AERIS cohort characteristics during clinical stability and at exacerbation. I also explore if participating in the research study was beneficial for the participants.

4.2 Results and comments

4.2.1 Cohort characteristics during clinical stability

At enrolment, the AERIS cohort represented a group of mature individuals with median age of 66.8 that were predominantly male. The cohort consisted of patients with severe airway obstruction as evidenced by a median FEV1 of 46.4%. These volunteers had a significant smoking history of 50.3 pack years and 43% of participants were still actively smoking at enrolment. This cohort of patients suffered from a median of 2 exacerbations in the year before the study, suggesting this cohort was enriched for the frequent exacerbator phenotype. (**Table 4.1**)

Table 4.1. Patient demographics and clinical characteristics of AERIS cohort at enrolment

	N	Median (IQR)
Age [α]	**127**	66.8(±8.6)
Smoking history (pack/years)	**127**	50.3(±28.2)
FEV1 (%)	**126**	46.4(25.0)
ΔFEV1(% of baseline)	**85**	4.93(21.05)
FEV1 reversibility	**105**	11.29(16.89)
KCO (mmol/kPa/min)	**123**	0.94(0.48)
TLCO(mmol/kPa/min)	**122**	4.47(2.32)
CAT	**126**	16.0(10.0)
EXACT-PRO	**101**	37.0(12.0)
BMI	**127**	27.0(6.7)
FFBM	**125**	48.9(21.5)
6MWT (distance in meters)	**125**	300(170)
Exacerbation rate 12 months before	**127**	2.0(2.0)
	N	**%**
Male	**68/127**	54%
Current smoker (Yes)	**54/127**	43%
Frequent exacerbators in Year 0*	**99/127**	78%

[α] reported as Mean(±SD); *frequent exacerbators in year prior to enrolment (Year 0) were defined as having 2 or more exacerbations.
For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

Sputum samples were collected monthly at routine follow up visits and at exacerbations. At the stable state 79.1% (n=959) of sputum samples were obtained. However, of these 952 samples were of sufficient weight for bacterial culture analysis. Overall, out of all stable visits 48.7% of sputum samples obtained (n=464 out of 952) were positive for bacteria by culture and 17.5% (18 out of 103) were positive for respiratory viruses. Antibiotics were administered prior to sample collection in only 1.1% (n=11) of stable samples.

In summary, the AERIS cohort represents a group of predominantly severe COPD patients with significant airflow obstruction and history of frequent exacerbations. There was a high prevalence of airway bacterial presence at stable state. My next assignment was to investigate general characteristics of the cohort during exacerbations.

4.2.2 Exacerbations in the AERIS cohort

To start with I analysed the total exacerbation rate over the first twelve months and found that there were 355 exacerbations in the AERIS cohort.

At all exacerbations there appeared to be a decline in physiological characteristics, namely, FEV1%, KCO and TLCO were lower than at enrolment (40.50, 0.85 and 4.15 respectively); whereas patient reported quality of life were raised as evidenced by the raise in CAT and EXACT-PRO scores , 22.0 and 329 respectively. Blood markers at exacerbations are presented and discussed in chapter 7.

Table 4.2 Clinical and physiological characteristics during exacerbations.

	N	Median (IQR)
FEV1 (%)	**322**	40.50(19.00)
KCO (mmol/kPa/min)	**261**	0.85(0.51)
TLCO(mmol/kPa/min)	**261**	4.15(2.46)
CAT	**346**	22.00(10.00)
EXACT-PRO	**329**	41.00(10.00)

For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

Sputum samples were collected during exacerbation visits with the majority of AECOPD samples (71%) collected within two days of the onset of exacerbation symptoms as assessed by a clinical team in response to an e-diary alert. 91.3% (n=324) of sputum samples were obtained during exacerbation visits but only 319 exacerbation samples were of sufficient weight for bacterial culture analysis. 8.6% (n=28) of volunteers received antibiotics prior to sample collection of exacerbation samples. 58.9% (n=188 out of 319) of exacerbation sputum samples were positive for bacteria by culture and 41.3% (n= 126 out of 305) were positive for respiratory viruses.

From the patients history the AERIS cohort represents a cohort of frequent exacerbators and this is supported by the prospective exacerbation frequency of the cohort (mean of 3.08 and median of 2.94). I therefore enquired how soon after the enrolment patients developed their first exacerbation of COPD. I thus conducted Kaplan Meyer test to generate a cohort overview of time to first exacerbation or to consent to withdrawal. This analysis demonstrated that approximately 50% of subjects either had their first exacerbation or withdrew their consent within 49 days. 19 individuals did not have an exacerbation episode in the first year (Year 1). Out of these 8 subjects

withdrew their consent prior to the end of Year 1 whilst 11 subjects had a full exacerbation-free year. (**Figure 4.1**)

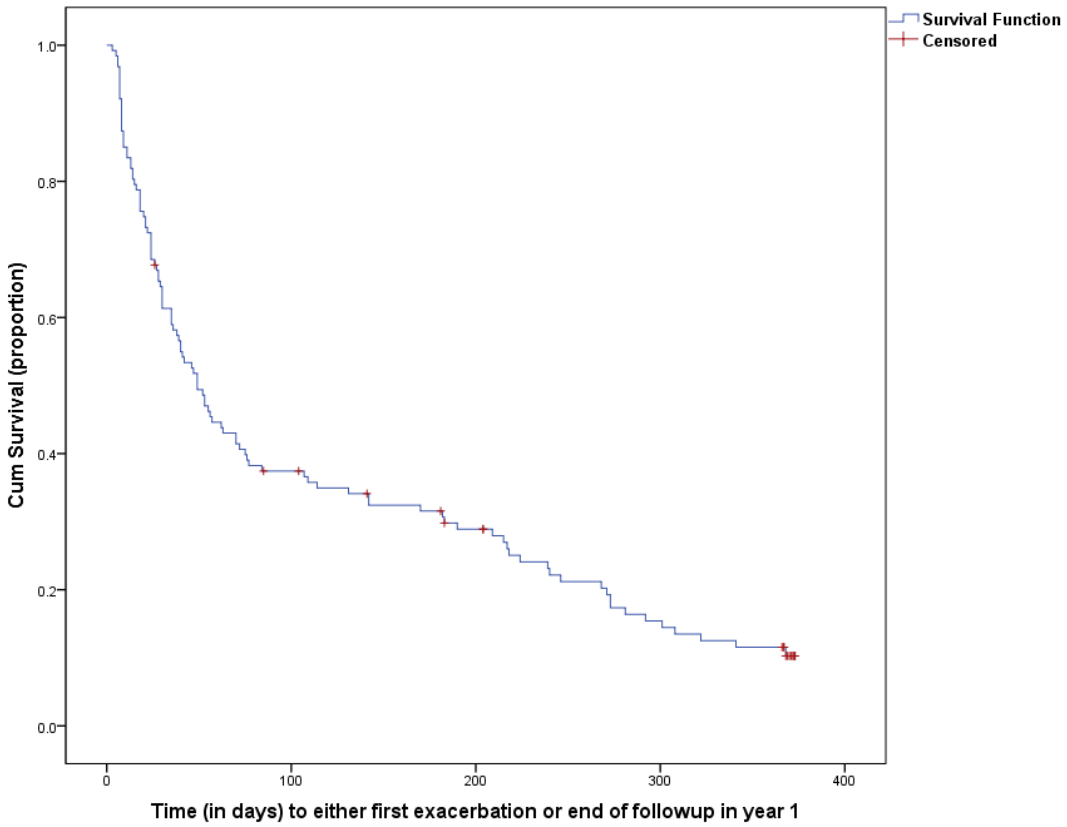
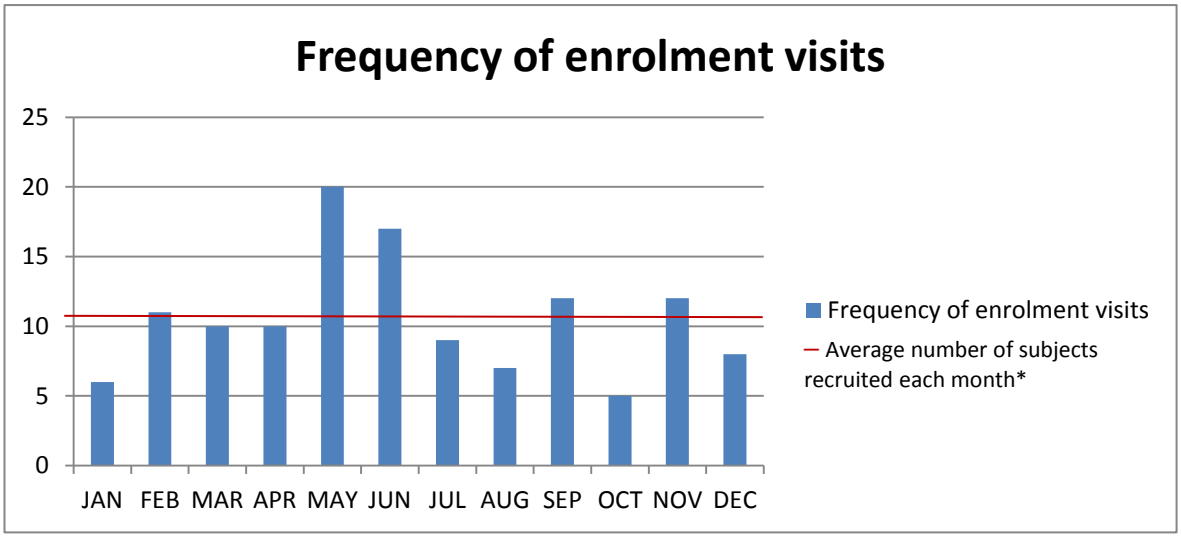


Figure 4.1 Kaplan-Meyer survival curve demonstrates an overview of time to either first exacerbation or till the end of observation period in 127 subjects. Median time to first exacerbation was 49 days. Censored indicate those patients who died during the study or otherwise withdrew their consent from the study.

One of the reasons that could explain such a volume of first exacerbations occurring in the first 49 days could be that patients were predominantly enrolled into the study immediately before or during high exacerbation seasons (i.e. Autumn and Winter) as reported previously.[275] In order to test this hypothesis I studied frequencies of enrolment visits per month. (**Figure 4.2**) The frequency of subjects recruited was relatively stable throughout the year with peaks seen in May and June which re not months associated with a high risk of exacerbations. Therefore seasonality of recruitment did not explain the reason why 50% of 1st exacerbations occurred within the first 49 days. A further hypothesis was that by study design, the AERIS was a cohort of frequent exacerbators (≥1 exacerbations over 12 months prior to study recruitment i.e. in Year 0)



*Average number of subject recruited each month if recruitment was even throughout the year

Figure 4.2 Bar chart representing the frequency of enrolment visits by month of enrolment

At enrolment there were 99 frequent exacerbators (2 or more exacerbations in the Year 0) and 28 non-frequent exacerbators (1 exacerbations in Year 0) in the cohort. The median time to first exacerbation was longer for non-frequent (114 days) than for frequent exacerbators (49 days). Thus the time to first exacerbation appears to have been driven by the large proportion of frequent exacerbators included in the cohort. (**Figure 4.3**)

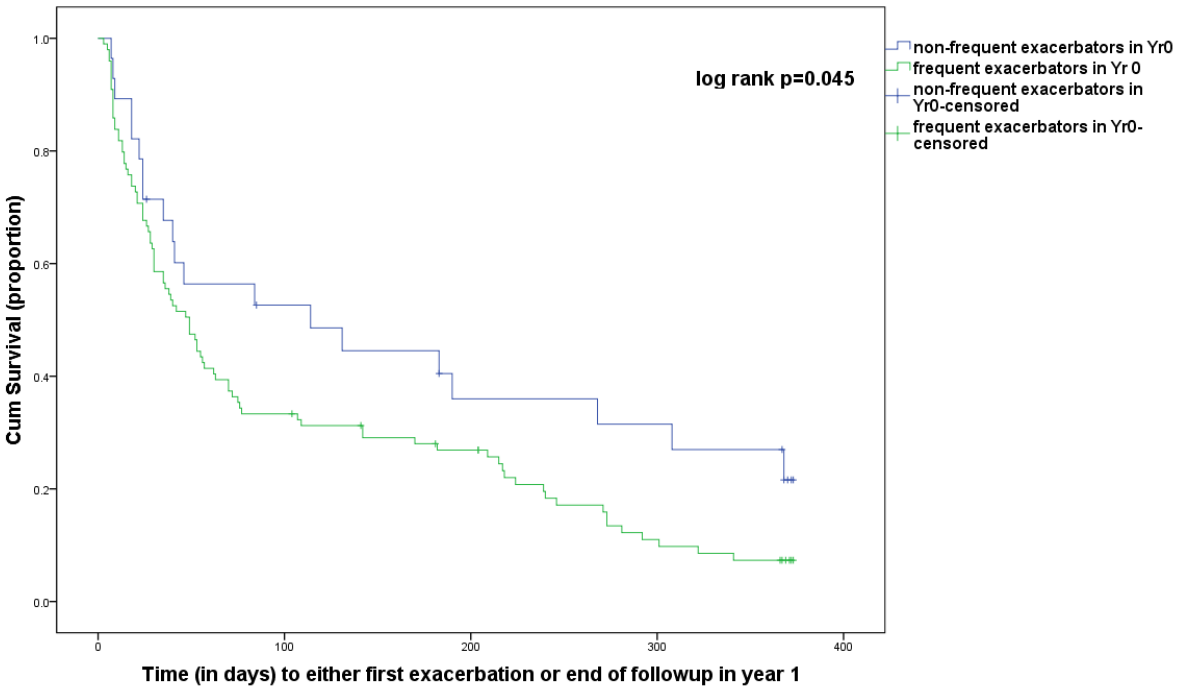


Figure 4.3 Kaplan-Meyer survival curve demonstrates an overview of time to either first exacerbation or till the end of observation period in frequent (n=99) and non-

frequent (n=28) exacerbators in a Year 0 groups. Median time to first exacerbation in frequent exacerbators was 49 days; in non-frequent exacerbators was 114 days.

Most patients in AERIS study reported that they enjoyed being part of the study thus implying the subjective benefit from participation in our project. I, therefore, focused my next analysis on a possible effect of study participation on exacerbation rate.

4.2.3 Clinical impact of AERIS participation on severe exacerbation rate

To begin with I analysed the difference between Year 0 and Year 1 total exacerbation rate and found that there was no statistically significant difference. In fact, when the data was expressed as means rather than medians, then near equivalence was observed between Y0 and Y1. (**Table 4.3**)

Table 4.3 Total exacerbation rates expressed over twelve months (Year 1)

		Year 0	Year 1	p-value
Total exacerbations frequency	**N**	399	355	0.402
Total exacerbation rate	**Mean**	3.14	3.09	
	Median(IQR)	2.00(2.00)	2.94(3.91)	
	Min	1.00	0.00	
	Max	14.00	13.77	

Data is non-parametric, mean reported along with median as more formative.

*To test for significance - Wilcoxon test applied.

In contrast, when exacerbations were analysed according to severity, less mild exacerbations of COPD were observed in the first year of the study. There was no statistically significant difference in moderate exacerbations between Y0 and Year 1. Importantly, there were fewer severe exacerbations in Year 1 compared to Year 0 despite a large outlier ($p<0.001$). (**Table 4.4)** Interestingly, the total number of exacerbations in Yr1 was 355 and Yr0 was 399, however, some patients were withdrew from the study in Yr1 thus affecting the final total exacerbation number.

Table 4.4 Exacerbation of COPD rates for the year before enrolment (Y0) and the 1st year of the AERIS study (Y1)

Severity of COPD exacerbations		Year 0	Year 1	p-value*
Severe	**Mean**	0.36	0.24	**<0.001**
	Median(IQR)	0.00(1.00)	0.00(0.00)	
	Min	0	0.00	
	Max	3	10.33	
Moderate	**Mean**	2.32	2.59	0.726
	Median(IQR)	2.00(2.00)	1.99(3.01)	
	Min	0	0.0 0	
	Max	14	11.77	
Mild	**Mean**	0.46	0.26	**0.018**
	Median(IQR)	0.00(0.00)	0.00(0.00)	
	Min	0	0.00	
	Max	10	2.96	
Total exacerbation rate	**Mean**	3.14	3.08	0.402
	Median(IQR)	2.00(2.00)	2.94(3.91)	
	Min	1.00	0.00	
	Max	14.00	13.77	

Data is non-parametric, mean reported along with median as median is not informative.
*To test for significance - Wilcoxon test applied.

In summary the rate of severe and mild exacerbations of COPD had significantly declined in the 1st year of the study compared with the year before the enrolment thus potentially implying that patients benefitted from participating in AERIS study.

4.3 Discussion

The AERIS cohort represents a population of COPD patients with severe airway obstruction and predominantly frequent exacerbators. The prevalence of airway bacterial presence was high during stable state and increased further during exacerbations of COPD. There was a noticeable increase in the detection of viruses at AECOPD. Half of the cohort had their 1st exacerbation within 49 days of study enrolment. This was predominantly driven by the subset of patients with frequent exacerbations.

By its longitudinal model AERIS study was similar to other longitudinal COPD studies[116, 276, 277] but also unique in its design[261]. To put the AERIS study in context, it is necessary to compare to other longitudinal COPD cohorts. The ECLIPSE (Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints) study was an observational COPD study designed to identify the parameters that predict disease progression in different COPD subtypes as well as to identify biomarkers that

may serve as surrogate end points.[276] The longitudinal design of the AERIS study was similar to ECLIPSE. Furthermore, the AERIS population was comparable in age, smoking history and degree of airway obstruction to the ECLIPSE cohort. However, there were some significant differences between the two studies. Patients in ECLIPSE were seen on quarterly basis and exacerbations were retrospectively documented but not reviewed and sampled in real time as in AERIS.[276] The ECLIPSE cohort also consisted from three subgroups: COPD (n=2164), smoking controls (n=337) and non-smoking controls (n=245), rather than COPD patients alone. The ECLIPSE COPD group consisted of subjects with COPD severity from GOLD stage 1(n=2) to GOLD stage 4.[278]

In contrast to ECLIPSE, the East London COPD study was designed to evaluate the causes and mechanisms of COPD exacerbations.[81, 116] Similar to the AERIS cohort, patients in the East London COPD cohort were recruited with moderate to very severe COPD (FEV1 <70% predicted and FEV1<80% predicted) and no other significant respiratory conditions.[49, 279] Patients in this study were asked to complete daily paper diaries in order to identify signs of exacerbations of COPD and were encouraged to contact the East London study team when they had noticed a deterioration of symptoms[80]. In the AERIS cohort, patients were monitored via e-diary by the clinical team and interactions were made by both patients and/or the clinical team to rule out exacerbations. Whilst both studies have clinical reviews within 48 hours of an exacerbation, the East London study only followed up patients monthly over the Winter season (October to March) with reduced follow-up of three-monthly over Summer period.[140]

Another longitudinal observational COPD study SPIROMICS was a multicentre observational study focused on identifying homogenous subgroups of COPD patients.[277] The population analysed consisted of either never smokers or current/former smokers with ≥20 pack year history with COPD.[280] There were four in-person annual clinics and quarterly calls were made to collect retrospective exacerbation data. [280] Patients in this study were not examined at the time of exacerbation. Sampling was performed at certain intervals, (during visits 1, 2 and 4 of annual in-person follow up) with induced sputum only collected at baseline. [277] The SPIROMICS cohort was comparable in age to the AERIS group, but consisted largely of patients with more moderate airway obstruction than the AERIS cohort. In addition, just over a quarter of SPIROMICS patients were current smokers compared to 43% in AERIS. However the biggest difference between these two cohorts is that the majority of SPIROMICS participants were non-frequent exacerbators.[280]

Similar to AERIS, the COPDMAP study was also a longitudinal observational two-year study. In contrast to AERIS, COPDMAP consisted of 370 subjects who attended scheduled visits only every 6 months when sputum and blood sampling took place.[281] In a subgroup of patients both baseline and exacerbations were studied. [179] Overall COPDMAP compared to AERIS cohort included a

slightly older population of COPD patients (70.2 and 66.8 respectively) with moderate airflow obstruction (post bronchodilator FEV1% 57.0 and 46.4 respectively). Despite the similar longitudinal designs of COPDMAP and AERIS these two studies are not fully comparable due to different inclusion criteria applied in each study. For COPDMAP, patients enrolled were on oral steroids and oral antibiotics and generally were also a infrequent exacerbators group (exacerbations 1.0(0.0-15.00) in the year prior to the study).[281]

The significance of the bacterial presence in stable COPD is in its association with the severity of COPD symptoms[157], raised local inflammatory process[49, 282], accelerated progression of COPD[80] and the severity of COPD exacerbations[49]. A considerable proportion of patients in the AERIS cohort had airway bacterial presence during stable disease. The prevalence of airway bacterial presence reported previously during clinically stable COPD ranged from 30.8% to 56.6% thus AERIS finding is in line with these data.[49, 80, 157,179, 283] COPDMAP reported bacterial colonisation in 52% of patients whereas a cross sectional study in Barcelona bacterial colonisation was reported in 48.7%. The prevalence of airway bacterial during clinical stability in East London cohort ranged from 30.8 to 56.6. The difference in reported range of bacterial isolation in these studies could be due the different methodology of bacterial detection applied in addition to the different number of typical airway bacteria reported in different studies. For example, Miravitlles et al in their study tested sputum for six isolates (*H. influenzae, H. parainfluenzae, S. pneumoniae, M. catarrhalis, P. aeruginosa* and *S. aureus*) using conventional quantitative bacterial microbiology method whereas in COPDMAP quantitative polymerase chain analysis was applied to test for three microorganisms (*H. influenzae, M.catarrhalis* and *S. pneumoniae*). During exacerbations previously reported prevalence of airway bacterial presence ranged from 35% to 71.6% thus AERIS finding is in keeping with the previously reported studies. [47, 124, 139, 179, 283] For instance, COPDMAP study reported that 78 out of 109 exacerbation event were found to be positive for bacterial pathogen, whereas bacterial presence in East London cohort was reported to be in 67.3 - 69.6% of exacerbations.[139, 179, 283]

Detection of respiratory viruses in COPD patients both during clinically stable disease and exacerbations was previously found to be associated with significant outcome measures. For instance, during clinical stability a persistent RSV isolation was associated with raised airway inflammation and an accelerated decline in FEV1.[167] Furthermore, at exacerbations respiratory viruses were found to be an important trigger of exacerbations and reported to be associated with longer time to recovery[140], HRV in particular was associated with raised lower airway inflammation[141], whereas co-infection of HRV and HI was associated with higher HI bacterial load[139]. Respiratory viruses were previously reported to be detected in 6-19% of patients with stable COPD[124, 138] and in 48.4 - 56% of patients during exacerbations.[124, 138] The prevalence of

respiratory virus in AERIS cohort both during clinical stability and exacerbations are in keeping with previously reported findings

In the AERIS study 50% of 1st exacerbations occurred in the first 49 days. This phenomenon was largely driven by frequent exacerbators group. Although there are studies looking into frequent exacerbators group and the stability of this phenotype,[118, 284] to my knowledge this is the first report to demonstrated that frequent exacerbators had an episode of exacerbations within such proximity to enrolment.

Exacerbation rate in AERIS cohort was relatively stable in the year before the study and the first year during the study. This finding is in line with previous studies that identified a previous history of exacerbations being a predictive factor of the susceptibility to future exacerbations.[118, 285] It is noteworthy however that there was a difference in the mild and severe exacerbations before and in the first year of the study. Namely, there were less mild exacerbations of COPD in the first year of the study. This was potentially due to a higher measurement error due to self reported nature of mild exacerbations prior to study enrolment whereas moderate and severe exacerbations had clinical expertise involved in diagnosis in Year 0 therefore the data was more rigorous. In addition, the rate of severe exacerbations of COPD significantly declined in the 1st year of the study compared with the year before enrolment. This phenomenon could be due to the early identification of symptoms and treatment and availability of clinical expertise that prevents an admission. Furthermore, patients in the AERIS cohort were trained how to early identify signs of COPD exacerbation and encouraged to discuss with the study doctor. This approach to management of COPD exacerbations supports previous report that an early detection and timely treatment of AECOPD were associated with better outcomes.[144] COPD management should be aimed at early detection via education on COPD symptoms and self-management plan.[286] Those patients who participated in research had direct access to a specialist clinical team to address their symptoms. Thus, study participants appeared to have benefitted from involvement in AERIS project.

In summary, in this chapter I have demonstrated that the AERIS study has a similar but unique design compared to other longitudinal cohorts. The AERIS cohort consists of predominantly severe COPD patients, the majority of whom were frequent exacerbators with a significant prevalence of airway infection during exacerbations. Whilst participation in the study did not appear to affect the number of exacerbations detected, there was an effect on the severity of exacerbation experienced by the patient.

In order to improve the management of COPD and particularly move closer to a personalised medicine approach in COPD a better understanding of markers of exacerbations, to identify

exacerbating state, is required. Therefore, in my next chapter I examined if using Southampton Sputum Colour chart to assess the sputum purulence could be useful in identifying patients with exacerbations and in particular those with airway infection presence.

Chapter 5 Sputum colour as a marker of inflammation and infection in COPD in the AERIS study

5.1 Introduction

Hippocrates of Kos is famous for the theory of the four humours (fluids): phlegm, black bile, yellow bile and blood. He believed that the human body is filled with these fluids and a delicate balance was required for the body to be healthy.[287] However, when these humours were out of balance, disease took over. The goal of medical management was to restore humoral equilibrium by adjusting diet, exercise and management of fluid evacuation.[287] Despite the move away from this historical way of practicing medicine, phlegm in the guise of sputum is still being studied in relation to respiratory conditions and health. Sputum assessment is a non-invasive method of assessing lung health, although some patients find the process of sputum expectoration somewhat uncomfortable.

The colour or purulence of sputum is defined by the presence of myeloperoxidase (MPO), an enzyme released by degranulating neutrophils [288]. The degree of purulence is positively associated with the amount of MPO in the sputum and, hence, is a marker of underlying neutrophilic inflammation[156], as well as predictive of the presence of bacteria.[154, 289]

The assessment of the degree of sputum purulence has been attempted previously using various assessment tools. These tools have ranged from using a descriptive approach such as “sputum colour purulent or not purulent” to quantitative methods with multi-coloured colour charts.[156, 158, 159] For example, in an attempt to assess sputum colour and correlate with underlying airway inflammation and bacterial presence, Stockley et al used a nine-point sputum chart on patients with only chronic bronchitis and bronchiectasis in an outpatient setting.[123, 156] Using these chart Stockley et al demonstrated that sputum colour was related to the underlying airway neutrophilic inflammation, furthermore, that the purulent sputum (graded 3-8) was associated with higher prevalence of airway bacteria compared to the mucoid sputum (graded 1-2).[123] Soler et al used the sputum colour to randomise patients with severe exacerbations of COPD to guide antibiotic treatment and assigned them to sputum purulent and non-purulent groups based on patients reported change in sputum colour.[158] Applying this descriptive approach Soler et al reported that 40% in purulent and 18% in non-purulent group yielded airway bacteria.[158] Allegra et al used a 10 colour Pantone catalogue chart to assess the purulence of sputum from patients with severe exacerbation of COPD.[159] Interestingly, Allegra et al reported a high yield of bacterial isolates in

both mucoid (colour 0-1) and purulent sputum (colour 2-9), in contrast to the report by Stockley et al.[123, 159] More recently, Daniels et al used a five-point sputum colour on patients with exacerbations of COPD and demonstrated an association between the purulent sputum (sputum colour 3-5) and bacterial presence.[160] In contrast, Brusse-Keiser et al in his study on severe exacerbations using a five-point sputum colour chart reported only a weak correlation between airway bacterial load and sputum colour, thus advocating against assessing sputum colour as a potential marker of bacterial infection.[161]

The assessment of the sputum purulence thus remains methodologically challenging with no universally accepted sputum colour chart and contradicting information on the association of increased sputum colour with airway bacterial presence. Furthermore, what remains unclear is how stable sputum colour is as a marker of airway inflammation and if a sputum colour chart provides any additional value to current symptoms based diagnosis of bacterial COPD exacerbations. In particular, I wished to assess if the objectively measured change in sputum colour at exacerbation from baseline is predictive of bacterial presence.

The overall aim of my project was to assess the clinical utility of biomarkers of infection and inflammation in COPD. In this chapter I examined if sputum neutrophilia was associated with PPM presence and whether this could be assessed without extensive laboratory analysis using the Southampton Sputum Colour (SSC) Chart.

5.2 Results and comments

Sputum colour is thought to be a marker of the underlying airway neutrophils present in the airway.[156] Thus prior to the sputum colour analyses, I examined the prevalence and proportion of sputum neutrophils in the AERIS cohort

5.2.1 Airway neutrophils in the AERIS cohort

5.2.1.1 Airway neutrophils during clinical stability

Initially, I studied the number of sputum samples obtained during clinical stability and found that there were 653 good quality samples over the first year with median sputum neutrophils % of 48.11(75.26). (**Figure 5.1**)

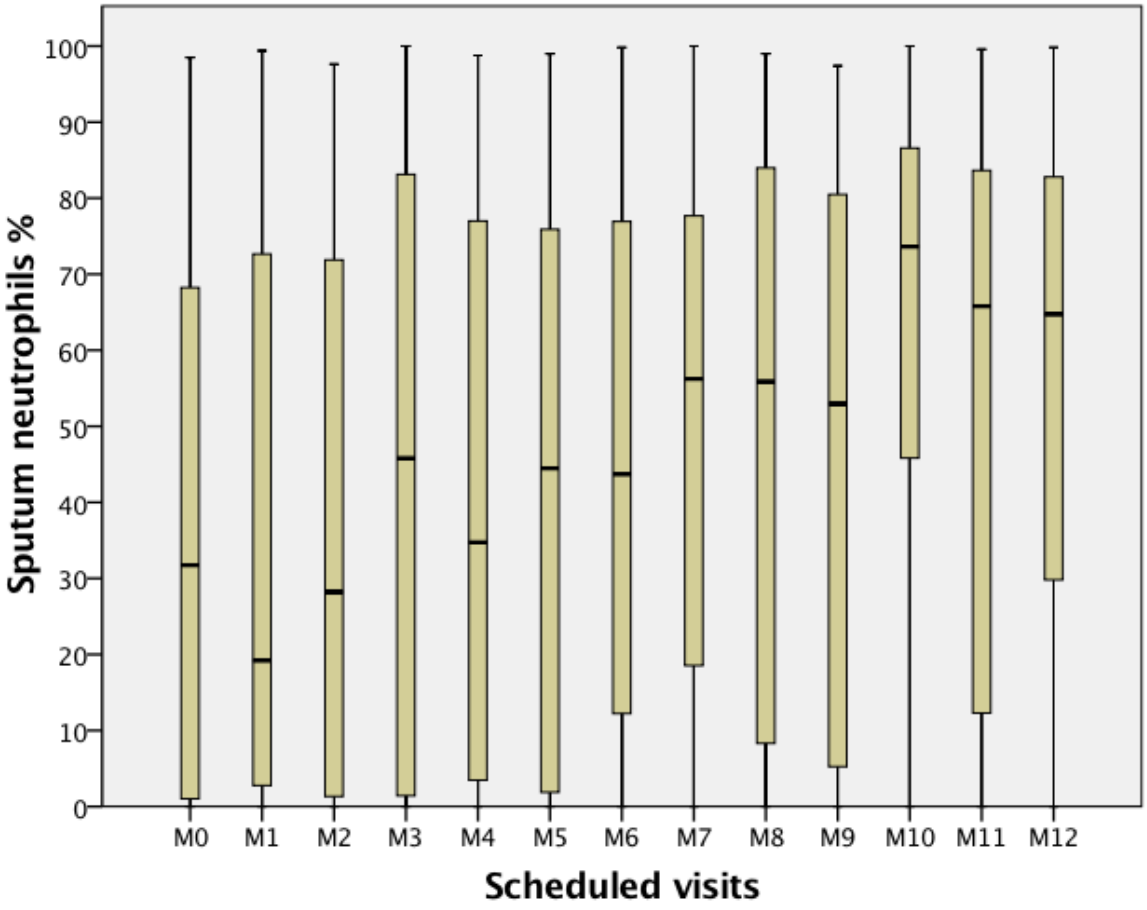


Figure 5.1 Box-and-whisker plots of sputum neutrophils% illustrating the spread over the first year when patients were deemed to be clinically stable (n=653)

To examine how reliable the percentage of sputum neutrophils were over the period of twelve months I conducted intra-class correlations (ICC) using STATA accounting for subjects multiple measurements and found that sputum neutrophil% was a moderately reliable measure over stable visits (ICC 0.51 95%CI 0.42; 0.60).

I further examined sputum neutrophil% at enrolment and found that out of 127 enrolment visits there were 69 samples with good quality sputum and median sputum neutrophil% at enrolment (47.47(71.13)) was similar to the sputum neutrophil% over all twelve months.

In order to explore that there were no significant differences between the groups included and excluded from the sputum neutrophil analyses I conducted the comparison between these two groups at enrolment. (**Table 5.1**) I found no significant differences between these two groups.

Table 5.1 Comparison of general characteristics between groups without and with sputum neutrophil % available at enrolment (excluded and included from the analysis)

	Excluded[α]		Included[β]		
	N	**Median(IQR)**	**N**	**Median(IQR)**	**P value[γ]**
Age*	58	66.33(±8.94)	69	67.22(8.37)	0.591
Pack year history	58	46.00(26.25)	69	49.00(29.00)	0.835
Current smoker (Y)	27	46.6%	27	39.1%	0.399
FEV1 (%)	57	45.00(25)	69	48.00(24)	0.360
Blood					
WBC* 10^9/L	58	7.30(2.23)	68	7.80(2.48)	0.216
Neutrophils* 10^9/L	58	4.80(1.53)	68	4.90(1.85)	0.317
CRP mg/L	58	5.00(5.25)	69	6.00(8.50)	0.118
Fibrinogen g/L	55	4.80(1.30)	59	5.00(0.90)	0.231
CAT	57	18.00(10)	69	16.00(11)	0.584
EXACT-PRO	46	37.00(12)	55	36.00(11)	0.743
Total exacerbation rate	58	2.16(3.23)	69	2.98(3.98)	0.217

[α] Excluded – group of patients with no good quality sputum data available; [β] Included – group of patients with good quality sputum data available; [γ]Mann Whitney test used for significance; * - parametric data and presented as mean(±SD)

For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

I then assessed if there was an association between airway neutrophils, markers of systemic inflammation, lung function and patient reported health status. I discovered no significant association between the sputum neutrophil%, markers of systemic inflammation, lung function or self-reported health status parameters. (**Table 5.2**)

Table 5.2. Relationship between sputum neutrophils % and other inflammatory markers and clinical features of at enrolment

	Sputum Neutrophils%	
	rho	**P value**
Post-bronchodilator FEV1 (%)	-0.135	0.376
Blood		
White blood cells10*9/L	0.045	0.768
Neutrophils 10*9/L	0.219	0.148
CRP mg/L	0.251	0.096
Fibrinogen g/L	0.160	0.293
Procalcitonin ng/ml	0.130	0.395
EXACT-PRO	0.006	0.971
CAT	0.109	0.476
Total exacerbation rate	0.112	0.462

For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

Sputum neutrophils>61% has previously been applied in an asthma population studies in order to identify patients with non-eosinophilic or neutrophilic airway inflammation.[174] Using the same cut off on the AERIS cohort I discovered that out of the 69 patients who produced good sputum samples at enrolment 27 (39.1%) had airway neutrophils ≥61%.

Applying the same cut off I examined if the groups with and without airway neutrophilia differ from each other at enrolment. (

Table 5.3) I found that at enrolment there were fewer active smokers among those with raised sputum neutrophils compared to those with lower sputum neutrophils, although the result did not reach significance (p=0.072). The neutrophilic group had significantly higher CRP levels compared to the non-neutrophilic. The patients with raised sputum neutrophils at enrolment had a significantly higher rate of exacerbations that were themselves neutrophilic compared to patients with lower sputum neutrophils (1.96(2.01) and 0.99(1.98) respectively, p=0.011) although there was no difference in total exacerbation rate over the first year between the groups.

Table 5.3 Characteristics of markers at enrolment for a full cohort and COPD phenotypes based on sputum neutrophils% (≥61)

Marker	Overall cohort		Non-neutrophilic		Neutrophilic		P value[Ω]
	n	Median (IQR)	n	Median (IQR)	n	Median (IQR)	
Age [α]	127	66.81(±8.61)	42	66.86(±6.81)	27	67.78(±10.46)	0.689
Smoking history (pack/years)	127	47.00(26.25)	42	50.5(33.25)	27	44.0(25.50)	0.184
Smoking status (Y)[γ μ]			20	47.6%	7	25.9%	0.072
6minute walk test	125	300.0(170)	42	308.0(186)	27	300(164)	0.936
Blood markers							
WBC*10^9/L	126	7.6(2.20)	41	7.7(2.85)	27	7.8(2.40)	0.740
Neutrophils*10^9/L	126	4.8(1.70)	41	4.8(1.80)	27	5.0(2.00)	0.459
Fibrinogen g/L	114	4.8(1.02)	38	4.75(1.15)	21	5.1(0.50)	0.101
Procalcitonin ng/ml	126	0.06(0.03)	42	0.06(0.02)	27	0.07(0.04)	0.491
CRP mh/L	127	5.0(8.00)	42	4.0(7.50)	27	9.0(12.00)	0.014
Sputum							
PPM presence n(%)			22	52.4%	15	55.6%	0.796
Spirometry							
FEV1 (%)	126	46.5(25)	42	50.0(24)	27	43.0(23)	0.319
ΔFEV1(L/year)	85	0.06(0.26)	29	0.03(0.24)	19	0.10(0.20)	0.118
Clinical markers							
CAT	126	16.0(10)	42	16.5(10)	27	16.0(11)	0.834
Exacerbation rate over the 1st year in the study	127	2.94(3.91)	42	1.99(3.90)	27	3.91(4.03)	0.372
Exacerbation rate with sputum neutrophilia in Year 1	127	0.99(1.98)	42	0.99(1.98)	27	1.96(2.01)	0.011

All continuous variables presented as median(IQR) unless stated otherwise. Mann-Whitney and Chi-Square tests used for significance unless stated otherwise; [Ω] significance test only for eosinophilic subgroups (excluding overall cohort variables); [α] reported as Mean(±SD); [β] reported as number and % within the subgroup; [μ] Fisher exact test reported for the significance. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

Having discovered that there was a significant difference in the neutrophilic exacerbation rate between the two neutrophil stratified groups I next examined if sputum neutrophils at enrolment can predict sputum neutrophilia at exacerbations.

Using all data available I conducted a ROC analysis to investigate if sputum neutrophil% at enrolment could predict raised neutrophils (≥61%) at exacerbations and found that there was a moderate association (AUC 0.643 95%CI 0.549; 0.738 p=0.004). The proportion of sputum neutrophils at enrolment was 56% sensitive and 65% specific in predicting exacerbations with airway neutrophilia. Furthermore, using all data and accounting for patients' multiple measurements, those volunteers with raised sputum neutrophils at enrolment were more likely to have raised sputum neutrophils at exacerbations although this has did not reach significance (OR 1.16 95%CI 0.99; 1.35 p=0.068).

Having examined the sputum neutrophils in the AERIS cohort during clinical stability I next proceeded to study the percentage of sputum neutrophils measured at exacerbations.

5.2.1.2 Airway neutrophils during exacerbations

As reported in Chapter 4 the AERIS cohort consisted of frequent exacerbators, with some subjects thus contributing towards the pool of exacerbation data more than once. To avoid skewing the data by including the same patient more than once I initially analysed only the 1st exacerbation from each subject, which totalled 108 unique events (out of 355 of total exacerbations). Out of 108 first exacerbations there were 61 subjects with good quality sputum samples obtained with median sputum neutrophils% of 74.00(47.11).

In order to ensure there were no significant differences between those included and excluded from the sputum neutrophil analyses I compared the two groups. *There was a trend towards lower FEV1% in the excluded patients compared to the included group, although this difference did not reach statistical significance (p=0.060).*. Fibrinogen was significantly higher in those included into the analyses compared to those who were not. There were no other significant differences between the two groups.

Table 5.4 Comparison of general characteristics between groups without and with sputum neutrophil% available at 1st exacerbation

	Excluded[α]		Included[β]		
	n	Median(IQR)	n	Median(IQR)	P value[γ]
FEV1 (%)	44	33.50(26)	53	44.00(18)	0.060
Blood					
WBC*10^9/L	44	8.10(3.60)	58	8.00(3.35)	0.829
Neutrophils*10^9/L	44	5.20(3.10)	58	5.35(3.20)	0.850
CRP mg/L	46	7.50(11.50)	57	10.00(26.50)	0.431
Fibrinogen g/L	46	4.90(1.33)	57	5.40(1.40)	0.023
CAT	45	19.00(13)	61	22.00(9)	0.427
EXACT-PRO	43	42.00(13)	54	41.00(10)	0.788

[α] Excluded – group of patients with no good quality sputum data available; [β] Included – group of patients with good quality sputum data available; [γ]Mann Whitney test used for significance. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

I further explored if there was an association between the sputum neutrophil% and other clinical parameters at first exacerbation. I found a weak but significant correlation between the sputum neutrophil% and WBC, blood neutrophils, CRP and fibrinogen. There was a negative weak association between the sputum neutrophil% and FEV1% although this was not significant (p=0.061). (**Table 5.5**)

Table 5.5. Relationship between sputum neutrophils % and other inflammatory markers and clinical features of at first exacerbations

	Sputum Neutrophils%	
	rho	**P value**
Post-bronchodilator FEV1 (%)	-0.259	0.061
Blood		
White blood cells *10^9/L	0.342	0.009
Neutrophils *10^9/L	0.442	0.001
CRP mg/L	0.467	<0.001
Fibrinogen g/L	0.275	0.039
Procalcitonin ng/ml	0.086	0.532
EXACT-PRO	-0.129	0.353
CAT	-0.049	0.706

For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

Applying the same cut off of ≥61% neutrophils to seek the prevalence of raised sputum neutrophils at exacerbation, a significant proportion of patients (63.9%) had sputum neutrophils ≥61% at their 1st exacerbation.

I next sought to explore the differences between the groups with and without raised sputum neutrophils at exacerbations. I discovered that those subjects with sputum neutrophils ≥61% at first exacerbation had a significantly enhanced systemic inflammatory profile (WBC, blood neutrophils, CRP) compared to those with sputum neutrophils<61%. (**Table 5.6**)

Table 5.6 Characteristics of markers at first exacerbations for the full cohort, sputum neutrophils≥61% and sputum neutrophils<61% groups.

Marker	Overall cohort		Non-neutrophilic		Neutrophilic	P value[Ω]
	n	Median (IQR)	n	Median (IQR)	n	Median (IQR)	
Blood markers							
WBC *10^9/L	102	8.00(3.53)	21	7.40(2.25)	37	8.80(3.75)	0.034
Neutrophils*10^9/L	102	5.30(3.20)	21	4.70(2.15)	37	5.60(3.75)	0.014
Fibrinogen g/L	103	5.10(1.40)	21	5.20(1.05)	36	5.50(1.87)	0.162
Procalcitonin ng/ml	99	0.07(0.04)	21	0.06(0.03)	34	0.08(0.05)	0.363
CRP mg/L	103	8.00(14.00)	21	5.0(10.50)	36	14.0(33.75)	0.004
Sputum							
PPM presence n(%)[§]	99	60 (60.6%)	13	59.1%	26	66.7%	0.554
Lung function							
FEV1 (%)	97	43.00(21)	20	45.5(19)	33	44.0(18)	0.349
Clinical markers							
CAT	106	21.00(11)	22	22.50(8)	39	21.0(10)	0.299
EXACT-PRO	97	41.00(11)	20	42.0(8)	34	40.0(12)	0.298

All continuous variables presented as median(IQR) unless stated otherwise. Mann-Whitney or Chi-Square tests used for significance unless stated otherwise; § Fisher Exact for significance test. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

5.2.1.3 Airway neutrophils and airway infection

As demonstrated in

Table 5.3 there was no difference in PPM presence between the groups with and without raised sputum neutrophils at enrolment. Contrastingly, at first exacerbation there was a trend towards greater prevalence of PPM among those with sputum neutrophils ≥61% than in those volunteers with sputum neutrophils<61% (66.7% and 59.1% respectively) although this difference was not statistically significant. (**Table 5.6**) However, when I re-examined the PPM prevalence using data from all exacerbations with good quality sputum data available for both sputum differential and microbiology (n=217) the prevalence of PPM in sputum neutrophils ≥61% group was significantly higher than in the group with sputum neutrophils<61% (67.9% and 48.1%, p=0.004). I therefore next examined if sputum neutrophils ≥61% could predict the presence of airway bacteria using all

available exacerbation data. Using a ROC analysis I discovered a significant moderate association between the sputum neutrophil% and airway bacterial presence at all exacerbations (AUC 0.673 95% CI 0.603; 0.744 p<0.001). Sputum neutrophils ≥61% was 72% sensitive and 47% specific in predicting airway bacterial presence at exacerbations. When using all exacerbations and accounting for subjects' multiple measurements I found there was a significant association between sputum neutrophilia and airway bacteria presence at exacerbations (OR 1.22 95%CI 1.06; 1.42 p=0.005).

5.2.1.4 Airway neutrophils and sputum colour

Having examined sputum data from the AERIS cohort and demonstrating an association between sputum neutrophil proportions and bacterial presence, I next studied if sputum colour derived using the Southampton Sputum Colour (SSC) chart is associated with airway neutrophilia. Using a Spearman's correlation, there was a weak but significant correlation between sputum colour and sputum neutrophil% at enrolment (rho 0.396 p=0.007). Similar analysis using data from only 1st exacerbations revealed a moderately strong association between sputum colour and sputum neutrophil % (rho 0.579, p<0.001).

5.2.2 Sputum Colour in the AERIS cohort

Sputum colour assessment using the SSC chart demonstrated a significant association with airway neutrophilia. Thus I further examined if sputum colour alone was associated with inflammation and, importantly, bacterial presence during clinical stability and at exacerbation.

5.2.2.1 Sputum colour during clinical stability

At enrolment, 87.4% of the AERIS cohort produced sputum samples. Sputum colour data was available for 99.1% of the available samples by the laboratory technicians. There were 17 subjects that had no sputum colour assessed at enrolment.

In order to assess if the group of subjects that had sputum colour assessed at enrolment ("included" group) was significantly different from the group that had no sputum colour data at enrolment ("excluded" group), I conducted a Mann-Whitney test to study general characteristics of both groups (**Table 5.7**). The group excluded from the analyses appeared to have less systemic inflammation as evidenced by lower blood white cell count and serum fibrinogen. There was a trend towards lower blood neutrophils and plasma CRP in the excluded group but these differences did not reach statistical significance.

Table 5.7 Comparison of general characteristics between groups without and with sputum colour data available at enrolment

		Excluded[α]		Included[β]		
		n	**Median(IQR)**	**n**	**Median(IQR)**	**P value[γ]**
	FEV1 (%)	16	38.00(24)	110	47.50(24)	0.158
Sputum						
	Neutrophils (%)	1	-	68	48.07(71.55)	0.393
Blood						
	WBC*10^9/L	17	6.40(2.50)	109	7.70(2.35)	0.006
	Neutrophils*10^9/L	17	4.20(1.50)	109	4.90(1.65)	0.055
	CRP mg/L	17	3.00(5.00)	110	5.00(7.25)	0.062
	Fibrinogen g/L	16	4.45(1.80)	98	4.90(0.90)	0.036
	PCT ng/ml	17	0.06(0.03)	109	0.06(0.03)	0.346
CAT		17	17.00(10)	109	16.00(11)	0.971
EXACT-PRO		13	38.00(14)	88	36.50(12)	0.776
Total exacerbation rate		17	2.00(2.51)	110	2.95(3.99)	0.228

[α] Excluded – group of patients with no sputum colour data available; [β] Included – group of patients with sputum samples available; [γ]Mann Whitney test used for significance. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

At stable visits over the subsequent 12 months (n=952) most subjects had a sputum colour grade between 1 and 3. (**Figure 5.2**, B) Among the group that had sputum colour assessed at enrolment most subjects (n=61/110) had sputum colour grade 2, fewer were graded 1 and 3 (n=23/110 and 23/110 respectively) and no subject had sputum colour grade 5 at enrolment (**Figure 5.2**, A).

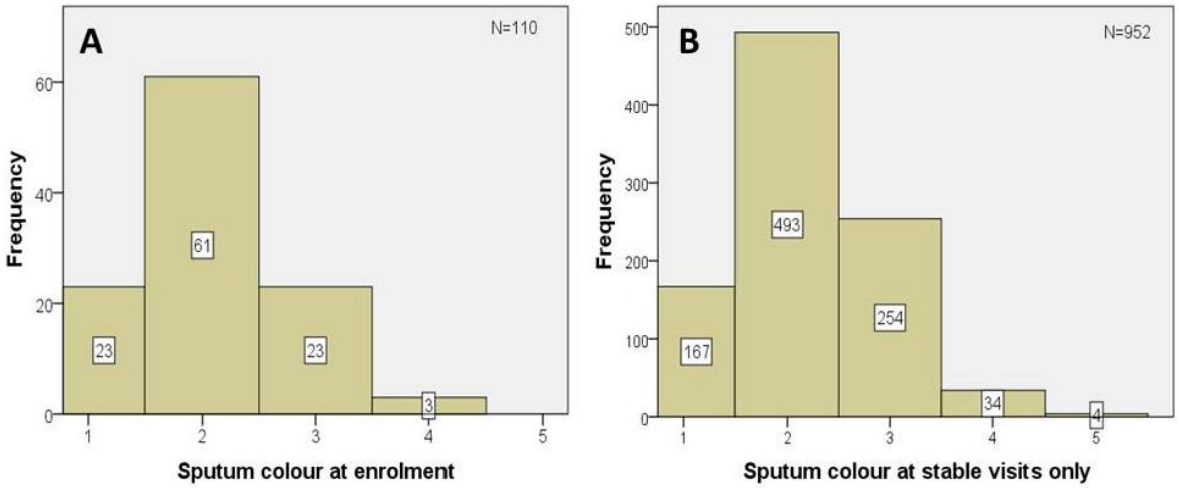


Figure 5.2 A - Histogram demonstrating the frequency of samples in each unit of sputum colour grade at enrolment; B - histogram illustrating the frequency of samples in each unit of sputum colour grade at all stable visits. N- total number of samples (N=110 and 952 at enrolment and all stable visits, respectively).

The differences in clinical characteristics between the sputum colour groups at enrolment are presented in **Table 5.9**. In addition, the sample size was small in sputum colour 4 thus no interquartile range was calculated. As expected, there was a significant difference in the sputum neutrophil% between sputum colour groups, otherwise, there did not appear to be clinically significant differences between patients with the different sputum colour grades at enrolment.

5.2.2.2 At exacerbation

In my initial analysis, from the 108 1st exacerbations, sputum colour was only available for 98 visits. To ensure there were no significant differences between those who did not have ("excluded") and those subjects who did have ("included") sputum colour assessed at the 1st exacerbation, a comparison of main clinical characteristics was conducted. No significant differences in clinical characteristics were found between the two groups. (**Table 5.8**)

Table 5.8 Comparison of general characteristics between groups without and with sputum colour data available at 1st exacerbations

	Excluded$^{\alpha}$		**Included$^{\beta}$**		
	n	**Median(IQR)**	**n**	**Median(IQR)**	**P value$^{\gamma}$**
FEV1 (%)	9	31.00(30)	88	43.00(21)	0.341
Sputum					

	Neutrophils (%)	-	-	61	74.00(47.11)	-
Blood						
	WBC*10^9/L	9	6.50(3.50)	93	8.20(3.40)	0.075
	Neutrophils*10^9/L	9	4.20(3.25)	93	5.40(3.15)	0.304
	CRP mg/L	10	7.00(11.50)	93	9.00(15.00)	0.562
	Fibrinogen g/L	10	4.65(1.70)	93	5.20(1.35)	0.346
	PCT ng/ml	9	0.07(0.04)	90	0.07(0.04)	0.592
CAT		10	20.50(19)	96	21.00(10)	0.897
EXACT-PRO		8	41.00(15)	89	41.00(11)	0.743

[α] Excluded – group of patients with no sputum colour data available; [β] Included – group of patients with sputum colour data available; [γ]Mann Whitney test used for significance. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

I next examined the frequency of the sputum colour grades at both 1st exacerbation and at all exacerbations (**Figure 5.3**). In the latter analysis of all exacerbations, each exacerbation was considered as an individual event. Out of all 1st exacerbations where sputum colour data was available (n=98) most subjects had sputum colour grade 2 and 3 (44/93 and 31/93 respectively). (**Figure 5.3, A**) When all exacerbations were analysed as individual events (n=317) there was a similar pattern in the frequency of the sputum colour grade. (**Figure 5.3**,**B**)

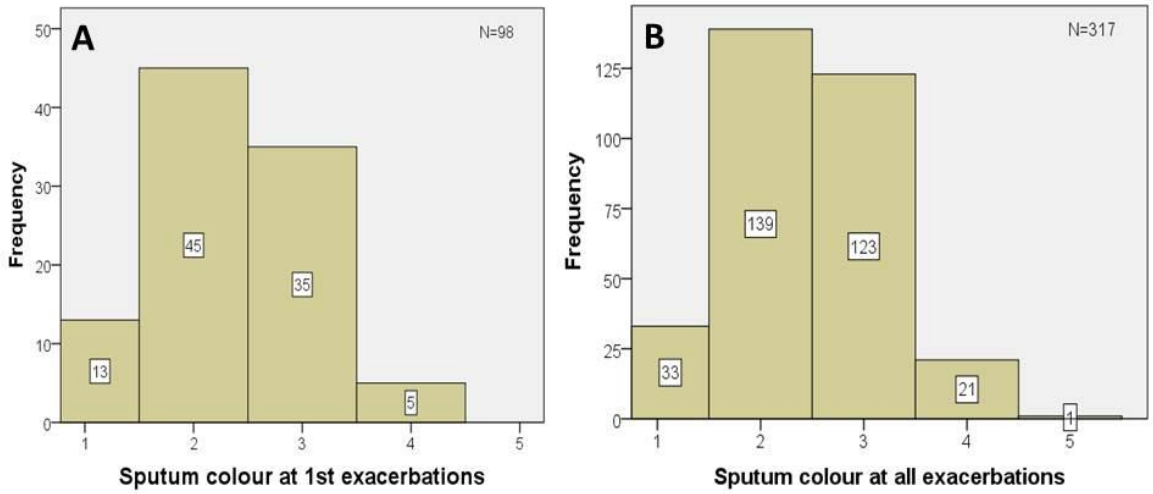


Figure 5.3. A - Histogram demonstrating the frequency of samples in each unit of sputum colour grade at 1st exacerbation with sputum data available; B - Histogram demonstrating the frequency of samples in each unit of sputum colour grade at all exacerbations with available sputum colour data. "N" denotes total number of samples.

There was a significant difference in the sputum neutrophil%, fibrinogen and CRP between patients with different sputum colours at 1st exacerbation with the trend of an increase in all of these biomarkers as the sputum colour increased. However, the levels of these inflammatory markers in sputum colour 4 were lower than sputum colour 3. (**Table 5.10**) This could potentially be due to a small sample size in group 4. No sputum samples of colour 5 were present at 1st exacerbation.

Table 5.9 Differences in clinical characteristics per sputum colour group at enrolment.

		Sputum colour at enrolment										
		1		**2**		**3**		**4**		**5**		
		n	**Median(IQR)**	**n**	**Median(IQR)**	**n**	**Median(IQR)**	**n**	**Median(IQR)**	**n**	**Median (IQR)**	**p-value**
	FEV1 (%)	17	54.00(27)	41	47.00(23)	14	39.00(26)	2	59.00(.)	-	-	0.434
Sputum												
	Neutrophils(%)	**8**	**8.48(55.87)**	**37**	**17.75(67.06)**	**20**	**68.24(43.09)**	**3**	**96.79(.)**	**-**	**-**	**0.020**
Blood												
	WBC*10^9/L	23	7.90(2.30)	60	7.80(2.28)	23	7.30(2.70)	3	7.40(.)	-	-	0.464
	Neutrophils*10^9/L	23	4.90(1.50)	60	4.90(1.68)	23	4.70(1.90)	3	5.40(.)	-	-	0.764
	CRP mg/L	23	6.00(7.00)	61	5.00(9.00)	23	6.00(8.00)	3	9.00(.)	-	-	0.701
	Fibrinogen g/L	21	4.70(1.40)	54	5.00(0.63)	20	4.80(1.43)	3	4.90(.)	-	-	0.732
	PCT ng/ml	22	0.06(0.03)	61	0.06(0.04)	23	0.07(0.02)	3	0.07(.)	-	-	0.512
CAT		23	16.00(12)	60	16.00(11)	23	18.00(17)	3	15.00(.)	-	-	0.569
EXACT-PRO		18	34.00(10)	46	37.00(14)	21	38.00(12)	3	28.00(.)	-	-	0.062
Total exacerbation rate		23	1.98(3.02)	61	2.98(3.99)	23	3.02(3.94)	3	3.98(.)	-	-	0.663

There were no samples in sputum colour 5. Sputum colour 4 sample size was low, hence, no IQR calculated; Kruskal Wallis test applied for significance. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

Table 5.10 Differences in clinical characteristics per sputum colour group at 1st exacerbations

		Sputum colour at 1st exacerbations										
		1		**2**		**3**		**4**		**5**		
		n	**Median(IQR)**	**n**	**Median(IQR)**	**n**	**Median(IQR)**	**n**	**Median(IQR)**	**n**	**Median(IQR)**	**p-value**
	FEV1 (%)	12	37.50(29)	43	43.00(19)	29	44.00(19)	4	36.00(21)	-	-	0.877
Sputum												
	Neutrophils (%)	**3**	**26.85(.)**	**23**	**58.23(47.19)**	**31**	**88.91(33.79)**	**4**	**97.48(5.11)**	**-**	**-**	**<0.001**
Blood												
	WBC*10^9/L	12	7.75(3.10)	43	7.90(3.40)	33	8.70(3.65)	5	9.30(6.05)	-	-	0.487
	Neutrophils*10^9/L	12	5.10(2.58)	43	5.00(2.70)	33	5.50(4.30)	5	7.20(6.55)	-	-	0.245
	CRP mg/L	**12**	**4.50(6.75)**	**44**	**6.50(12.00)**	**32**	**16.50(48.75)**	**5**	**8.00(45.50)**	**-**	**-**	**<0.001**
	Fibrinogen g/L	**13**	**4.10(1.60)**	**42**	**5.15(0.95)**	**33**	**5.60(2.05)**	**5**	**4.70(1.30)**	**-**	**-**	**<0.001**
	PCT ng/ml	11	0.07(0.04)	44	0.07(0.03)	30	0.08(0.05)	5	0.08(0.05)	-	-	0.366
CAT		13	19.00(12)	43	20.00(10)	35	22.00(8)	5	19.00(15)	-	-	0.610
EXACT-PRO		12	39.00(9)	42	40.00(9)	31	42.00(10)	4	37.00(15)	-	-	0.233

There were no samples in sputum colour 5; Sputum colour 4 sample size was low, hence, no IQR calculated; Kruskal Wallis test applied for significance. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

As demonstrated, there was a significant difference in the airway neutrophil% between the sputum colour groups at 1st exacerbation. Thus I examined what the likelihood of an increase in sputum colour predicting airway neutrophilia was (as defined by sputum neutrophils% ≥61%) when the data from all exacerbations was applied. Using logistic regression on all exacerbations and after adjusting for subjects with more than one exacerbation, the odds of exacerbations being neutrophilic (sputum neutrophils<61%) at all exacerbations was raised for sputum colour groups 2, 3 and 4 versus sputum colour 1 (OR 3.46 95%CI 0.34; 34.97 p=0.293, OR 17.61 95% CI 1.62; 190.99 p= 0.018 and 132.24, 4.24; 5124.30, 0.005 respectively) although the OR for sputum colour 2 versus sputum colour 1 was not statistically significant (p=0.293). This result was probably expected as the median sputum neutrophil% in sputum colour 2 group was <61% (median 58.23(47.19)) thus the sputum colour 2 could not predict sputum neutrophilia (p=0.239).

To summarise, airway neutrophils significantly differed between the sputum colour groups and increased with the sputum colour grade at both enrolment and exacerbation. At exacerbations sputum colour 3 and 4 were significantly predictive of airway neutrophilia. There also appeared to be significant differences in systemic inflammatory markers between sputum colour categories at exacerbation although this relationship was not present at enrolment. So, how does analysing sputum colour as a categorical variable relate to airway infection?

5.2.2.3 Sputum colour and airway infection

I first examined if PPM presence in sputum by culture differed between sputum colour groups at enrolment and found no significant difference in sputum PPM presence between patients with the different sputum colour grades. (**Table 5.11**)

5.2.2.3.1 Sputum colour and bacterial presence at enrolment

There was a trend towards higher prevalence of airway bacteria at enrolment with increasing sputum colour, although this relationship was not statistically significant. The airway bacterial presence at enrolment was 56.5% and greater for the sputum colour of ≥3. Singh et al in their study analysed sputum samples collected from all stable visits.[290] I therefore conducted an additional analysis of all stable visits where sputum colour was reported over the 1st year (n=952). The result demonstrated a significant difference in airway PPM presence detected by culture between all five sputum colour grades (p=0.004), but this analysis did not account for repeated measurements. When the repeated subject effect was accounted for, PPM presence was 29.8% higher with each unit increase in sputum colour at all stable visits (OR 1.298 95% CI 1.02; 1.65 p=0.033).

5.2.2.3.2 Sputum colour and bacterial presence at exacerbations

I next studied if PPM differed between sputum colour grades at exacerbation and found a significant difference in PPM presence between groups at those 1st exacerbations with data available. PPM prevalence was higher in those groups with an increase in sputum colour. However, it is important to note that the sample size in sputum groups 4 and 5 was small. (**Figure 5.4**, **Table 5.11**) There was also a significant difference in PPM presence between the sputum colour grades when all exacerbations were considered. . When the effect of subjects with repeated exacerbations was accounted for, the odds of PPM being present at exacerbation as detected by culture was found to be 2.7 times higher for every 1 unit increase in sputum colour (OR 2.70 CI 1.60; 4.57 p<0.001). The odds of PPM being present at exacerbation as detected by PCR also followed a similar pattern (OR 2.02 CI 1.18; 3.44 p= 0.010). I further studied what colour score better predicts PPM presence and revealed an association between the Sputum colour and PPM presence detected by culture at exacerbations (AUC 0.607, CI 0.544; 0.670 p=0.001). Sputum colour grade ≥3 at exacerbations was 69.0% sensitive and 49.7% specific when airway PPMs were detected by culture. There was a weaker association between the Sputum Colour and PPM presence detected by PCR (AUC 0.577, CI 0.510; 0.643 p= 0.030). Sputum colour ≥3 was 52.0% sensitive and 63.0% specific in identifying PPM presence by PCR.

Table 5.11. PPM presence detected by culture in each sputum colour group at enrolment, 1st exacerbations and all exacerbations, with data available

	Enrolment	**1st exacerbations**	**All exacerbations**
Sputum colour	**n/N [α] (%)**	**n/N (%)**	**n/N (%)**
1	11/22 (50.00)	5/13 (38.50)	15/33 (45.50)
2	30/61 (49.20)	24/45 (53.30)	71/139 (51.10)
3	13/23 (56.50)	27/35 (77.10)	83/123 (67.50)
4	2/3 (66.70)	4/5 (80.00)	16/21 (76.20)
5	0/0	0/0	1/1 (100.00)
P value[β]	0.897	0.033	0.007

[α] n/N defined as n= number of PPM+ve samples, N = number of total samples in the sputum colour group; [β] Fisher's exact test applied in view of a small sample size in certain groups for significance between PPM+ve and PPM-ve groups at different clinical states.

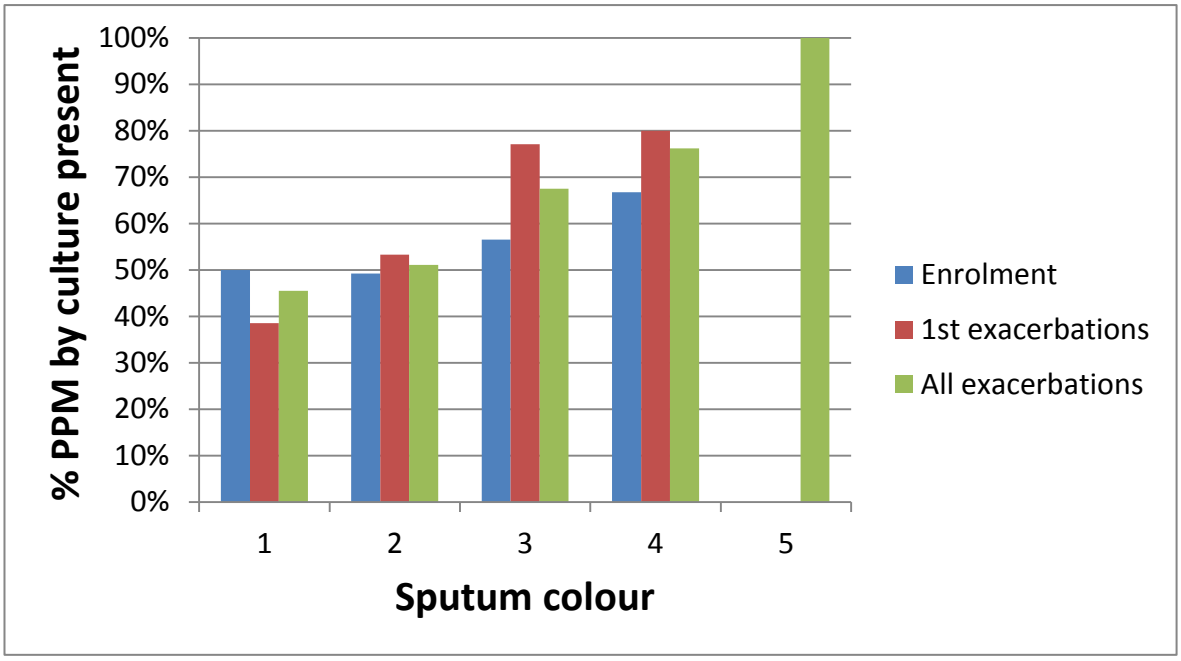


Figure 5.4. Cluster bar chart illustrating % PPM present detected by culture in each sputum colour group at enrolment, 1st exacerbations and all exacerbations with data available.

5.2.2.3.3 Sputum colour and Respiratory viruses

Papi et al reported that purulent sputum was more prevalent in viral positive compared to viral negative exacerbations.[124] In light of this report I examined if this was the case in the AERIS cohort. I first investigated the prevalence of respiratory viruses in each sputum colour group.

Although, sputum colour grade tended to decrease as respiratory viruses prevalence increased at all exacerbations, this association was not statistically significant. At 1st exacerbations and enrolment there were no viruses present in sputum samples of grade 4 and there were no samples produced in sputum colour grade 5 at both visits. (**Figure 5.5**, **Table 5.12**) Applying logistic regression and adjusting for subjects' repeated measurements, the odds of respiratory viruses being present were 33% lower for every unit increase in sputum colour (OR 0.67 CI 0.46; 0.98 p=0.037). It therefore appears that the presence of virus has little impact on sputum colour as assessed by the SSC chart.

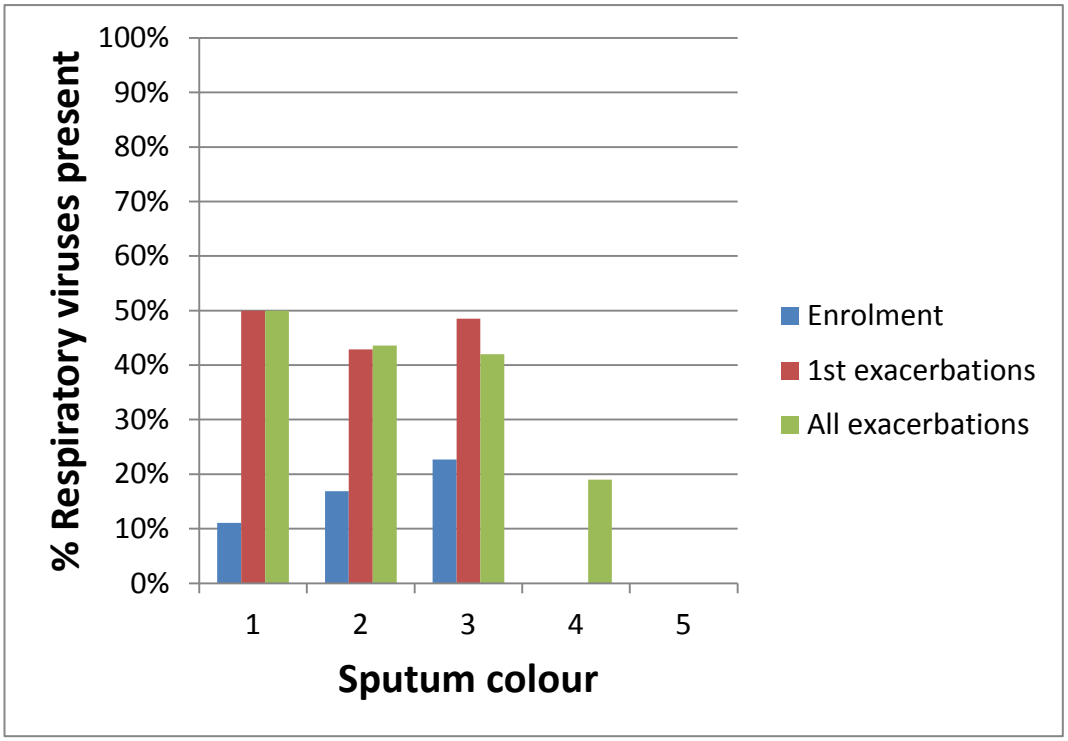


Figure 5.5. Cluster bar chart illustrating % Respiratory viruses present in each sputum colour group at enrolment, 1st exacerbations and all exacerbations, with data available.

Table 5.12. Presence of respiratory viruses in each sputum colour group at enrolment, 1st exacerbations and all exacerbations, with data available

	Enrolment	**1st exacerbations**	**All exacerbations**
Sputum colour	**n/N$^{\alpha}$ (%)**	**n/N (%)**	**n/N (%)**
1	2/18 (11.10)	5/10 (50.00)	14/28 (50.00)
2	10/59 (16.90)	18/42 (42.90)	58/133 (43.60)
3	5/22 (22.70)	16/33 (48.50)	50/119 (42.00)
4	0/3 (0)	0/5 (0)	4/21 (19.00)
5	0/0	0/0	0/1 (0)
P value$^{\beta}$	0.776	0.155	0.227

$^{\alpha}$ n/N defined as n= number of PPM+ve samples, N = number of total samples in the sputum colour group; $^{\beta}$ Fisher's exact test applied in view of a small sample size in certain groups for significance between ReV+ve and ReV-ve groups at different clinical states.

5.2.2.3.4 Change in sputum colour and airway infection

Having examined an association between airway bacterial and viral infection and sputum colour grade in different clinical states, I asked if a change in sputum colour between enrolment and exacerbation was associated with the presence of airway infection.

Therefore, analysis of the change in sputum colour between 1st exacerbation and enrolment (n=93) carried out and revealed that in 39.8% of subjects there was no change in sputum colour, in 38.7% it was reported as more purulent at exacerbations and in 21.5% the sputum colour appeared to be less purulent at exacerbations. (**Table 12**, **Figure 9**) Analysis of the change in sputum colour between all exacerbations and enrolment (n=294) showed a similar pattern. In 43.54% of cases no change, 38.77% worsening and 17.73% an improvement in sputum colour was found. (**Table 12**, **Figure 9**)

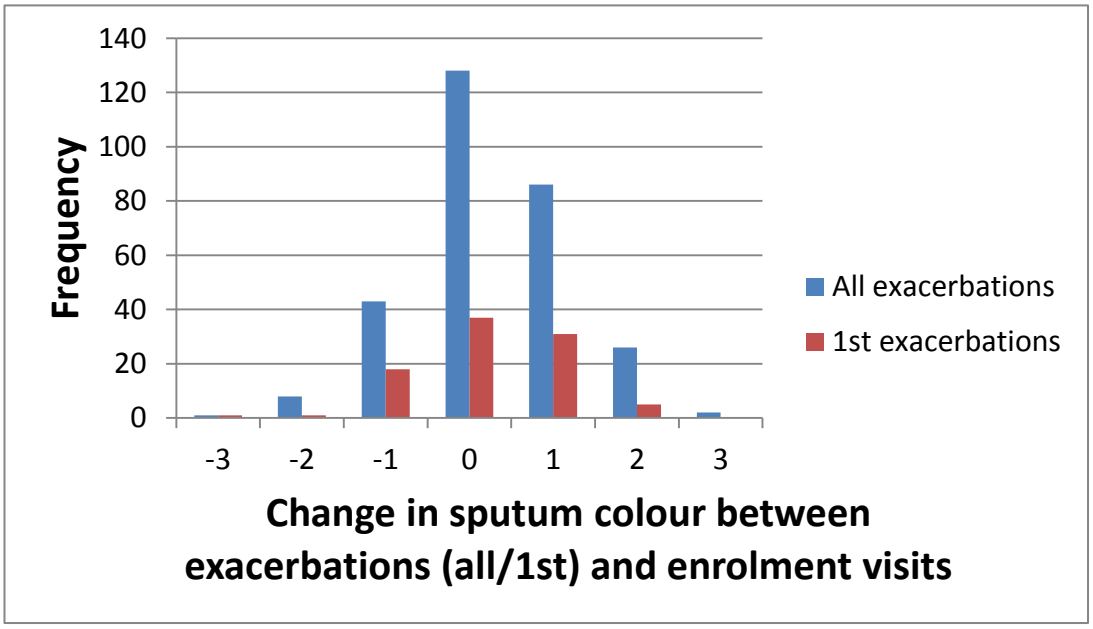


Figure 5.6. Cluster chart illustrating the number of cases per unit of sputum colour change between exacerbations and enrolment.

Table 5.13. Frequency and percentage of cases per unit of change in sputum colour between exacerbations (all/1st) and enrolment.

Δ sputum colour	All exacerbations & enrolment (n/N)	%	1st exacerbations & enrolment (n/N)	%
-3	1/294	0.34	1/93	1.08
-2	8/294	2.72	1/93	1.08
-1	43/294	14.63	18/93	19.35
0	128/294	43.54	37/93	39.78
1	86/294	29.25	31/93	33.33
2	26/294	8.84	5/93	5.38
3	2/294	0.68	-	-

Having studied the prevalence of exacerbation cases per each unit of change in sputum colour, I next examined the prevalence of PPM presence at 1st exacerbations per unit change in sputum colour. The larger the scale of positive change in the sputum colour the higher the proportion of PPM present at 1st exacerbations (p=0.014). Similar analysis for all exacerbations mirrored the pattern of airway bacterial presence at all exacerbations per unit of sputum colour change (p=0.001). (**Table 13**, **Figure 10**) Likewise, I studied the prevalence of respiratory viruses per each unit of sputum colour change and revealed that the distribution of respiratory viruses per unit of change in sputum colour appeared to be in the opposite direction to the PPM although changes were not statistically significant at either 1st exacerbation or all exacerbations (p=0.966 and p=0.286, respectively). (**Table 13**, **Figure 10**)

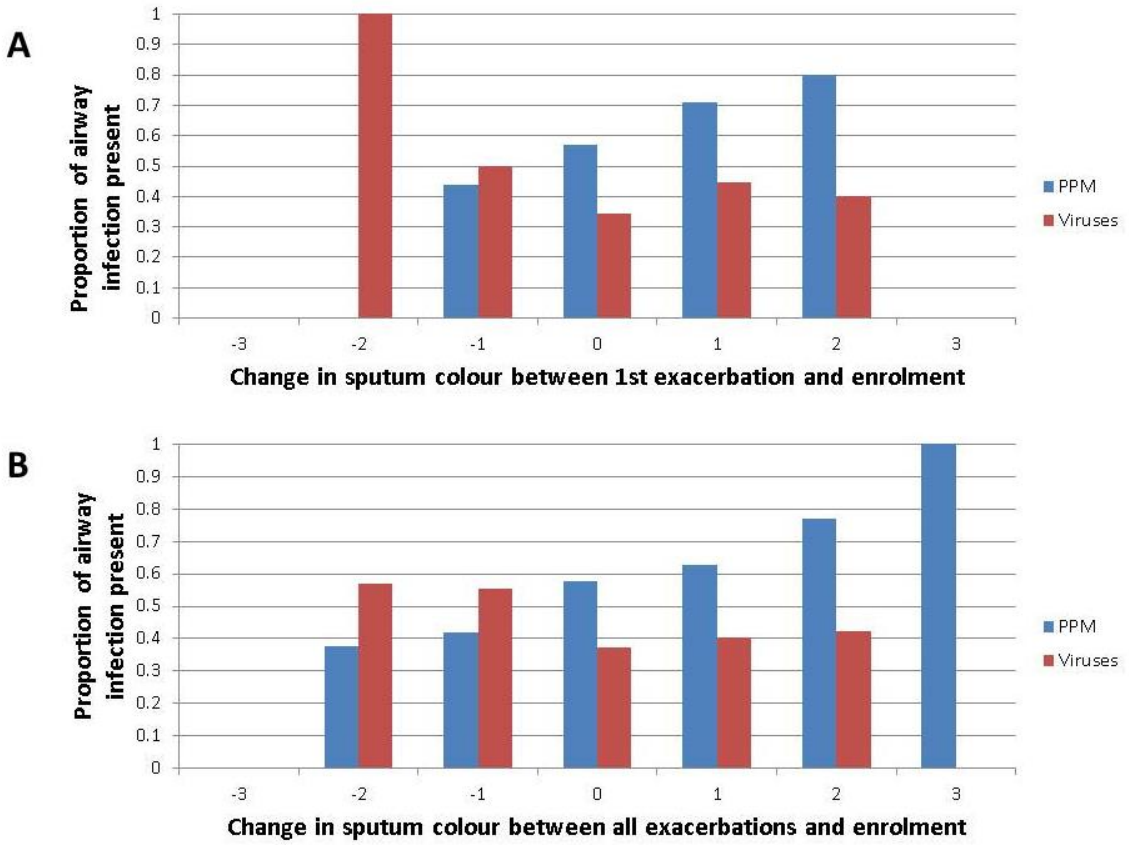


Figure 5.7. A - Cluster bar chart illustrating presence of PPM and respiratory viruses at 1st exacerbations per unit of change in sputum colour between 1st exacerbations and enrolment; B - Cluster bar chart illustrating presence of PPM and respiratory viruses at all exacerbations per unit of change in sputum colour between all exacerbations and enrolment.

Table 5.14. Proportion of airway infection present at exacerbations per unit change in sputum between exacerbations (all/1st) and enrolment.

	PPM				**Respiratory viruses**			
Δ sputum colour	**All exacerbations & enrolment**		**1st exacerbations & enrolment**		**All exacerbations & enrolment**		**1st exacerbations & enrolment**	
	n	**mean**	**n**	**mean**	**n**	**mean**	**N**	**mean**
-3	1	0	1	0	1	0	1	0
-2	8	0.375	1	0	8	0.571	1	1.00
-1	43	0.419	18	0.444	43	0.553	18	0.50
0	128	0.578	37	0.568	128	0.371	37	0.343
1	86	0.628	31	0.71	86	0.402	31	0.448
2	26	0.769	5	0.8	26	0.423	5	0.40
3	2	1	-	-	2	0	-	-
P value		0.001		0.014		0.286		0.966

To summarise, there appears to be an association between the sputum colour assessed by laboratory staff using the 5-coloured SSC chart and airway bacterial presence at exacerbation. Although our patients did not report the change in sputum using the SSC, an increase in sputum colour was reported by patients daily via e-diary (Yes/No) and in conjunction with other major and minor symptoms a clinical diagnosis of exacerbation was made by a clinical research fellow within 72 hours of symptom onset (described in more detail in Methods section).

In the next section I examined if the airway bacterial presence was associated with patients' reported change in sputum colour (yes/no) and if the likelihood of bacterial presence was the same as when the colour was assessed by laboratory staff using the SSC chart.

5.2.2.4 Sputum colour at exacerbations: likelihood of bacterial presence by technician assessment versus patients' report

The change in sputum colour was reported in 58.9% (209 out of 355) of all exacerbation visits. In 34.9% of these visits (73 out of 209) an increase in sputum colour was reported.

Sputum colour was assessed by laboratory staff in 89.3% of exacerbation visits (317 samples out of 355 exacerbation visits) or 97.8% of all samples obtained at these visits (317 out of 324 of samples obtained at exacerbations).

Using logistic regression and taking subjects with more than one exacerbation into account there was no significant association between patient reported change in sputum colour and PPM presence (OR 1.43, 95% CI 0.62; 3.27, p=0.392). Darker sputum colour reported by technicians using the SSC chart was associated with a higher likelihood of PPM presence (OR 2.70, 95%CI 1.60; 4.57, p=<0.001).

5.2.2.4.1 Summary

The use of the SSC chart reported by trained technicians was significantly associated with airway bacterial presence. In contrast, an increase in sputum colour reported by patients did not show significant associations.

5.3 Discussion

In the present chapter sputum colour as a biomarker was examined overtime during clinical stability and at specific time points such as exacerbations and stable visits. I have demonstrated that sputum colour, as assessed by trained technicians using the SSC chart, was associated with underlying airway neutrophilia at both clinical stability and during exacerbations. At exacerbation, the sputum colour was associated with airway and systemic inflammation. In addition, there was a strong association with airway bacterial presence. Furthermore, a greater positive change in sputum colour was associated with an increased prevalence of bacterial detection at exacerbations, which on its own has a potentially significant implication for the management of COPD exacerbations and antibiotic guidance.

Neutrophilic inflammation is a prominent feature of COPD thus I demonstrated that the AERIS cohort has expectedly high prevalence of airway neutrophilia both during clinical stability and exacerbations.[33, 121] Furthermore, airway neutrophilia was associated with higher systemic inflammatory response, particularly at exacerbations as reported previously.[121] I demonstrated that airway neutrophilia was associated with higher bacterial presence at exacerbations in line with previous authors.[46, 47]

Airway neutrophilia is a hallmark of COPD[61] that is elevated during exacerbations[168] especially when airway bacteria are present[124, 291]. However, direct assessment of airway inflammometry is laborious and requires a specialised laboratory team to process and analyse sputum samples. Anthonisen et al was the first group to attempt to classify the severity of COPD exacerbations and defined types of exacerbations according to the presence of dyspnoea, sputum purulence and volume.[143] Since the intensity of sputum purulence is defined by MPO presence[156], evaluation of sputum colour appeared to be an easy and useful tool. Various sputum colour assessments have been previously studied across a range of respiratory conditions including acute bronchitis, bronchiectasis and COPD.[154, 156, 159, 289, 292] Sputum colour assessments ranged from a simple description of the colour to a visual representation of colour grades that can be objectively compared to the actual sputum colour. [154, 156, 159, 289, 292] Due to financial and intellectual property concerns regarding previously published colour charts, our group designed the five-grade SSC chart for the objective evaluation of the degree of sputum purulence. We designed a five–colour chart which we felt was easier to use than 8 or 10 graded charts and, thus, more practical. I demonstrated that the sputum colour was significantly associated with and predictive of the airway neutrophilic inflammation, in line with previous studies[48, 156, 159] supporting the validity of the SSC chart. Sputum colour at exacerbation was also positively associated with markers of systemic inflammation, again similar to previously reported studies[123, 158, 160].

Sputum colour at stable state did not significantly change over time, however, the reliability of sputum colour as a marker of airway neutrophilia, as measured by ICC, was weak. This could be explained by the fact that the sputum colour chart is only a 5-point scale, so 1 point changes might be considered a large difference by ICC. Weak reliability could also be explained by the intra-individual variability over time. Having examined all stable visits over 12 months for individuals with at least 3 samples (n=107) in 43% of subjects sputum colour varied by a maximum of 1 unit (out of 4) while in 57% of subjects sputum colour varied by at least of 2 units.

I did observe that sputum colour at exacerbation was associated with and is predictive of the airway bacterial presence at exacerbation, in line with previous studies.[123, 154, 293] Stockley et al reported a favourable predictive potential of sputum purulence and presence of bacteria in the airways at exacerbations[123] which was subsequently confirmed in bronchoalveolar lavage by Soler et al.[293] However, in contrast to the AERIS cohort Stockley et al recruited only patients with the history of chronic bronchitis thus excluding an important group of COPD patients with emphysema.[123] Furthermore, patients were enrolled from the primary care had FEV1 performed when possible, therefore, the accuracy of the COPD diagnosis (spirometrically supported FEV1/FVC <0.7) made in the primary care was not confirmed. In addition, Stockley et al examined an association between the purulent (grade 3-8) and mucoid (grade 1-2) groups using a nine-point sputum chart compared to the analysis of the five-graded analyses presented in this chapter. Despite of the robustness of the lower airway secretion collection method (via protected specimen brush) used in Soler et al cohort, the study significantly differed methodologically from the AERIS in that it consisted of only severe exacerbations, sputum purulence was based solely on patients' report and no numerical assessment of sputum purulence was performed. [293]

To my knowledge this is the first report on the relationship between the objective numeric change in sputum colour and bacterial presence at exacerbation. The change in sputum colour was derived from a difference between the sputum colour at exacerbations and enrolment. Even though just over 60% of patients had no objective change or a negative change in sputum colour at exacerbation, the higher the positive change in sputum colour the greater the likelihood of the bacterial presence. Soler et al previously studied a qualitative change in sputum colour and its association with bacterial presence. The change in sputum from uncoloured to yellow/green was regarded as "purulent sputum" and was reported to be predictive of bacterial presence.[158] However, Soler et al examined only severe exacerbations of COPD and no other changes in sputum colour were considered, such as darkened sputum colour, "no change" in sputum colour between stable and exacerbation visits or controversially "improved"/negative changes were not previously studied. Thus there is an ongoing question whether the bacterial presence in the airways is a trigger of an exacerbation and needs to be treated or is just an associated event.

In this light another question on the association between respiratory viruses and sputum colour also arises. Papi et al studied bacterial, viral, bacterial-viral and "no pathogen" exacerbations in a cohort of patients hospitalised with exacerbation of COPD.[124] They reported that the prevalence of sputum purulence was higher in infected compared to non-infected exacerbations, and, interestingly, this included exacerbations with respiratory viruses present. Furthermore, the authors postulated that viral presence was associated with airway eosinophilia.[124] In my initial analysis there did not appear to be an association between respiratory viruses and sputum colour at exacerbation. However, when corrected for repeated exacerbations by the same subject, there was a statistically significant negative relationship (p=0.037) between sputum colour and respiratory virus presence at exacerbations. This finding may potentially be due to the different airway inflammation pathway, i.e. eosinophilic[124], caused by respiratory viruses in the absence of bacteria. Hence, higher respiratory viral presence may have been associated with a lighter sputum colour at exacerbations. Therefore, there appeared to be limited evidence of the relationship between sputum colour and respiratory viruses in the AERIS study. The relationship between respiratory viruses and eosinophilic inflammation is presented and discussed in the next chapter.

Previous studies reported higher bacterial presence in stable COPD. [157, 290] Singh et al reported a significantly higher bacterial load in darker sputum colour during clinical stability[290]. In their study Miravitlles et al demonstrated significant association between both the presence of bacteria and high bacterial load with the darker sputum colour and severity of dyspnoea at a stable state.[157] Miravitlles et al also reported sputum colour of ≥3, "dark yellow", was associated with an 80% prevalence of bacterial presence.[157] Admittedly, the sputum colour tool used by Miravitlles et al in their cross-sectional study was different to our approach but their sputum colour scale had the same description of colours to ours (from 1-"white" to 5-"greeninsh"). Interestingly, at enrolment I found no statistically significant relationship between sputum colour and bacterial presence. Despite this relationship was not being statistically significant, the prevalence of airway bacteria at enrolment was higher as sputum colour increased. However, an additional analysis of all stable visits, where sputum colour was reported over the 1st year (n=952) and with the subject repeated measurements accounted for, demonstrated that PPM presence was 29.8% higher with each unit increase in sputum colour at all stable visits (p=0.033). It is noteworthy, that there is a methodological difference between our study and the studies done by Singh et al and Miravitlles et al. In the first study bacterial detection was limited to three pathogens (*H. influenzae, S. pneumoniae, M. catarrhalis),* in the study by Miravitlles et al additional pathogens were investigated (*H. influenzae, H. parainfluenzae, S. pneumoniae, M. catarrhalis, P. aeruginosa*, enterobacteria and/or *S. aureus),* whereas in the AERIS project the focus was on five main bacteria *(H. influenzae, M. catarrhalis, S. pneumoniae, P. aeruginosa* and *S. aureus)*.

As opposed to conventional culture methods, bacterial presence detected by PCR was higher as per earlier studies[294], due to the more sensitive nature of the PCR test. There is emerging evidence that using PCR is somewhat superior to conventional culture in identifying airway bacterial presence[283, 295]. Despite this, the clinical application of molecular detection of airway bacterial infection is still largely unknown as clinically relevant thresholds of detection are yet to be established. Thus the use of PCR to detect bacteria is currently not widely used in the clinical setting. Hence, for the purposes of this thesis I have focused more on the clinically relevant bacterial culture data.

It was evident that sputum colour at exacerbation as assessed by laboratory staff against the colour chart was significantly associated with airway bacterial presence, unlike the change in sputum colour reported by patients. Does this really mean that the change in sputum colour reported by patients is not a reliable marker of a bacterial presence? Perhaps this conclusion may be too premature, as the absence of a significant relationship between patients reported sputum colour change and bacterial presence in the AERIS study may be contributed to by other factors. For instance, this may suggest that it is better to use sputum colour captured using a five-colour change which is related to bacterial presence than a binary report of an increase in sputum colour at exacerbations, and perhaps patients should use the sputum colour chart as in previous studies[156, 160, 289] . Furthermore, laboratory technicians were trained in using a five-colour chart in the same laboratory setting that had a largely static environment (i.e.. lighting), whereas patients were trained to report a change in colour, that differed from the baseline sputum colour, without a visual platform. In addition, there may have been cases where sputum colour change occurred more gradually, thus, not reported as such on the day of exacerbation. Another possible explanation for this non-significant relationship in patient reported colour change could be that a much smaller number (n=209) of patients reported change in sputum colour than technicians' reported sputum colour (n=317). In an ideal setting I would have liked to conduct a study where sputum colour was graded by both technicians and patients using SSC chart to examine the association between these reports and their relationship with airway bacterial presence.

These analyses are not without limitations. Firstly, the study design may have impacted the results. The primary objective of the AERIS study was to assess the incidence of all cause exacerbations of COPD and the contribution of airway infection to exacerbations of COPD[261], thus, the study was neither designed nor powered to investigate the association between the sputum colour grades, inflammatory markers and the bacterial presence. Hence, the sample size for some of the analyses is small and further studies are required to support the abovementioned results.

Furthermore, there was only 1 sample in sputum colour 5 at exacerbations thus sputum colour 5 was impossible to analyse along with other sputum colour samples. This might be due to the fact that the sputum colour chart, specifically the tone of the colour 5 as "greenish" was not accurately selected, thus leading to the lower sputum colour 5 sample size. Equally, this could be explained by the fact that our cohort represented both emphysematous and bronchitic patients unlike in the previous studies where only chronic bronchitis patients were recruited. [123, 156] Moreover, vast majority of our patients with exacerbations were managed early and monitored closely thus perhaps augmenting the natural process of an enhanced airway inflammation via increased myeloperoxidase level in response to airway bacterial presence. In an ideal setting for assessing the sputum purulence I would design a gadget to screen the sputum purulence that would be programmed to detect different shades of the sputum colour and categorise these into scores objectively. I would still consider limiting the number of sputum colour to five as we effectively are looking for mucoid and purulent samples.

Study methods were aimed to achieve the primary objective, thus when the sputum sample weighed less than 0.1g the sample was prioritised for microbiology analyses, thus, fewer sputum samples were processed for sputum differential cell counts. In addition, PPM by PCR analysis was performed on raw samples stored during the sputum processing. Due to the small sputum sample size received from some subjects, fewer samples were available for PPM by PCR analysis, thus leading to the discrepancy in the number of microbiology samples processed for culture and PCR analyses.

Not all limitations were related to the study design and methods. There were analytical issues related to the availability of data at the time of PhD analyses. There were only binominal variables for bacterial presence available at the time of PhD analyses and not bacterial load. Therefore, the concept that the sputum colour reflects the activity of the underlying inflammatory mediators[156] that in turn is further stimulated by the bacterial load in the airway[48, 293] was not possible to assess in this thesis.

In summary, this chapter has highlighted the relationships between airway neutrophilia, sputum colour and the nature of airway exacerbations. Primarily, I have provided evidence that the SSC chart can be reliably used to assess sputum colour as it was demonstrated to be a surrogate marker of underlying neutrophilic inflammation. Furthermore, the analysis of sputum colour by the Southampton sputum colour chart may help to indicate airway bacterial presence at exacerbation and this is of a potential relevance for the prescription of antibiotics. This is the first report of the association between the numeric change in sputum colour and airway bacterial presence. Specifically, the quantified change in sputum colour from baseline to exacerbation is a

useful factor in increasing our understanding of underling infective impact during exacerbations and maybe more useful than the sputum colour score alone at the time of exacerbation. Therefore, in the next chapter I studied whether the cellularity of the sputum could identify patients with different clinical features that may potentially indicate a more tailored approach to management of COPD exacerbations.

Chapter 6 Impact and associations of eosinophilic inflammation in COPD

6.1 Background

Eosinophils, coarsely granular cells, were first described by Paul Ehrlich in 1879.[296, 297] Ehrlich first introduced the term "eosinophil" to describe cells with "alpha" granules. The latter he speculated were responsible for the conditions such as asthma, skin diseases and reaction to medications.[297] As highlighted in the last chapter, over the years COPD was thought to be more associated with neutrophilic airway inflammation, with eosinophilic inflammation in COPD being considered controversial and thought to be more of a hallmark of asthma in the past. However, there is a volume of evidence emerging to support that eosinophilic inflammation is a distinct COPD phenotype. Sputum eosinophils >3% have been reported to be an important marker of airway inflammation in COPD and a good indicator of steroid treatment response.[175, 177] In particular, an increase in exacerbation rate when inhaled corticosteroids were withdrawn in WISDOM study. Sputum sampling is however not a clinically available test thus previous studies explored a more widely available marker of airway eosinophilia. These studies revealed that blood eosinophils are associated and predictive of sputum eosinophilia.[47, 197, 298] Blood eosinophils were found to be predictive of the response to oral[253, 254] and inhaled corticosteroid therapy.[299] Recent reports on the interventional studies support the evidence of significant contribution of eosinophilic phenotype in COPD exacerbations by its response to the treatment with Mepolizumab (anti-Interleukin 5).[300] The reference to the raised blood eosinophils as eosinophilic inflammation in COPD has been previous made by some authors.[195] Although, it is noteworthy that previously reported cut offs are still within normal range for blood eosinophils[47, 195, 197, 198], the normal range that was derived from the mean ±2SD of the presumed healthy population. However, it is apparent that subgroup of patients with raised systemic markers differs from others, namely, has a better response to ICS treatment.[206, 301] Therefore, for the simplicity I used the term "systemic eosinophilic inflammation" with the reference to the blood eosinophils ≥2%.

Hence eosinophilic inflammation is an important COPD endotype but little is known about its stability over time, its relationships to the inflammatory nature and aetiology of exacerbations. To improve understanding of eosinophilic inflammation in COPD, I examined these factors in the AERIS cohort in this chapter.

6.1.1 Outline of the eosinophils in AERIS cohort analyses

This chapter is divided into two main sections: airway and systemic eosinophilic inflammation. Analyses firstly examined characteristics of eosinophilic and non-eosinophilic groups at enrolment and 1st exacerbations. I then examined if longitudinal airway eosinophilia in COPD exists and if it persists over time, and how those with eosinophilia over time differ from those without. Finally I studied the role of seasonality and airway infection on eosinophilic inflammation.

6.2 Results

6.2.1 Airway eosinophilic inflammation in AERIS cohort

Airway eosinophilic inflammation was defined as sputum eosinophils % >3% in line with previous studies.[47, 175, 177, 182, 206] Only good quality sputum samples from stable visits with squamous cells <30% were included in the airway eosinophilia analyses.

6.2.1.1 Airway eosinophilia at stable state

To begin with, I examined the number of good quality sputum samples available during stable visits and found that there were 653 high quality samples available from stable visits over the 1st year. The median (IQR) % of eosinophils in these samples was 1.38 (4.49). (**Figure 6.1**)

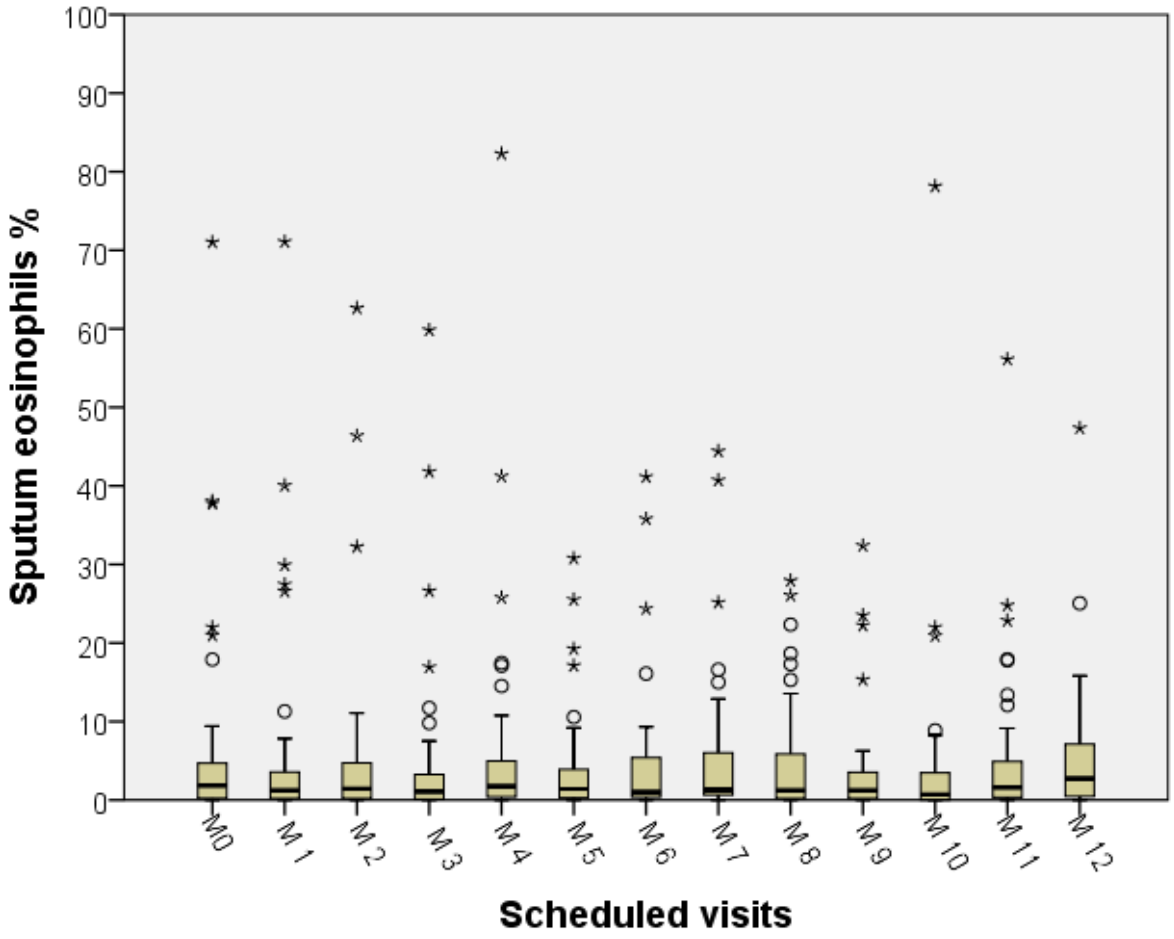


Figure 6.1 Box-and-whisker plots of sputum eosinophils% illustrating that the spread of sputum eosinophils per each month when patients were deemed clinically stable. N=653

In my initial analysis, at enrolment out of 127 subjects only 69 had a good quality sputum sample with median (IQR) sputum eosinophils % of 1.93 (5.09). In order to examine that a group included into my analysis was not significantly dissimilar from the group excluded, I conducted a comparison analysis between these two groups. This analysis demonstrated no statistically significant differences between excluded and included in analysis groups, apart from blood eosinophils (0.20(0.20) and 0.20(0.18) respectively, p=0.027). However, the median value of blood eosinophils in both groups was similar. (**Table 6.1**)

Table 6.1 Comparison of general characteristics between groups without and with sputum eosinophils % available at enrolment

	Excluded[α]		Included[β]		
	n	**Median(IQR)**	**n**	**Median(IQR)**	**P value[γ]**
Age*	58	66.33(±8.94)	69	67.22(8.37)	0.591
Pack year history	58	46.00(26.25)	69	49.00(29.00)	0.835
FEV1 (%)	57	45.00(25)	69	48.00(24)	0.360
FEV1 reversibility (L)	50	0.13(0.17)	59	0.11(0.16)	0.386
Blood					
WBC *10^9/L	58	7.30(2.23)	68	7.80(2.48)	0.216
Neutrophils*10^9/L	58	4.80(1.53)	68	4.90(1.85)	0.317
Eosinophils*10^9/L	58	0.20(0.20)	68	0.20(0.18)	0.027
CRP mg/L	58	5.00(5.25)	69	6.00(8.50)	0.118
Fibrinogen g/L	55	4.80(1.30)	59	5.00(0.90)	0.231
CAT	57	18.00(10)	69	16.00(11)	0.584
EXACT-PRO	46	37.00(12)	55	36.00(11)	0.743
Total exacerbation rate	58	2.16(3.23)	69	2.98(3.98)	0.217

[α] Excluded – group of patients with no good quality sputum data available; [β] Included – group of patients with good quality sputum data available; [γ]Mann Whitney test used for significance; * - data parametric and presented as mean(±SD). For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

At enrolment, the prevalence of airway eosinophilia (>3%) was 34.8% (n=24), among those with sputum eosinophilia the median (IQR) sputum eosinophil % at enrolment was 6.96(10.88). In order to examine if patients with and without airway eosinophilia were different at recruitment, subjects with good quality sputum samples at enrolment (n=69) were divided into two groups: eosinophilic and non-eosinophilic based on sputum eosinophilia of >3% and ≤3% respectively. I discovered that those in the eosinophilic group had a less significant smoking pack year history, contained fewer active smokers, had lower levels of WBC, blood neutrophils and fibrinogen, and higher blood eosinophils at enrolment compared to the non-eosinophilic group at enrolment. CAT score was lower in those with eosinophilic airway inflammation at enrolment but it did not reach significance (p= 0.071). There was no significant difference in ICS use between the eosinophilic and non-eosinophilic groups. (**Table 6.2**)

The total exacerbation rate was not significantly different between those with and without airway eosinophilia at enrolment. However, exacerbations associated with raised sputum eosinophils % was significantly higher in those who had airway eosinophilia at enrolment (p=0.045). (**Table 6.2**) This suggests that while airway eosinophilia does not predispose individuals to frequent total exacerbations, when they do exacerbate, they are likely to be associated with airway eosinophilia. So I examined if the odds of an exacerbation having airway eosinophilia was different between

those with and without airway eosinophilia at enrolment and found it was 3.82 times higher if airway eosinophilia was present at enrolment for that individual, however, this was of borderline significance (CI 0.93; 15.75; p=0.063).

In summary, over third of patients had sputum eosinophilia at enrolment. Airway eosinophilia at enrolment was associated with lower systemic inflammatory inflammation (WBC, blood neutrophils and fibrinogen) and higher blood eosinophilic inflammation compared to the non-eosinophilic group at enrolment. Those with airway eosinophilia at enrolment had higher prevalence of airway eosinophilia at 1st exacerbations than those without. Although of a borderline significance, I demonstrated that airway eosinophilia at enrolment did appear to predict airway eosinophilia at exacerbations.

At this point I moved on to the next step, the analysis of exacerbations with and without airway eosinophilia.

Table 6.2 Characteristics of markers at ENROLMENT for a full cohort and COPD phenotypes based on eosinophils% in SPUTUM (>3%)

Marker	Overall cohort		Non-Eosinophilic		Eosinophilic		P value[Ω]
	Median (IQR)	n	Median (IQR)	n	Median (IQR)	n	
Age [α]	66.81(±8.61)	127	66.62(±8.29)	45	68.33(±8.58)	24	0.636
Smoking history (pack/years)	47.00(26.25)	127	53.00(26.50)	45	41.50(19.75)	24	0.012
Smoking status[γ μ]	54(42.5%)	127	23(51.1%)	45	4(16.7%)	24	<0.01
Use of ICS at enrolment (within subgroups) [β μ ¥]	111(87.4%)		38(86.4%)		23(95.8%)		0.407
Beclomethasone equivalent dose	1000(1200.00)	127	1000(1200.00)	32	800(600.00)	19	0.135
BMI	27.04(6.69)	127	27.16(7.15)	45	27.19(8.18)	24	0.687
6MWT	300.00(170)	125	300.00(168)	45	351.00(198)	24	0.212
Blood markers							
WBC*10^9/L	7.60(2.20)	126	8.20(2.32)	44	6.55(1.75)	24	0.001
Eosinophils *10^9/L	0.20(0.20)	126	0.20(0.20)	44	0.30(0.18)	24	<0.001
Neutrophils *10^9/L	4.80(1.70)	126	5.25(1.67)	44	3.80(1.93)	24	<0.001
Fibrinogen g/L	4.80(1.02)	114	5.10(1.15)	40	4.70(0.70)	19	0.040
Procalcitonin ng/ml	0.06(0.03)	126	0.06(0.02)	45	0.07(0.04)	24	0.029
CRP mg/L	5.00(8.00)	127	6.00(10.00)	45	5.50(8.00)	24	0.324
Sputum							
%Eosinophils in sputum	1.93(5.09)	69	0.48(1.89)	45	6.96(10.88)	24	-
%Neutrophils in sputum	47.02(71.22)	69	17.75(69.10)	45	59.86(49.66)	24	0.132
PPM presence n(%)	57(51.8%)		24(53.3%)		13(54.2%)		0.947
Virus presence n(%)	52(17.2%)	302	10(22.2%)	45	5(20.8%)	24	0.894
Spirometry							
FEV1 (%)	46.50(25)	126	50.00(24)	45	44.50(22)	24	0.562
FEV1 (L)	1.13(0.66)	126	1.16(0.64)	45	1.12(0.66)	24	0.748
FEV1 reversibility (% of preBDFEV1)[€]	11.29(16.89)	109	8.29(21.39)	38	8.86(13.27)	21	0.698
FEV1 reversibility (L)	0.12(0.17)	109	0.11(0.17)	38	0.12(0.15)	21	0.552
ΔFEV1(% of baseline)[ξ]	4.93(21.05)	85	6.85(19.09)	32	-0.22(17.64)	16	0.269
ΔFEV1(L/year)	0.06(0.26)	85	0.09(0.24)	32	-0.01(0.18)	16	0.309
RV (L)	3.23(1.25)	116	3.22(1.34)	42	3.13(1.17)	24	0.405
TLC (L)	6.34(1.84)	116	6.44(1.71)	42	6.01(2.60))	24	0.405
TLCO (mmol/kPa/min)	4.47(2.32)	121	4.40(2.41)	43	4.49(2.02)	24	0.647
KCO (mmol/kPa/min)	0.94(0.48)	121	0.92(0.47)	43	1.04(0.50)	24	0.596
Clinical markers							
CAT	16.00(10)	126	17.00(11)	45	14.00(11)	24	0.071
EXACT-PRO	37.00(12)	101	36.00(10)	35	36.00(16)	20	0.180
mMRC dyspnoea score	4.00(1)	127	4.00(1)	45	4.00(0)	24	0.337
Exacerbations rate 12 months before the study	2.00(2.00)	127	3.00(2.50)	45	2.00(1.00)	24	0.568
Exacerbation rate over the 1st year in the study	2.94(3.91)	127	2.99(3.98)	45	1.99(3.89)	24	0.301
Exacerbation rate with sputum eosinophilia in Year 1	0.00(0.97)	127	0.00(0.00)	45	0.49(1.00)	24	0.045

All continuous variable presented as median(IQR) unless stated otherwise. Mann-Whitney and Chi-Square tests used for significance unless stated otherwise; [Ω] significance test only for eosinophilic subgroups (excluding overall cohort variables); [α] reported as Mean(±SD); [β] reported as number and % within the subgroup; [μ] Fisher exact test reported for the significance.
For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

6.2.1.2 Airway eosinophilia at exacerbations

I found that a total of 218 exacerbation events had good quality sputum samples available over the 1st year. In order to avoid data skewing by repeated measure from same group of patients, an initial cross sectional analysis was performed on 1st exacerbations where good quality sputum data was collected (n=61). To ensure the group included in analysis is representative of the rest of the 1st exacerbations excluded from the analysis, I compared main clinical characteristics between the two groups and found that the group included in analysis had higher fibrinogen levels compared to the group excluded (5.40(1.40) and 4.90(1.33) respectively, p=0.023) and no significant differences in other systemic inflammatory markers. (**Table 6.3**)

Table 6.3 Comparison of general characteristics between groups without and with sputum eosinophils % available at 1st exacerbations

		Excluded[α]		Included[β]		
		n	**Median(IQR)**	**n**	**Median(IQR)**	**P value[γ]**
FEV1 (%)		44	33.50(26)	53	44.00(18)	0.060
Blood						
	WBC*10^9/L	44	8.10(3.60)	58	8.00(3.35)	0.829
	Neutrophils*10^9/L	44	5.20(3.10)	58	5.35(3.20)	0.850
	Eosinophils*10^9/L	44	0.20(0.20)	58	0.20(0.20)	0.793
	CRP mg/L	46	7.50(11.50)	57	10.00(26.50)	0.431
	Fibrinogen g/L	46	4.90(1.33)	57	5.40(1.40)	0.023
CAT		45	19.00(13)	61	22.00(9)	0.427
EXACT-PRO		43	42.00(13)	54	41.00(10)	0.788

[α] Excluded – group of patients with no good quality sputum data available; [β] Included – group of patients with good quality sputum data available; [γ]Mann Whitney test used for significance. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

Of the 61 1st exacerbations with quality sputum, 23.0% (n=14) were found to have airway eosinophils >3%. To examine any differences between subjects with and without airway eosinophilia at 1st exacerbation, I compared the two groups and found that those who had airway eosinophils >3% at 1st exacerbations had a higher blood eosinophil count, lower CRP and lower sputum neutrophils %. Blood neutrophils level was also slightly higher in the non-eosinophilic group but this did not reach significance. (**Table 6.4**)

Table 6.4 Characteristics of markers at 1st EXACERBATIONS for the full cohort and high and low sputum eosinophilia (>3% or ≤3%)(n=61)

Marker	Overall cohort		Non-Eosinophilic (n=47)		Eosinophilic (n=14)		P value[Ω]
	Median (IQR)	n	Median (IQR)	n	Median (IQR)	n	
Blood markers							
WBC*10^9/L	8.00(3.53)	102	7.95(3.50)	46	8.35(3.13)	12	0.387
Eosinophils *10^9/L	0.20(0.20)	102	0.20(0.20)	46	0.35(0.45)	12	0.018
Neutrophils *10^9/L	5.30(3.20)	102	5.45(3.30)	46	4.85(2.22)	12	0.077
Fibrinogen g/L	5.10(1.40)	103	5.40(1.58)	46	5.20(1.30)	11	0.357
Procalcitonin ng/ml	0.07(0.04)	99	0.07(0.04)	43	0.08(0.05)	12	0.589
CRP mg/L	8.00(14.00)	103	12.50(28.00)	46	4.00(4.00)	11	0.014
Sputum							
%Eosinophils in sputum	1.20(2.38)	61	0.60(1.60)	47	5.79(16.14)	14	-
%Neutrophils in sputum	74.00(47.11)	61	87.8(41.29)	47	57.4(34.09)	14	0.016
PPM presence n(%)[§]	60 (60.6%)	99	34(72.3%)		5(35.7%)		0.024
Viruses presence n(%)	39(43.3%)	90	21(46.7%)	45	6(50.0%)	12	0.837
Lung function							
FEV1 (%)	43.00(21)	97	45.00(18)	40	43.00(18)	13	0.942
FEV1 (L)	1.02(0.62)	97	1.18(0.57)	40	1.01(0.61)	13	0.780
Clinical markers							
CAT	21.00(11)	106	22.00(9)	47	19.00(9)	14	0.239
EXACT-PRO	41.00(11)	97	41.00(11)	40	40.50(7)	14	0.874

All continuous variable presented as median(IQR) unless stated otherwise. Mann-Whitney or Chi-Square tests used for significance unless stated otherwise; [§] Fisher Exact for significance test. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

To summarise, a quarter of patients were found to have airway eosinophilia (>3%) at first exacerbation. Airway eosinophilia was associated with higher blood eosinophils, lower CRP and lower sputum neutrophils.

Having established that groups with and without airway eosinophilia differed at enrolment and at exacerbation, I next asked if airway eosinophilia was reliably detected over time in stable disease and if exacerbations associated with sputum eosinophilia could be predicted by airway eosinophilia at enrolment.

6.2.1.3 Change in airway eosinophils over time

Firstly I examined if there was a consistency between stable visits over time and then between stable and exacerbation states.

To study if there was a dynamic change over time, I compared sputum eosinophils % at enrolment and at Month 12 and found that sputum eosinophils % did not significantly change over time (Median (IQR) 1.93(5.09) and 1.06(7.07) respectively, p-value = 0.510). With an aim to examine reliability of sputum eosinophils (%) over the 1st year I conducted an intraclass correlation coefficient analysis and found that reliability was moderate. (ICC 0.47, CI 0.37; 0.57 p=0.050)

Applying paired analysis, I investigated if there was a significant difference in sputum eosinophils (%) between enrolment and 1st exacerbations with sputum data available. I found that sputum eosinophils were decreased at the 1st exacerbation compared to enrolment (Median (IQR) 1.97 (4.43) and 0.82 (2.33), p = 0.003).

Thus, I demonstrated that a group with airway eosinophilia differed in clinical parameters from a group without airway eosinophilia at a single clinical time point (enrolment or 1st exacerbation) and significantly differed between these two clinical states. However, the question remained is airway eosinophilia a discreet and persistent phenotype that present differently both at clinical state and at exacerbations?

6.2.1.4 Longitudinal groups with airway eosinophilia

To investigate the stability of airway eosinophilia over time I divided subjects into three groups: predominantly, intermittent and rarely eosinophilic groups.

Predominantly airway eosinophilia group (PAE) was defined as sputum eosinophilia at either all stable visits, or all but 1 visit where the sputum eosinophils were ≤3%; the rarely airway eosinophilia (RAE) group was defined as sputum eosinophils ≤3% at all visits, or all but 1 visit where the sputum eosinophils were >3%; the IAE group was defined when none of the abovementioned criteria were met. Only those subjects who had at least 3 stable visits with good quality sputum data (3 out of 13) over 12 months were included in the group analyses (n= 80). (

Table 6.5)

Table 6.5 . Number of good quality sputum samples and cumulative % per each subject over the 12 months

N of samples	N of subjects	Cumulative %
13	1	0.8
12	3	3.1
11	3	5.5
10	6	10.2
9	5	14.2
8	9	21.3
7	11	29.9
6	10	37.8
5	11	46.5
4	7	52.0
3	14	63.0
2	15	74.8
1	16	87.4
0	16	100.0

Only individuals with 3 or more samples were included into the longitudinal analyses

63% of subjects (n=80) had at least 3 stable visits with good quality sputum and were included into longitudinal analyses. The remaining 37% of subjects (n=47) did not meet the criteria and were excluded from the analyses.

To ensure there was no significant difference between those included and excluded from the longitudinal analyses the comparison analysis was performed. It was found that the excluded group had a lower prevalence of blood eosinophils (count and %) and lower follow up in years. The summary of descriptive characteristics is presented in **Table 6.6**.

Table 6.6. Characteristics of an overall cohort, and groups excluded and included in the longitudinal analyses based on sputum eosinophils % at enrolment.

	Excluded		Included		P-value
	N	**Median (IQR)**	**N**	**Median (IQR)**	
Age [α]	**47**	66.11(±8.87)	**80**	67.23(±8.48)	0.431
Male[β]	**22/47**	46.80%	**46/80**	57.50%	0.243
Current smoker (Yes) [β]	**20/47**	42.60%	**34/80**	42.50	0.995
Smoking history (pack/years) [α]	**47**	44.19(±19.21)	**80**	53.86(±31.87)	0.079
Use of ICS at enrolment[β]	**40/47**	85.10	**71/79**	89.90%	0.424
Beclomethasone equivalent dose	**38**	900(1200.00)	**69**	1000(1400.00)	0.748
FEV1 (%)	**46**	45.50(29)	**80**	47.00(23)	0.706
Sputum Eosinophils (%)	**10**	1.97(6.68)	**59**	1.93(4.55)	0.218
Blood Eosinophils *10^9/L	**47**	0.20(0.20)	**79**	0.20(0.20)	0.008
Eosinophils (%)	**47**	2.02(2.20)	**79**	3.16(2.78)	0.004
CAT	**46**	16.00(10)	**80**	16.00(11)	0.796
EXACT-PRO	**35**	38.00(12)	**66**	36.00(11)	0.565
BMI	**47**	27.47(7.98)	**80**	26.89(7.24)	0.916
6MWT (distance in meters)	**45**	275(206)	**80**	318(156)	0.111
Exacerbation rate 12 months before	**47**	2.00(3.00)	**80**	3.00(2.00)	0.971
Exacerbation rate in Year 1	**47**	1.99(4.99)	**80**	2.99(3.91)	0.104
Follow up in years[Y]	**47**	0.83(±0.30)	**80**	0.98 (±0.12)	0.010

All continuous variable presented as median(IQR) unless stated otherwise. Mann-Whitney and Chi-Square tests used for significance unless stated otherwise, [α] reported as Mean(±SD); [β] Data presented as n/N(%) n=number of subjects, N=total number of subjects; [Y]Data is non-parametric but presented as Mean (SD) as deemed more representative. Median (IQR) for the overall cohort, excluded and included in the analysis 1.00(±0.02), 1.00(±0.45) and 1.01(±0.02) respectively. Mann Whitney test for significance was applied. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

6.2.1.4.1 Airway eosinophils in longitudinal groups at enrolment

I further assessed the prevalence of longitudinal airway eosinophilia phenotype in AERIS cohort and discovered that out of 80 subjects who had at least 3 good quality sputum at stable visits over the period of 12 months, 21% (n=17) had predominantly (PAE), 36% (n=29) intermittently (IAE) and 43% (n=34) rarely (RAE) airway eosinophilia over the 12 months. Interestingly, similar to the eosinophilic and non-eosinophilic airway groups at enrolment, a similar pattern was observed concerning active smoking in the longitudinal groups. In particular, there were fewer active smokers in those with the predominantly eosinophilic airway inflammation compared to those with intermittently and rarely. There was a significant difference in WBC, blood neutrophils and

CRP with the lowest median in the predominantly eosinophilic group and highest in the rarely group, inverse results observed for blood eosinophils. There was also a trend towards significance in fibrinogen level between the three groups with the highest value among those in the rarely group. Although there was no difference in the total exacerbation rates between the three groups, those in the predominantly eosinophilic group had a higher rate of exacerbations associated with airway eosinophilia than those who in rarely eosinophilic group. (**Table 6.7**)

Table 6.7 Characteristics of longitudinal COPD groups based on eosinophils% in SPUTUM at ENROLMENT

	Predominantly		**Intermittently**		**Rarely**		
Marker	**Median(IQR)**	**n**	**Median(IQR)**	**n**	**Median(IQR)**	**n**	**p-value**
Age $^{\alpha}$	66.29(±6.90)	17	68.41(±9.83)	29	66.68(8.08)	34	0.417
Smoking history (pack/years)	43.00(34.08)	17	50.00(28.00)	29	52.25(27.38)	34	0.564
Smoking status $^{\gamma\,\mu}$	3(17.6%)	17	12(41.4%)	29	19(55.9%)	34	0.031
Use of ICS at enrolment $^{\mu}$	15(88.2%)	17	26(89.7%)	29	29(87.9%)	33	1.000
Beclomethasone equivalent dose	1000(1200.00)	15	800(1600.00)	25	1000(1400.00)	29	0.722
Blood							
WBC*10^9/L	6.50(1.00)	17	7.50(2.50)	29	8.20(2.20)	33	0.001
Eosinophils *10^9/L	0.30(0.40)	17	0.20(0.10)	29	0.20(0.20)	33	0.001
Neutrophils *10^9/L	3.80(1.45)	17	4.70(1.40)	29	5.20(1.40)	33	<0.001
Fibrinogen g/L	4.55(1.30)	14	4.95(1.48)	26	5.10(0.68)	30	0.072
Procalcitonin ng/ml	0.06(0.03)	17	0.06(0.03)	29	0.06(0.03)	34	0.884
CRP mg/L	2.00(6.50)	17	6.00(7.50)	29	7.00(8.75)	34	0.018
Sputum							
Eosinophils %	7.36(17.07)	12	2.41(4.21)	22	0.22(1.13)	25	
PPM presence n(%)	10(58.8%)	17	15(53.6%)	28	20(62.5%)	32	0.782
Viruses presence n(%)	4(23.5%)	17	3(11.1%)	27	7(21.9%)	32	0.470
Lung function							
FEV1 (%)	46.00(30)	17	50.00(20)	29	44.50(23)	34	0.403
FEV1 reversibility (L)	0.12(0.11)	14	0.10(0.11)	26	0.12(0.17)	30	0.336
ΔFEV1(L/year)	-0.05(0.27)	14	0.04(0.21)	22	0.09(0.20)	26	0.224
BMI	25.32(3.66)	17	27.21(7.14)	29	27.52(9.03)	34	0.082
6MWT	328.00(192)	17	311.00(158)	29	303.50(152)	34	0.568
CAT	16.00(10)	17	17.00(11)	29	16.50(10)	34	0.673
EXACT-PRO	37.00(9)	15	36.00(11)	25	37.50(17)	26	0.553
mMRC dyspnoea score	4.00(1)	17	4.00(2)	29	4.00(0)	34	0.487
Exacerbations rate 12 months before the study	3.00(3.00)	17	2.00(2.50)	29	3.00(2.00)	34	0.669
Exacerbation rate over the 1st year in the study	2.98(3.79)	17	2.99(3.96)	29	3.01(3.91)	34	0.961
Exacerbation rate with sputum eosinophilia over 1st year $^{\Psi}$	1.07(±1.09)	17	0.66(±0.97)	29	0.27(±0.79)	34	0.001

All continuous variable presented as median(IQR) unless stated otherwise. Mann-Whitney and Chi-Square tests used for significance unless stated otherwise; $^{\alpha}$ Presented as Mean(SD); $^{\mu}$ Fisher exact test applied for significance; $^{\Psi}$ Exacerbation rates for exacerbations with sputum eosinophilia, variable is non-parametric but presented as mean(SD) as median not informative. Median(IQR) for the predominant, intermittent and rarely groups 1.00(1.00), 0.00(0.99) and 0.00(0.00) respectively. Kruskall Wallis test used for significance. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

Having discovered the significant difference in the rate of exacerbations associated with airway eosinophilia between the longitudinal groups (**Table 6.7**), I investigated if the likelihood of an exacerbation being eosinophilic is higher in the predominantly eosinophilic compared to other groups applying all exacerbations with available sputum samples. I applied logistic regression analyses to adjust for the effect of multiple measurements and discovered that the odds of an exacerbation being eosinophilic was 84% lower in the intermittent than predominant group (OR 0.16, CI0.03; 0.97, p=0.046). There was no such association between the rarely and the predominant groups. (OR 1.11, CI 0.25; 4.90, p=0.892).

I also investigated if sputum eosinophils at enrolment could be a marker of predominantly airway eosinophilia group over time and found that sputum eosinophils% at enrolment were predictive of those with the predominantly airway eosinophilia over time (AUC 0.894, CI 0.7888; 0.999 p<0.001).

Thus, it does appear that at enrolment patients with predominant, intermittently and rarely airway eosinophilia differ in both the systemic markers of inflammation and the exacerbation rate with airway eosinophilia in the subsequent year. Furthermore, the persistence of eosinophilic airway inflammation could be predicted at enrolment. Due to these differences in the stable state, I next asked if this underlying phenotype also differed at exacerbation.

6.2.1.4.2 Airway eosinophilia in longitudinal groups at exacerbations

I examined if the airway longitudinal groups previously defined differed at 1st exacerbation and found no significant clinical differences at 1st exacerbations across the longitudinal eosinophilic groups. (**Table 6.8**)

Table 6.8 Characteristics of longitudinal COPD groups based on eosinophils% in SPUTUM at 1st EXACERBATIONS

Marker	Predominantly		Intermittently		Rarely		
	Median(IQR)	n	Median(IQR)	n	Median(IQR)	n	p-value
Blood markers							
WBC*10^9/L	7.90(3.60)	17	8.90(3.33)	22	7.40(3.10)	29	0.186
Eosinophils*10^9/L	0.20(0.35)	17	0.20(0.13)	22	0.10(0.30)	29	0.648
Neutrophils *10^9/L	5.30(2.95)	17	5.55(2.70)	22	5.10(2.70)	29	0.229
Fibrinogen g/L	5.50(1.68)	16	5.25(1.23)	22	4.80(0.95)	29	0.303
Procalcitonin ng/ml	0.07(0.04)	15	0.07(0.05)	22	0.06(0.05)	27	0.735
CRP mg/L	5.00(12.00)	17	7.00(22.00)	21	8.00(11.00)	30	0.302
Sputum							
%Eosinophils in sputum	2.25(7.19)	10	0.90(1.97)	12	0.82(2.57)	17	0.506
PPM presence n(%)$^{\mu}$	10(66.7%)	15	12(60.0%)	20	16(57.1%)	28	0.896
Viruses presence n(%)	5(35.7%)	14	9(47.4%)	19	9(36.0%)	25	0.704
Lung function							
FEV1 (%)	40.00(18)	16	44.00(17)	19	43.00(19)	27	0.530
FEV1 (L)	1.00(0.61)	16	1.17(0.68)	19	1.01(0.58)	27	0.666
Clinical markers							
CAT	19.00(10)	17	20.00(14)	22	22.00(10)	28	0.232
EXACT-PRO	41.00(5)	16	40.00(14)	21	44.00(13)	27	0.107

All continuous variable presented as median(IQR) unless stated otherwise. Mann-Whitney and Chi-Square tests used for significance unless stated otherwise; $^{\alpha}$ Presented as Mean(SD); $^{\mu}$ Fisher exact test applied for significance. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

6.2.1.5 Seasonality and airway eosinophilia

As exacerbations are more common in the Winter than in the Summer,[275, 279] seasonality may have a potential effect on the exacerbation phenotype. Thus I enquired if there was an impact of a season on exacerbations with sputum eosinophilia. To investigate an impact of seasonality on exacerbations I divided the year into 2 seasons, each containing six months: one containing winter and the other summer months. For the simplicity I defined them as Winter (October-March) and Summer (April – September) seasons as previously published by our group[275].

I found no significant difference in the proportion of exacerbations associated with airway eosinophilia between Winter and Summer seasons (28 out of 130 (21.5%) and 24 out of 88 (27.3%) respectively, p=0.330). Using all exacerbations with good quality sputum data available and having adjusted for subjects repeated measurements, I examined if the odds of exacerbations being eosinophilic differed between Winter and Summer seasons. I found no effect of seasonality on sputum eosinophilia at exacerbations in Winter compared to Summer (OR 1.94, CI 0.82; 4.58, p value = 0.129). (**Table 6.19**)

In summary, there did not appear to be any difference between the three longitudinal groups present at 1st exacerbations nor was there an association of exacerbations with airway eosinophilia and seasonality. As exacerbations frequently have an infectious aetiology [275], I next investigated if there was an association between airway infection and airway eosinophilia.

6.2.1.6 Airway infection and airway eosinophils

There was no significant difference in PPM prevalence between those without and with airway eosinophilia at enrolment (53.3% and 54.2%, p=0.947 respectively) and likewise in respiratory viruses (22.2% and 20.8% respectively, p=0.894). (**Table 6.2**)

Similar cross sectional analysis of the group with airway eosinophilia had significantly lower PPM presence compared to the group without sputum eosinophils at first exacerbation (35.7% and 72.3% respectively, p=0.024). (**Table 6.4**) A more sophisticated logistic regression analysis allowing for exacerbations for all subjects with repeated measurements revealed that among those who had airway eosinophilia at exacerbation the presence of airway bacteria was 78% lower than in those without airway eosinophilia at exacerbations (OR 0.22, CI 0.09; 0.55, p=0.001). In contrast, there was no significant difference in the presence of respiratory viruses between these two groups at 1st exacerbation (50.0% and 46.7% respectively, p=0.837). (**Table 6.4**)

Similarly to the cross sectional analysis, I then examined if there was a difference in the prevalence of airway infection between the predominantly, intermittent and rarely airway eosinophilic groups (defined at enrolment) and found no significant difference in either airway bacterial (58.8%, 53.6% and 62.5% respectively, p=0.782) or viral (23.5%, 11.1% and 21.9% respectively, p=0.470) presence. (**Table 6.7**) Likewise, at 1st exacerbation there was no significant difference between the three longitudinal groups in the prevalence of bacterial (66.7%, 60.0% and 57.1% respectively, p=0.896) and viral infections (35.7%, 47.4% and 36.0% respectively, p=0.704). (**Table 6.8**) However, given there were more exacerbations with sputum data available, I examined all available data with correction for patients' repeated measures and discovered no significant association in airway bacterial presence between the three longitudinal eosinophil groups at all exacerbations (RAE vs PAE OR 0.425, CI 0.12; 1.53, p= 0.192, IAE vs PAE OR1.40, CI 0.37; 5.30, p=0.619).

I studied if there was a seasonality effect on airway bacterial presence at exacerbations among each longitudinal group and discovered that there was a seasonal variation in PPM presence in the predominantly eosinophilic group with PPM presence higher in the Winter season than in Summer. There was a similar trend in the rarely airway eosinophilia group although this did not

reach significance, and no significant difference in PPM presence between Winter and Summer in the intermittently airway eosinophilic group was observed. (**Table 6.9**)

Table 6.9 Prevalence and odd ratios of PPM in the longitudinal airway eosinophilic groups per season at exacerbations in each group

	Summer	Winter	OR, CI, p value
Predominantly eosinophilic	11/23 (47.8%)	28/40 (70.0%)	9.01, 1.05; 77.15, 0.045
Intermittently eosinophilic	20/32 (62.5%)	29/40 (72.5%)	1.59, 0.53; 4.73, 0.407
Rarely eosinophilic	11/30 (36.7%)	23/41 (56.1%)	3.96, 0.90; 17.38, 0.069

Data presented as n/N (%), n= number of, N = total number in the group

I further investigated the odds of respiratory viruses present in those with and without sputum eosinophilia at enrolment and exacerbation and found that there was no relationship between viral presence and sputum eosinophilia at enrolment (OR 0.92, CI 0.28; 3.09, p=0.894) and at exacerbations among those with sputum eosinophilia viral presence was 50% lower compared to those with no sputum eosinophilia, but the results did not reach statistical significance. (OR 0.50, CI 0.23; 1.10, p=0.083)

To summarise, those who had raised airway eosinophilia demonstrated different characteristics to the group without airway eosinophilia both at enrolment and exacerbation. Furthermore, patients with predominantly airway eosinophilia were also different from the intermittent and rarely groups in lower systemic inflammatory profile and higher systemic eosinophils in PAE. Both airway eosinophilia at enrolment and predominantly airway eosinophilia groups had higher exacerbation rates associated with airway eosinophilia. Airway eosinophilia at 1st exacerbation was associated with a lower prevalence of airway bacteria. Although there was no difference in PPM prevalence between the three longitudinal groups at exacerbation when the entire year was considered, PPM prevalence did appear to be higher in the Winter than in the Summer at exacerbations only in predominantly eosinophilic group. If proven in larger trials, this information could potentially be critical when clinical decisions on antibiotic therapy are being made. These findings may have a potential impact on the management of exacerbations of COPD in those who are known to be predominantly eosinophilic or those with airway eosinophilia at exacerbation, particularly when seasonality is taken into consideration.

It is, however, critical to note that, firstly, we have no readily available access to the sputum test for differential cells in current clinical practice and, secondly, the statistical significance observed may have been skewed by insufficient sample size. Although the knowledge on eosinophilic airway inflammation is vital further steps are required (larger study and the availability of sputum

differential test in routine clinical medicine) before these observations could be translated into daily practice.

6.2.2 Systemic eosinophilic inflammation in AERIS cohort

The issue with small sample size was partly related to the sputum sample collection methods and, frequently, to the quality of sample. As sputum cell differentials are not routinely available in current clinical practice, a more easily accessible marker of airway eosinophilic inflammation was examined. I demonstrated above that those with airway eosinophilia had higher blood eosinophils both at enrolment and exacerbation. Raised blood eosinophils (≥2%) have been previously postulated to be a good biomarker of sputum eosinophilia.[47, 197] Therefore, in this section I examined if those patients with blood eosinophils of ≥2% represented a clinically different group of COPD patients.

6.2.2.1 Association between sputum and blood eosinophils

Blood eosinophils (% and count) displayed moderately strong positive correlations with sputum eosinophils (rho 0.463 and 0.581 respectively at enrolment, $p<0.001$) and blood eosinophils were predictive of sputum eosinophilia (AUC 0.850, 95%CI 0.750; 0.951 and AUC 0.768, 95%CI 0.651; 0.884 respectively). (**Figure 6.2**)

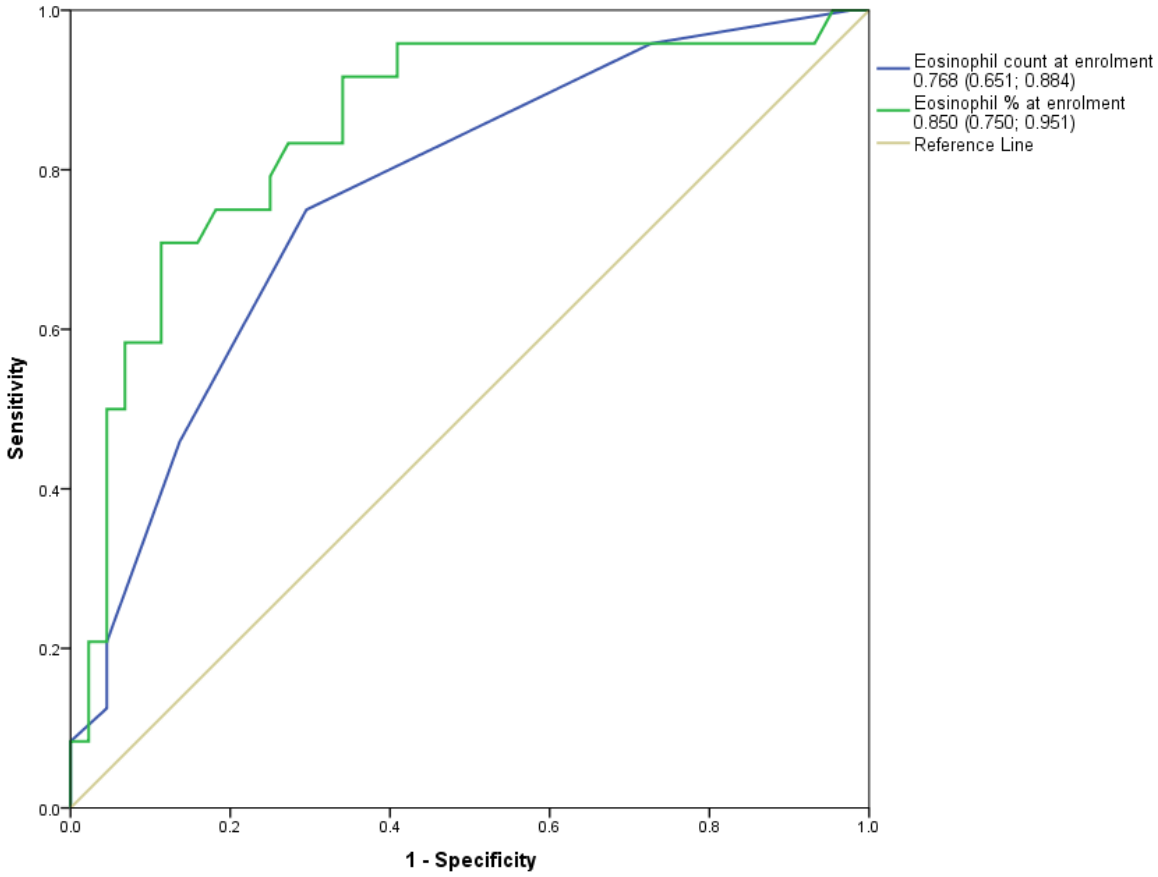


Figure 6.2 Receiver operating characteristic curve with area under the curve (95% confidence interval) illustrating blood eosinophil count and blood eosinophil % at enrolment positively predicting sputum eosinophilia >3% at enrolment.

At exacerbation, blood eosinophils (count and %) were predictive of sputum eosinophilia (>3%) (AUC 0.724 CI 0.642; 0.8066 and 0.728, 0.644; 0.812 respectively). (**Figure 6.3**)

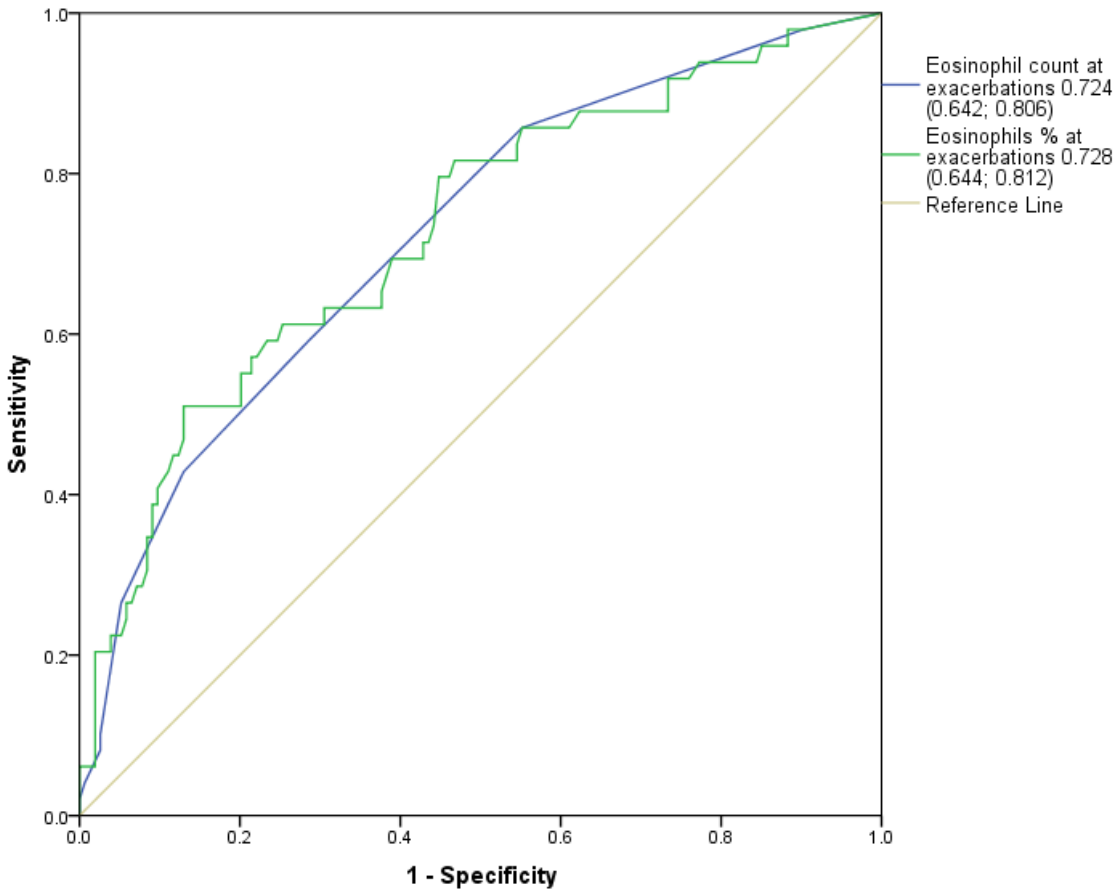


Figure 6.3 Receiver operating characteristic curve with area under the curve (95% confidence interval) illustrating blood eosinophil count and blood eosinophil % at exacerbations positively predicting sputum eosinophilia >3% at exacerbations.

When investigated how sensitive is the blood eosinophil ≥2% cut off to predict sputum eosinophilia (>3%) I found that this corresponded with a sensitivity of 95.8% and specificity of 31.8% at enrolment. I also explored whether blood eosinophil absolute count rather than percentage could offer a similar sensitivity and specificity. The blood eosinophil count was reported up to one decimal by the haematology lab, therefore it was not possible to apply the cut off of 150 cells/uL as previously reported.[195, 302] I therefore applied the cut off of ≥200 cells/uL and found that it offered similar sensitivity to the ≥2% blood eosinophils (95.8%), but lower specificity (27.3%).

Having established that airway eosinophils were associated with blood eosinophils and blood eosinophils at ≥ 2% were predictive of sputum eosinophils >3%, I next examined how those patients with raised blood eosinophils differed from others.

6.2.2.2 Systemic eosinophilic inflammation at stable state

I assessed the prevalence of eosinophilic inflammation in AERIS cohort applying these previously published cut off (blood eosinophils ≥2%)[47] and found that at all stable state visits with valid data (n=460) the prevalence of blood eosinophilia was 65.4% (n=301). The overall blood eosinophils' level expressed as median (IQR) for the whole cohort was 0.20(0.20). The distribution of blood eosinophils in each month over 12 months is presented in **Figure 6.4**.

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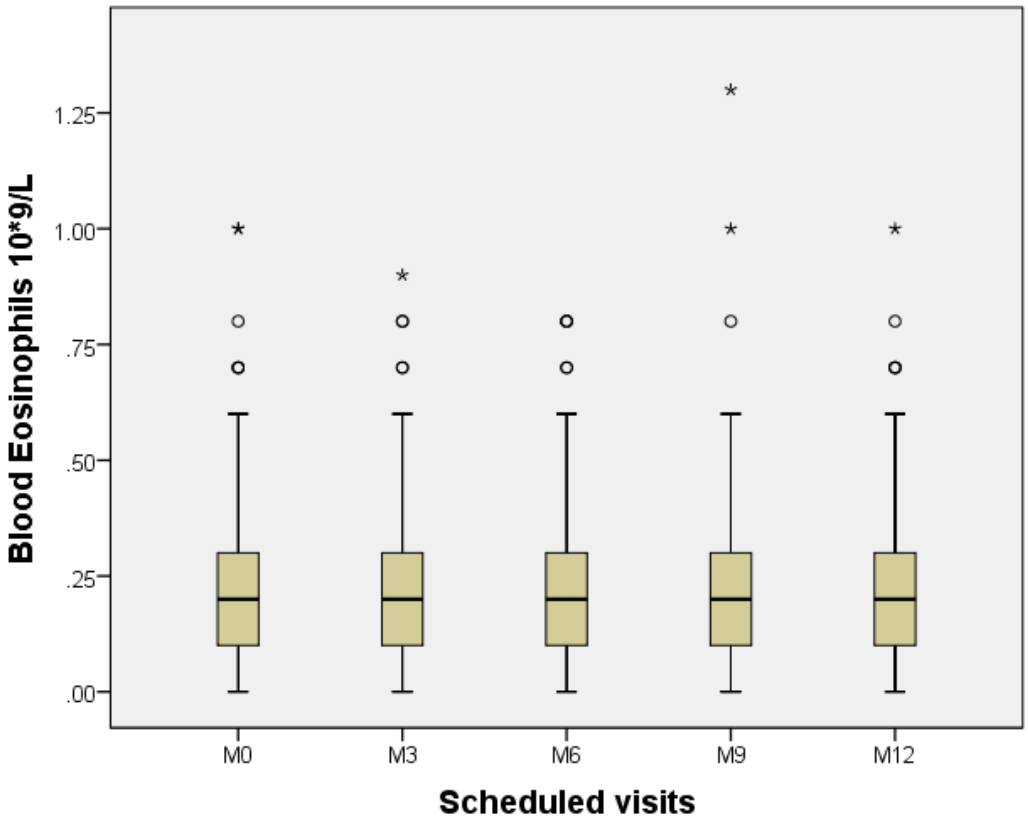


Figure 6.4 Box-and-whisker plots of blood eosinophil count illustrating the spread of blood eosinophil count at quarterly samplings when patients were deemed to be clinically stable.

I further assessed the prevalence of eosinophilic inflammation in AERIS cohort only at enrolment and found that 68.3% (n=86) had raised eosinophils ≥2% with median (IQR) of 0.30(0.20).

At enrolment those with raised blood eosinophils ≥2% had lower blood neutrophils and higher sputum eosinophils compared to the non-eosinophilic group. There was a trend towards lower WBC level in the eosinophilic group at enrolment (p=0.074). (**Table 6.10**)

Table 6.10 Characteristics at ENROLMENT for the full cohort and COPD phenotypes based on eosinophils% in BLOOD

Marker	Overall cohort (n=127)		Non-Eosinophilic (n=40)		Eosinophilic (n=86)		P value[Ω]
	Median (IQR)	n	Median (IQR)	n	Median (IQR)	n	
Age [α]	66.81(±8.61)	127	66.55(±8.50)	40	67.08(±8.65)	86	0.883
Smoking history (pack/years)	47.00(26.25)	127	46.00(30.25)	40	47.63(26.44)	86	0.679
Smoking status [μ]	54(42.5%)	127	19(47.5%)		35(40.7%)		0.563
Use of ICS at enrolment [β μ]	110(87.3%)		34(85.0%)		75(88.2%)		0.614
BMI	27.04(6.69)	127	28.07(8.29)	40	26.73(5.81)	86	0.285
6MWT	300.00(170)	125	300.00(158)	39	300.00(184)	85	0.607
Blood markers							
WBC*10^9/L	7.60(2.20)	126	7.75(2.40)	40	7.50(2.13)	86	0.074
Eosinophils *10^9/L	0.20(0.20)	126	0.10(0.00)	40	0.30(0.20)	86	-
Neutrophils *10^9/L	4.80(1.70)	126	5.15(2.10)	40	4.75(1.60)	86	0.005
Fibrinogen g/L	4.80(1.02)	114	4.65(1.27)	36	4.95(0.92)	78	0.180
Procalcitonin ng/ml	0.06(0.03)	126	0.06(0.03)	40	0.06(0.03)	85	0.192
CRP mg/L	5.00(8.00)	127	5.00(4.00)	40	5.00(8.25)	86	0.427
Sputum							
%Eosinophils in sputum	1.93(5.09)	69	0.25(1.04)	15	2.62(5.71)	53	0.001
PPM presence n(%)	57(51.8%)		18(60.0%)		39(49.4%)		0.321
Spirometry							
FEV1 (%)	46.50(25)	126	53.00(25)	39	44.00(24)	86	0.125
FEV1 (L)	1.13(0.66)	126	1.23(0.71)	39	1.07(0.58)	86	0.444
FEV1 reversibility (% of preBDFEV1)[€]	11.29(16.89)	109	10.45(19.52)	36	11.59(14.55)	72	0.492
FEV1 reversibility (L)	0.12(0.17)	109	0.11(0.15)	36	0.13(0.15)	72	0.492
ΔFEV1(% of baseline)[ξ]	4.93(21.05)	85	6.76(23.37)	24	4.56(22.17)	60	0.343
ΔFEV1(L/year)	0.06(0.26)	85	0.06(0.27)	24	0.05(0.26)	60	0.778
RV (L)	3.23(1.25)	116	3.51(1.36)	35	3.21(1.34)	80	0.839
TLC (L)	6.34(1.84)	116	6.14(1.60)	35	6.34(1.97)	80	0.181
TLCO (mmol/kPa/min)	4.47(2.32)	121	4.84(2.15)	37	4.40(2.33)	83	0.905
KCO (mmol/kPa/min)	0.94(0.48)	121	0.99(0.42)	37	0.89(0.49)	83	0.389
Clinical markers							
CAT	16.00(10)	126	18.00(12)	39	16.00(9)	86	0.377
EXACT-PRO	37.00(12)	101	36.00(12)	29	37.00(12)	71	0.528

mMRC dyspnoea score	4.00(1)	127	4.00(2)	40	4.00(0)	86	0.136
Exacerbations rate 12 months before the study	2.00(2.00)	127	2.50(3.75)	40	2.00(2.00)	86	0.776
Exacerbation rate over the 1st year in the study	2.94(3.91)	127	2.00(3.87)	40	2.98(3.99)	86	0.488

All continuous variable presented as median(IQR) unless stated otherwise. Mann-Whitney and Chi-Square tests used for significance unless stated otherwise; $^{\Omega}$ significance test only for eosinophilic subgroups (excluding an overall cohort variables); $^{\mu}$ Fisher Exact's Test used for significance; $^{\alpha}$ reported as Mean(±SD); $^{\beta}$ reported as number and % within the subgroup; $^{\mu}$ Fisher exact test reported for the significance. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

There was no difference in the total exacerbation rates between these two groups (0.488). However, the rate of exacerbations associated with raised blood eosinophils was higher in the eosinophilic group (p<0.001). (**Table 6.11**)

Table 6.11 Total and eosinophilic exacerbation rates for those without and with systemic eosinophilic inflammation

	Eosinophils at enrolment*		
	<2%	**≥2%**	**P value****
Total exacerbation rate	2.98(3.99)	2.00(3.87)	0.488
Eosinophilic exacerbation rate	1.00(2.99)	0.00(0.99)	<0.001

*Exacerbation rate presented as median (IQR); **Mann-Whitney test applied to test significance

To investigate if raised blood eosinophils at enrolment were predictive of raised blood eosinophils at exacerbation, a logistic regression analysis was performed which allowed accounting for subjects multiple measurements using data from all exacerbations. The odds of an exacerbation associated with blood eosinophilia were 9.6 times higher in the group with blood eosinophils ≥2% compared to the group with low blood eosinophils at enrolment (OR 9.60, CI 4.23; 21.77, p<0.001).

Thus the prevalence of blood eosinophils≥2% is higher at the stable state. Those with raised blood eosinophils differed from the <2% blood eosinophils group only in blood neutrophils and sputum eosinophils %. Furthermore, raised blood eosinophils at enrolment are predictive of raised blood eosinophilia at exacerbations. I further studied the characteristics of the group with and without raised blood eosinophils at exacerbations.

6.2.2.3 Systemic eosinophilic inflammation at exacerbations

Across all exacerbations with valid data (n=355) the prevalence of blood eosinophils ≥2% was 51.1% (n=167). Out of all 1st exacerbations with valid data (n=102), 53.9% (n=55) were found to have blood eosinophils ≥2% . At 1st exacerbation, the group with raised blood eosinophils had significantly lower WBC, blood neutrophils, fibrinogen, CRP, airway neutrophilia and higher sputum eosinophils% compared to the group without raised blood eosinophils. (**Table 6.12**)

Table 6.12 Characteristics at 1st EXACERBATIONS for the full cohort and COPD phenotypes based on eosinophils% in BLOOD

Marker	Overall cohort (n=108)		Non-Eosinophilic (n=47)		Eosinophilic (n=55)		P value$^{\Omega}$
	Median (IQR)	n	Median (IQR)	n	Median (IQR)	n	
Blood markers							
WBC*10^9/L	8.00(3.53)	102	9.40(4.50)	47	7.40(2.40)	55	0.004
Eosinophils *10^9/L	0.20(0.20)	102	0.10(0.10)	47	0.30(0.30)	55	-
Neutrophils*10^9/L	5.30(3.20)	102	6.80(3.60)	47	4.40(1.60)	55	<0.001
Fibrinogen g/L	5.10(1.40)	103	5.40(1.80)	47	4.80(1.40)	53	0.009
Procalcitonin ng/ml	0.07(0.04)	99	0.07(0.04)	45	0.07(0.04)	51	0.306
CRP mg/L	8.00(14.00)	103	13.00(32.00)	47	6.50(9.75)	54	0.003
Sputum							
%Eosinophils in sputum	1.20(2.38)	61	0.40(1.10)	24	1.75(3.16)	34	0.003
%Neutrophils in sputum	74.00(47.11)	61	93.19(30.82)	24	62.87(64.58)	34	0.002
PPM presence n(%)	60(60.6%)	108	28(70.00%)	40	30(55.6%)	54	0.154
Viruses presence n(%)	39(43.3%)	90	17(45.9%)	37	19(39.6%)	48	0.556
Spirometry							
FEV1 (%)	43.00(21)	97	40.00(24)	40	43.00(20)	52	0.449
TLCO (mmol/kPa/min)	4.27(2.35)	80	3.67(2.60)	31	4.43(2.61)	44	0.366
KCO (mmol/kPa/min)	0.87(0.50)	80	0.83(0.48)	31	0.93(0.43)	44	0.386
Clinical markers							
CAT	21.00(11)	106	21.50(12)	46	20.50(10)	54	0.860
EXACT-PRO	41.00(11)	97	41.00(12)	42	41.00(10)	49	0.994

All continuous variable presented as median(IQR) unless stated otherwise. Mann-Whitney test for continuous variables or Chi-Square test for categorical variables used for significance. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

To sum up, patients with raised blood eosinophils significantly differed in their airway and systemic inflammatory profiles from the group without raised blood eosinophils at 1st exacerbations.

To assess if there was a change in blood eosinophils over time and between two clinical states I conducted my next analyses.

6.2.2.4 Change in Systemic eosinophilic inflammation over time

When compared blood eosinophil count between enrolment and Month 12, no significant difference was found (0.20(0.30) and 0.20(0.20) respectively, p=0.679) thus suggesting that blood eosinophils level was stable. In order to assess this hypothesis of the stability of blood eosinophilia overtime, I analysed the reliability of the marker in capturing a phenotype over time and found blood eosinophils (count and %) to be relatively stable within individuals across the 12 months (ICC 0.66, CI 0.58; 0.74 and ICC 0.69 CI 0.62; 0.76, respectively).

When compared blood eosinophil (count) between the enrolment and the 1st exacerbation for each patient I found a significant difference between the two clinical states, although the magnitude of the difference was small. To illustrate this difference, the mean is presented alongside the median and IQR (mean, median(IQR) 0.25, 0.20(0.20), and 0.26, 0.20(0.20), for enrolment and 1st exacerbation respectively p=0.001).

In summary, there was a significant prevalence of patients with raised blood eosinophils both at stable state and at exacerbations. Raised eosinophils at enrolment were predictive of raised eosinophils at exacerbations. Furthermore, those with blood eosinophils ≥2% significantly differed in the airway and systemic inflammatory profiles from those with blood eosinophils<2% at exacerbations.

Blood eosinophils are relatively stable marker over time thus If the group with and without raised blood eosinophils differed from each other at a single time point, how do those with the predominantly raised blood eosinophils over time behave?

6.2.2.5 Longitudinal groups with raised blood eosinophils

Similarly to the sputum eosinophil analysis, to investigate the stability of blood eosinophilia over time I again divided subjects into three groups: predominantly, intermittent and rarely eosinophilic groups.

Predominantly blood eosinophils ≥2% (PBE) group was defined as raised blood eosinophils at either all stable visits, or all but 1 visit where the blood eosinophils were <2%; the rarely blood eosinophilia (RBE) group was defined as blood eosinophils <2% at all visits, or all but 1 visit where the blood eosinophils were ≥2%; the intermittent blood eosinophilia (IBE) group was defined when none of the abovementioned criteria were met. Only those subjects who had at least 3 stable visits with valid blood results (3 out of 5 potential) over 12 months were included in the group analyses (n=99). (**Table 6.13**)

Table 6.13 Number of valid blood samples and cumulative % per each subject over 12 months

N of samples	N of subjects	Cumulative %
5	40	31.5
4	39	62.2
3	20	78.0
2	16	90.6
1	12	100.0
0	0	100.0

Only individuals with 3 or more samples were included into the longitudinal analyses

78% of subjects (n=99) had 3 or more valid blood samples available over the first year and were included into the longitudinal analysis. In order to ensure the group included into the analysis did not significantly differ from the group excluded I compared the two groups and found that there were significant differences for the prevalence in raised blood eosinophils, 6MWT, exacerbation rate in Year 1 and follow up. (**Table 6.14**)

Table 6.14. Characteristics of the groups excluded and included in the longitudinal analyses at ENROLMENT

		Excluded		Included	P-value
	N	Median (IQR)	N	Median (IQR)	
Age [α]	28	67.61(±7.80)	99	66.59(±8.85)	0.673
Sex (male)	13	46.40%	55	55.60%	0.393
Current smoker [γ] **(Yes)**	11	39.30%	43	43.40%	0.695
Smoking history (pack/years) [α]	28	44.21(±17.71)	99	52.00(±30.34)	0.263
Use of ICS at enrolment [¥]	26	92.90%	84	85.70%	0.52
FEV1 (%)	27	42.00(24)	99	47.00(25)	0.782
ΔFEV1(% of baseline) [ξ]	6	5.74(20.78)	79	4.93(21.62)	0.864
FEV1 reversibility (% of preBDFEV1) [€]	22	8.56(16.77)	87	11.59(18.26)	0.385
KCO (mmol/kPa/min)	27	0.86(0.41)	94	0.98(0.48)	0.220
Sputum neutrophils (%)	19	55.51(66.02)	70	38.03(70.65)	0.261
Sputum eosinophils (%)	19	1.93(3.77)	70	1.63(5.17)	0.733
Sputum eosinophilia (>3%) Yes	6	31.60%	23	32.90%	0.916
Blood eosinophils *10^9/L	28	0.20(0.20)	98	0.20(0.15)	0.090
Blood eosinophils (%)	28	2.16(3.11)	98	3.05(3.01)	0.102
Blood eosinophilia (≥2%)	15	53.60%	71	72.40%	0.058
PPM presence (Yes)	13	54.20%	44	51.20%	0.795
Viruses presence	4	20.00%	14	16.90%	0.741
CAT	27	20.00(13)	99	16.00(10)	0.096
EXACT-PRO	18	38.50(8)	83	36.00(14)	0.149
BMI	28	28.42(7.57)	99	26.68(6.39)	0.080
FFBM	28	46.50(20.03)	97	49.30(21.70)	0.526
6MWT (distance in meters)	28	228.50(130)	97	324.00(169)	0.002
Exacerbation rate 12 months before	28	3.00(3.00)	99	2.00(2.00)	0.215
Exacerbation rate in Year 1	28	3.99(6.70)	99	1.99(3.02)	0.078
Exacerbation rate with blood eosinophilia in Year 1	28	1.34(3.32)	99	0.99(1.99)	0.407
Follow up in years	28	0.58(0.57)	99	1.01(0.02)	<0.001

[α] reported as Mean(±SD); [γ] smoking status report based derived from ATS Q7A4; [¥]ICS use were coded as "Yes" if one of the following medications/inhalers was on the list (SYMBICORT, SERETIDE, QVAR, FOSTAIR, BECLOMETHASONE, BECLAMETHOSONE/FORMOTEROL, BECLOMETHASONE dipropiionate, CLENIL, FLUTICASONE/SALMETEROL, BUDESONIDE/FORMOTEROL

[ξ] calculated as FEV1 at month 12 * 100 / FEV1 at enrolment; [€] calculated as (postBDFEV1 - preBDFEV1) / preBDFEV1 * 100 *Mann-Whitney U test used to test differences between those included and excluded for continuous variables, Chi-Square for categorical. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

I further categorised the group into three longitudinal eosinophilic phenotypic groups and found that out of the 99 patients with sufficient data, 57 (57.58%) were PBE, 16 (16.16%) IBE and 26 (26.26%) RBE over the 12 months.

6.2.2.5.1 Systemic eosinophilic inflammation in longitudinal groups at enrolment

There were significant differences between the three longitudinal groups at enrolment, namely, higher sputum eosinophils% and lower blood neutrophils in PBE patients. Furthermore, patients in the RBE group were slightly younger than those in the PBE and IBE groups. WBC was higher in the RBE group and lowest in the PBE group, however this did not reach significance. (**Table 6.15**)

Table 6.15 Characteristics of markers of longitudinal COPD groups based on eosinophils% in BLOOD at ENROLMENT

Marker	Predominantly Median(IQR)	n	Intermittently Median(IQR)	n	Rarely Median(IQR)	n	p-value
Age $^{\alpha}$	67.96(±9.05)	57	67.94(±6.87)	16	62.73(±8.63)	26	0.034
Smoking history (pack/years)	49.00(±30.13)	57	53.00(±25.16)	16	45.50(±21.13)	26	0.098
Smoking status $^{\gamma\,\mu}$	24(42.1%)	57	4(25.0%)	16	15(57.7%)	26	0.125
Use of ICS at enrolment $^{\mu}$	49(87.5%)	56	15(93.8%)	16	21(80.8%)	26	0.506
Beclomethasone equivalent dose	800(1600)	49	1600(1200)	15	800(500	20	0.08
Blood							
WBC *10^9/L	7.30(2.00)	57	7.65(1.65)	16	8.30(3.30)	25	0.06
Eosinophils *10^9/L	0.30(0.20)	57	0.20(0.10)	16	0.10(0.10)	25	-
Neutrophils *10^9/L	4.30(1.75)	57	4.80(0.50)	16	5.20(2.55	25	0.03
Fibrinogen g/L	4.80(0.90)	51	4.75(0.98)	14	4.70(1.40)	23	0.905
Procalcitonin ng/ml	0.06(0.03)	56	0.05(0.03)	16	0.06(0.03)	26	0.824
CRP mg/L	5.00(7.00)	57	5.00(5.75)	16	4.00(7.50)	26	0.966
Sputum							
Eosinophils %	3.22(7.44)	35	1.81(2.12)	5	0.25(1.45)	15	0.007
PPM presence n(%)	26(50.0%)	52	6(50.0%)	12	12(54.5%)	22	0.935
Viruses presence n(%) $^{\mu}$	10(20%)	50	0(0%)	12	4(19.0%)	21	0.288
Lung function							
FEV1 (%)	47.00(26)	57	44.00(23)	16	48.00(25)	26	0.663
FEV1 reversibility (L)	0.13(0.13)	49	0.13(0.19)	14	0.10(0.14)	24	0.404
ΔFEV1(L/year)	0.07(0.28)	45	0.13(0.23)	15	0.05(0.25)	19	0.559
BMI	25.88(4.73)	57	27.93(5.69)	16	28.15(8.15)	26	0.280
6MWT	326.50(164)	56	322.50(172)	16	300.00(199)	25	0.914
CAT	16.00(9)	57	20.50(13)	16	15.50(10)	26	0.101
EXACT-PRO	36.00(15)	46	36.50(7)	14	36.00(14)	23	0.607
mMRC dyspnoea score	4.00(1)	57	4.00(0)	16	4.00(1)	26	0.808
Exacerbations rate 12 months before the study	2.00(2.00)	57	2.00(2.75)	16	2.00(2.50)	26	0.941
Exacerbation rate over the 1st year in the study	2.04(3.95)	57	1.02(2.00)	16	2.47(3.22)	26	0.197
Exacerbation rate with blood eosinophilia over 1st year $^{\Psi}$	1.80(±1.62)	57	0.62(±0.89)	16	0.53(±0.75)	26	<0.001

All continuous variable presented as median(IQR) unless stated otherwise. Mann-Whitney and Chi-Square tests used for significance unless stated otherwise; $^{\alpha}$ Presented as Mean(SD); $^{\mu}$ Fisher exact test applied for significance; $^{\Psi}$ Exacerbation rates for exacerbations with blood eosinophilia, variable is non-parametric but presented as mean(SD) as median not informative. Median(IQR) for the predominant, intermittent and rarely groups 1.38(2.98), 0.00(0.99) and

0.00(0.99) respectively. Kruskall Wallis test used for significance. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

Similar to the groups with and without raised blood eosinophils at enrolment I found no significant differences in the total exacerbation rate (median(IQR)) between the longitudinal groups (p=0.197) but there was a significant difference in the rate of eosinophil associated exacerbations (p<0.001). It is noteworthy that in the PBE group the total exacerbation rate was 2.04(3.95), but this rate of exacerbation was dominated by those associated with blood eosinophilia (eosinophilic exacerbation rate 1.38(2.98)), whereas this was not the case in the IBE and RBE groups. (**Table 6.16**)

Table 6.16 Exacerbation rates in groups based on BLOOD eosinophilia in Year 1.

	Overall cohort	**Predominant eosinophilic**	**Intermittent eosinophilic**	**Rarely eosinophilic**	**p-value***
	n=127	**n=57**	**n=16**	**n=26**	
Total Exacerbation rate		2.04(3.95)	1.02(2.00)	2.47(3.22)	0.197
Eosinophilic (blood) exacerbation rate		1.38(2.98)	0.00(0.99)	0.00(0.99)	<0.001

Rates are presented as median(IQR)

*Kruskall-Wallis test for significance

Thus, I analysed if the odds of an exacerbation being eosinophilic was different between the longitudinal blood eosinophil groups and discovered that the odds of an exacerbation being associated with raised eosinophils (≥2%) was 12.00 times higher in the PBE group compared to the RBE group (OR 12.00, CI 4.85; 29.71, p< 0.001).

I further examined whether blood eosinophils assessed at enrolment was a useful predictor of being predominantly eosinophilic over time (using a different categorisation for the groups which excluded enrolment). Raised blood eosinophils (% and count) at enrolment were predictive of being in the PBE group over the next 12 months (AUC 0.841, p<0.001, 95%CI 0.755; 0.928 and AUC 0.806, p<0.001, 95%CI 0.710; 0.901 respectively). (**Figure 6.5**) A blood eosinophil cut off of ≥2% was 84.3% sensitive and 51.9% specific in identifying those in the PBE group over the next 12 months.

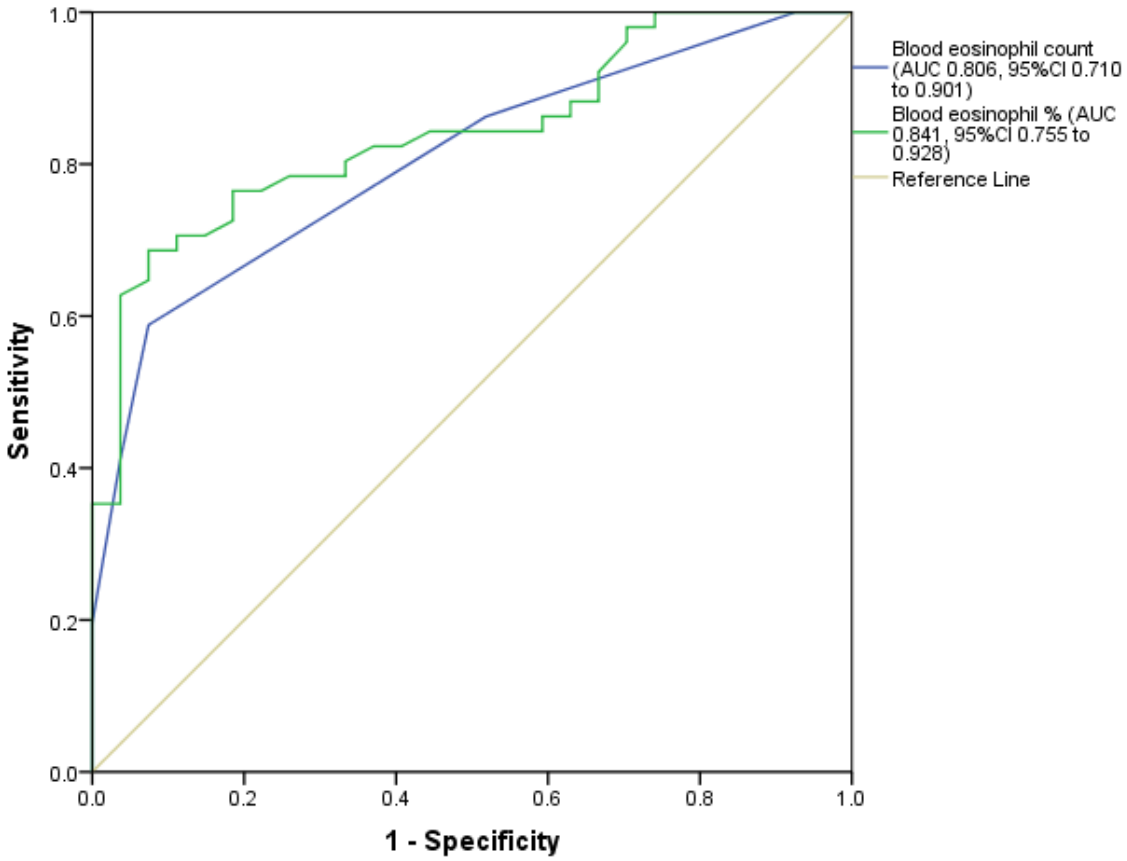


Figure 6.5. Receiver operating characteristic curve for blood eosinophils (count and %) at enrolment predicting the predominantly eosinophilic group over 12 months following enrolment) (n=78).

I inferred that raised blood eosinophils at stable state, both over time and at enrolment, were predictive of raised blood eosinophils at exacerbation. In addition, a group with predominantly raised blood eosinophils could be predicted at enrolment.

I next investigated if these longitudinal groups presented differently during exacerbations.

6.2.2.5.2 Systemic eosinophilic inflammation in longitudinal groups at exacerbations

When analysing only 1st exacerbations with available data I found that the longitudinal eosinophilic groups did not appear to differ symptomatically at first exacerbation (CAT and EXACT-PRO). However, there was a significant difference in blood neutrophils at exacerbation between these groups (p= 0.045) and in airway bacterial presence with the highest being in the rarely group at 1st exacerbations. (**Table 6.17**)

Table 6.17 Characteristics of longitudinal COPD groups based on eosinophils% in BLOOD at 1st EXACERBATIONS

Marker	Predominantly		Intermittently		Rarely		
	Median(IQR)	n	Median(IQR)	n	Median(IQR)	n	p-value
Blood markers							
WBC*10^9/L	7.30(3.23)	48	8.95(2.55)	12	8.70(4.25)	25	0.349
Eosinophils *10^9/L	0.30(0.28)	48	0.15(0.10)	12	0.10(0.15)	25	-
Neutrophils *10^9/L	4.45(2.98)	48	5.55(2.58)	12	5.60(4.05)	25	0.045
Fibrinogen g/L	5.10(1.60)	46	5.50(2.25)	12	4.80(1.80)	25	0.135
Procalcitonin ng/ml	0.07(0.04)	44	0.07(0.04)	12	0.06(0.03)	26	0.972
CRP mg/L	7.00(13.00)	47	26.50(49.75)	12	7.00(12.50)	25	0.087
Sputum							
%Eosinophils in sputum	1.48(3.56)	28	1.40(3.23)	9	0.62(2.29)	15	0.273
PPM presence n(%)$^{\mu}$	27(57.4%)	47	6(54.5%)	11	19(86.4%)	22	0.044
Viruses presence n(%)$^{\mu}$	14(33.3%)	42	4(40.0%)	10	12(54.5%)	22	0.235
Lung function							
FEV1 (%)	42.50(20)	44	41.50(18)	12	48.50(30)	22	0.432
TLCO (mmol/kPa/min)	4.07(2.38)	37	4.39(2.60)	10	4.79(4.09)	19	0.418
KCO (mmol/kPa/min)	0.85(0.35)	37	0.91(0.33)	10	1.02(0.55)	19	0.314
Clinical markers							
CAT	19.00(8)	47	22.00(17)	13	22.00(12)	26	0.915
EXACT-PRO	39.50(7)	44	40.00(10)	12	43.00(13)	23	0.142

All continuous variable presented as median(IQR) unless stated otherwise. Mann-Whitney and Chi-Square tests used for significance unless stated otherwise; $^{\alpha}$ Presented as Mean(SD); $^{\mu}$ Fisher exact test applied for significance. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

6.2.2.6 Seasonality of systemic eosinophilic inflammation

Exacerbations were overall more common in the Winter (October to March) than in the Summer season (April to September) (217(61.1%) and 138 (38.9%) respectively). A larger proportion of exacerbations had blood eosinophilia in the Summer season compared to the Winter season, although the total number of eosinophilic exacerbations (≥2% in blood) per month remained similar throughout the year. (**Table 6.18**; **Figure 6.6**)

Table 6.18 Proportion of exacerbations in each season. Association between the exacerbation type and season

	Summer		Winter		
	n	%	n	%	P value*
Exacerbations EOS (blood)	82	62.6%	85	43.4%	0.001
Exacerbations EOS (sputum)	24	27.3%	28	21.5%	0.330

*Chi-Square test; µSummer season defined as April-September, Winter as October-March

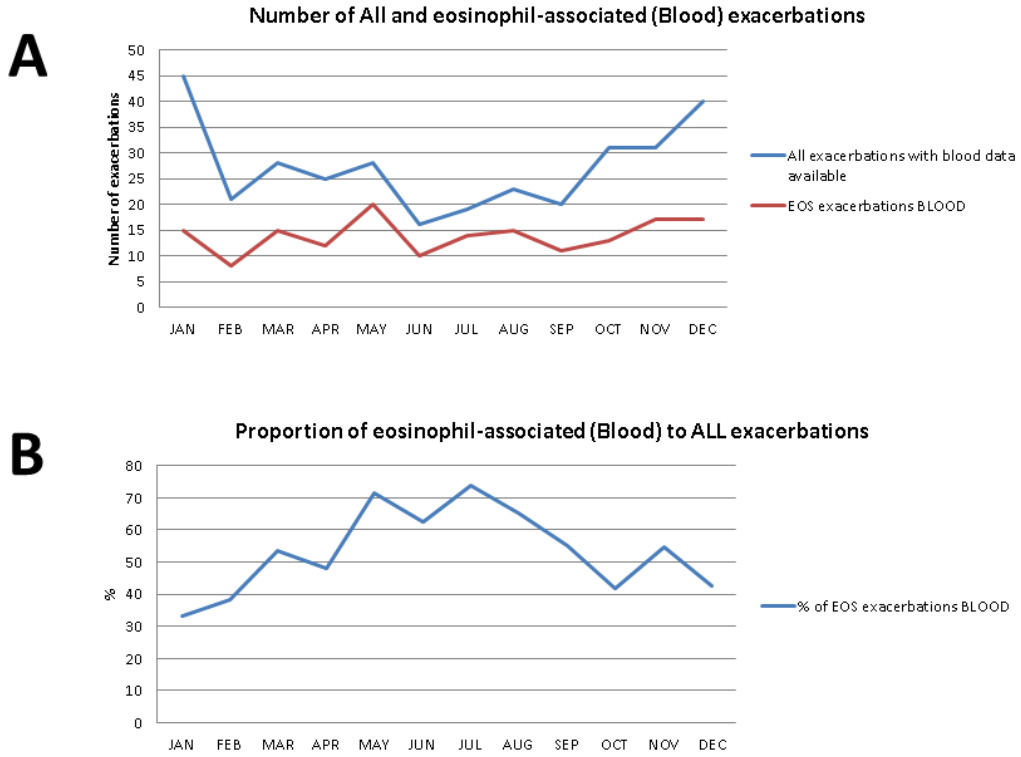


Figure 6.6 Seasonal distribution of total and eosinophil-associated exacerbations. **A** -Number of total and eosinophil associated exacerbations. **B** - Proportion of eosinophil associated exacerbations defined as eosinophil associated exacerbation to total exacerbation rates in the predominantly, intermittent and rarely groups.

This increase in the proportion of eosinophil associated exacerbations does not account for subject effect and the contribution of multiple exacerbations by some subjects. Therefore I next studied the effect of seasonality on the odds of an exacerbation having blood eosinophils ≥2% present, accounting for subject effect. The odds of an exacerbation having blood eosinophilia were 2.57 times higher in the Summer season than in Winter (95% CI 1.44; 4.60). (**Table 6.19**)

Table 6.19 The odds of eosinophilic inflammation at exacerbation in summer compared to winter

Exacerbations	N of exacerbations	N of individuals	Odds Ratio	95% CI	P value
Eosinophil associated (BLOOD)	327	104	2.57	1.44; 4.60	0.001
Eosinophil associated (SPUTUM)	218	91	1.94	0.82; 4.58	0.129

*Conditional logistic regression including subject as a random effect

Having discovered that being in the PBE group (compared to RBE) and the Summer season (compared to Winter) were both strongly associated with increased odds of eosinophil ≥2% at exacerbation, I considered which of these factors was most important. Both longitudinal group and season were independently associated with the odds of an exacerbation having eosinophils ≥2% (OR 12.58, CI 4.97; 31.84, p<0.001 and OR 2.58, CI 1.34; 4.96, p=0.004 for eosinophilic group and season respectively). I also examined if the same applied for the single measurement of eosinophils at enrolment and likewise found that both factors were independently associated with the odds of an exacerbation having eosinophils ≥2%. (OR 9.36, CI 4.07; 21.45, p<0.001 and OR 2.32, CI 1.32; 4.10 p=0.004 respectively).

I then analysed the odds of exacerbations having eosinophils ≥2% by season for the three longitudinal eosinophilic groups separately. In the PBE group the odds of an exacerbation having eosinophils ≥2% was 3.01 times higher in the Summer than in the Winter season (OR 4.01, CI 1.59; 10.14, p=0.003). No such association was found for the IBE and RBE groups (OR 1.71, CI 0.27; 10.92, p=0.568 and 1.33, 0.41; 4.33, 0.636 respectively).

6.2.2.7 Airway infection and blood eosinophils

This increase in the number of exacerbations associated with eosinophils <2% during Winter may indicate underlying changes in the lung microbiome as a result of the known seasonal effects of viral and bacterial infections. I therefore examined whether the association between seasonality and prevalence of raised blood eosinophils at exacerbation persisted when accounting for PPM presence at exacerbation. The association between seasonality and odds of eosinophilia at exacerbation did not diminish when accounting for presence of PPMs (OR 2.39, CI 1.28; 4.49, p= 0.007).

PPMs were present in 58.8% of all exacerbations with sputum data (n=320) and 60.6% at 1st exacerbations (n=99). At 1st exacerbations I found no difference in PPM presence between the blood eosinophils<2% and ≥2% groups. However, when all exacerbations with microbiology data were taken into account (n=300) there was a significant difference in PPM presence between those with and without raised blood eosinophils ≥2% (51.9% and 66.2%, p=0.012). I further studied PPM prevalence at exacerbation in the three longitudinal eosinophilic groups and found a significant difference in PPM prevalence at the 1st exacerbation; PPMs were more prevalent in those subjects who were in the RBE group (86.4%) compared to the other two groups (PBE and IBE, 57.4% and 54.5% respectively, p= 0.044). (**Table 6.17**)There was no such association in stable state (enrolment) microbiology found between the three groups (p= 0.935). **(Table 6.15**)

Respiratory viruses were detected at 41.3% of all exacerbations (n=305) and 43.3% of 1st exacerbations (n=90). There was no significant difference between the longitudinal groups in the prevalence of respiratory viruses either at enrolment or 1st exacerbation.

The odds of having PPM present at an exacerbation was 75% lower in the PBE group compared to the RBE group but this association was of borderline significance. (OR 0.25, CI 0.06; 1.09; p=0.065) Out of all exacerbations, the odds of PPM presence were 45% lower in exacerbations with blood eosinophils ≥2% compared to exacerbations without blood eosinophils <2% (OR 0.551, CI 0.345; 0.879, p= 0.012). However when adjusted for repeated measures (subject effect) a similar trend persisted but did not reach significance (OR 0.50 CI 0.242; 1.048, p= 0.067). I also examined if blood eosinophils at enrolment were associated with PPM presence at exacerbation and found that there did not appear to be such a relationship (OR 0.92, CI 0.29; 2.88, p= 0.880). However, the odds of having PPM presence at exacerbation were higher in Winter than Summer (OR 2.51, CI 1.27; 4.96, p=0.008). This seasonal effect was only apparent in the PBE group, odds of PPM greater in Winter than in Summer (OR 4.74, CI 1.43;15.71, p=0.011). No seasonal variation in PPM at exacerbation was seen In the IBE and RBE groups (OR 4.42, CI 0.00; 3476.74, p=0.662 and OR 1.15, CI 0.29;4.56, p=0.838 respectively). (**Table 6.20**)

Table 6.20 Prevalence and odd ratios of PPM in the longitudinal blood eosinophilic groups per season at exacerbations in each group

	Summer	**Winter**	**OR, CI, p value**
Predominantly eosinophilic	29/60 (48.3%)	57/91 (62.6%)	4.74, 1.43;15.71, 0.011
Intermittently eosinophilic	3/4 (75.0%)	14/19(73.7%)	4.42, 0.00; 3476.74, 0.662
Rarely eosinophilic	19/27(70.4%)	31/42(73.8%)	1.15, 0.29;4.56, 0.838

Data presented as n/N (%), n= number of, N = total number in the group

To summarise, blood eosinophils ≥2% is highly prevalent both at the stable and exacerbation state. Raised blood eosinophils were associated with and predictive of airway eosinophilia >3%. Blood eosinophils at enrolment predicted both the longitudinal stable state eosinophil phenotype across the year, and increased risk of eosinophil-associated inflammation at exacerbation. Furthermore, the PBE group was highly predictive of exacerbations being associated with raised blood eosinophils. The likelihood of an exacerbation with raised blood eosinophils was higher in the Summer than in the Winter season. Higher eosinophils at exacerbation and predominantly raised blood eosinophils at stable visits were associated with a lower risk of bacterial presence at exacerbation. In particular, I demonstrated a seasonal effect on bacterial presence in the PBE group, namely, bacterial infection at exacerbation was higher in the Winter than Summer in patients in this longitudinal group.

6.3 Discussion

The main findings in this chapter were that significant proportion of COPD patients presented with airway and systemic eosinophilic inflammation. Eosinophilic inflammation, in both airway and systemic strata, was a stable longitudinal phenotype, which could be predicted over 12 months by an initial blood level measurement. I report for the first time that eosinophilic inflammation is prevalent in both airway and systemic compartments at exacerbation in those patients with predominantly raised eosinophils over time. In addition, exacerbations associated with raised blood eosinophils were seasonal with a larger proportion of exacerbations occurring in the Summer season. I also presented evidence that airway and blood eosinophil-associated exacerbations are less frequently associated with airway bacterial infection. Furthermore, the presence of airway bacteria was more common in the Winter than in the Summer, but only in the PAE and PBE groups. Contrastingly, the prevalence of airway bacterial infection at exacerbations was higher in the longitudinal RBE compared to the IBE and PBE groups. These findings have potential implications for future therapeutic clinical trials and eosinophil targeted treatment with the view of a stratified approach to patients' care.

6.3.1 Airway eosinophilic inflammation

To my knowledge this is a first description of the persistency of raised airway eosinophils in this well characterised COPD cohort and the prevalence of eosinophil associated exacerbations among those with airway eosinophilia at a stable state. Furthermore, this is a first description of the seasonal variability of the airway bacterial presence in the group with the predominantly raised sputum eosinophils over time.

I demonstrated that 34.8% of patients had airway eosinophilia at enrolment. This finding is consistent with the previous reports on the prevalence of airway eosinophilia in COPD.[175, 303] With regards to the persistency of airway eosinophilia over time, I believe my findings are the first description of a longitudinal airway eosinophilia in COPD. I demonstrated that 21% of AERIS population presented with the predominantly airway eosinophilia over twelve months. In severe asthma cohort McGrath et al previously described persistency of airway eosinophilia (≥2%) over time and reported that 22% had airway eosinophilia at all visits (persistent), 31% intermittently (at least on 1 occasion) and 47% had no eosinophilia at every occasion.[304] In the AERIS cohort those patients in the PAE group had lower systemic inflammatory profile but higher level of blood eosinophils at enrolment compared to the intermittently and rarely groups.

The prevalence of eosinophil associated exacerbations was higher among those with airway eosinophilia at enrolment and those with the predominantly raised sputum eosinophils over time. This finding demonstrate that the stability of the eosinophilic phenotype (defined during clinical stability) that persists into a different inflammatory state (from stable to exacerbation), in spite of the airway bacterial presence.[47, 59, 295] It is known that airway infection plays a vital part in COPD exacerbations[124, 283] and bacterial presence is associated with increased airway neutrophils. Interestingly.

There were 14 patients who had raised airway eosinophils at both enrolment and 1st exacerbation. In these patients, there was a trend towards higher PPM at 1st exacerbation than at enrolment (50% v 35.7%) although this difference did not attain statistical significance (95% CI -20.2; 44.5).

When only exacerbation events were investigated, my findings confirmed recent report that airway eosinophilia is inversely correlated with airway bacterial pathogens at exacerbations[179]. Moreover, across all the longitudinal airway groups I found no significant difference in the presence of airway bacteria. Interestingly, among the group with predominantly airway eosinophilia airway bacteria were more likely in the Winter than Summer season at all exacerbations. This phenomenon was not apparent in the IAE group or RAE group.

In the sensitivity analysis of longitudinal groups, I found that there was difference in the level of blood eosinophils and follow up years in the excluded group. The number of sputum samples collected is the factor accountable for the inclusion into longitudinal analysis, patients who subsequently withdrew from the study had less than three visits thus were not included in the longitudinal analysis. This explains the difference in the follow up in years between those included

and excluded from the analysis. Excluded group comprised of patients who either did not produce sputum sample, or the weight of the produced sputum sample was insufficient, or the quality of produced sputum was deemed to be poor.

Among patients with airway eosinophilia I found that there were fewer active smokers at enrolment both in the cross sectional and in the longitudinal analyses. This finding is in line with previous studies on asthma patients where a similar observation was reported that smokers with asthma had lower airway eosinophilia and higher airway neutrophilia compared to asthmatics who were non-smokers.[305, 306]

6.3.2 Systemic eosinophilic inflammation

I believe this is the first report of eosinophil associated exacerbations and impact of the seasonality in longitudinal blood eosinophilic groups. The seasonal effect on exacerbations of COPD was previously described and reported that exacerbations were more common in the Winter than in the Summer months.[127, 128, 307, 308] In this analysis I saw a similar seasonal pattern for all exacerbations but when focused on exacerbations associated with blood eosinophils ≥2% I report that a larger proportion of exacerbations were associated with blood eosinophils ≥2% in the Summer (April-September) than the Winter (October-March). Moreover, I report that both the group with the predominantly raised blood eosinophils and Summer season were independently associated with the presence of eosinophilia at exacerbations.

In the AERIS study, the odds of blood eosinophils ≥2% at exacerbation were 12 times higher in subjects who had raised eosinophils over time. I also demonstrated that blood eosinophils ≥2% at exacerbations was strongly associated with blood eosinophils ≥2% at enrolment, which supports earlier reported findings.[253, 254]

It was previously reported that blood eosinophils ≥2% identified sputum eosinophilia at exacerbations (>3%) and it was 90% sensitive and 60% specific.[47] I applied the same cut off for blood eosinophilia at enrolment in this analysis and it corresponded with 3% sputum eosinophilia with similar sensitivity but lower specificity (sensitivity 95.8% and specificity 31.8%). The difference in sensitivity could be explained by the low numbers of patients with good quality sputum. Another reason for this discrepancy in specificity might be due to the difference in clinical states at the time of analysis; Bafadhel et al conducted the analysis at exacerbations and in AERIS study I tested the cut off during clinical stability. I found that at exacerbations ≥2% blood eosinophils were 79.60% sensitive and 53.90% specific.

As I was unable to base my sensitivity analysis on ≥150cells/uL cut off I conducted a sensitivity analyses with the ≥200cells/uL cut off and found that apart from the significant differences between the three groups in age and smoking history, there were no other significant findings compared to when the ≥2% blood eosinophilia cut off was applied.

It was previously reported that subjects with persistently raised blood eosinophils demonstrated less severe COPD at baseline (higher FEV1, lower SGRQ and mMRC scores, slower emphysema progression).[195] In the AERIS cohort I found a small but significant difference in age, and expected variations in blood neutrophils and the percentage of sputum eosinophils across the groups, otherwise these groups were clinically indistinguishable at enrolment. Particularly those with the predominantly raised blood eosinophils were slightly older than rarely group of patients (p=0.034) and there were trends towards a lower prevalence of current smokers but with a greater smoking history in the predominantly group. Singh et al based on ECLIPSE cohort reported that patients with persistently raised blood eosinophils ≥2% were older and fewer current smokers.[195] Patients excluded due to fewer stable state sampling points were as expected more frequent exacerbators.

Bacteria play an important role in exacerbations of COPD.[122, 124, 129-131] Bacterial exacerbation had been previously reported to be rarely associated with sputum eosinophilic exacerbation.[47] I investigated the prevalence of PPMs at exacerbation in the prospective eosinophilic groups and found that PPMs were far less common in the predominant group compared to the rarely eosinophilic group, and while the magnitude of this difference was large (75% less likely), it did not quite reach statistical significance (p=0.065). When I analysed PPM presence at exacerbation in combination with blood eosinophilia at exacerbation, I found presence of eosinophilia to be associated with reduced odds of PPM presence, and again while the magnitude of the difference was large (50% less likely) this did not quite reach statistical significance when the subject effect was adjusted for (p=0.067). This is consistent with the previous study on the association between eosinophilic inflammation by blood and sputum and lower bacterial presence[179] Therefore, understanding the clinical phenotype of stable inflammation may be a useful tool to stratify bacterial aetiology of exacerbations and hence antibiotic use.

In the cohort, 43.3% of 1st exacerbations were associated with respiratory viruses, corresponding with previously reported findings.[124, 309] There was no significant difference in the prevalence of respiratory viruses between the three longitudinal blood groups at enrolment or at 1st exacerbations. Papi et al reported airway eosinophilic inflammation to be a good predictor of viral infections. In their study only severe exacerbations were included whereas in this study data from the spectrum of mild, moderate and severe exacerbations was collected.[124] In the study by

Bafadhel et al viral presence(%) was not reported to be higher in the eosinophil- cluster compared to the bacteria-, pauciinflammatory-, virus-predominant at exacerbations.[47] Further studies across the disease spectrum are required to ascertain the mechanisms linking infection and inflammatory patterns of disease. It is noteworthy, that there was no significant difference in use of inhaled corticosteroids and bronchial reversibility across groups, similar to previously reported studies.[47, 254]

Airway and systemic compartments

To identify longitudinal groups with eosinophilic inflammation I used same method for airway and systemic compartments. The rationale of the method to describe the longitudinal eosinophilic phenotype was to focus the analysis on subjects who were predominantly eosinophilic (allowance of one non-eosinophilic event) as opposed to persistently (all visits were eosinophilic). This rule, was felt, represents a more pragmatic and "real world" approach. Applying this rule, I demonstrated that 21% and 57.58% (sputum and blood respectively) of subjects in AERIS cohort had predominantly raised eosinophils over the period of 12 months. Singh et al studied longitudinal eosinophilic phenotype and reported that 37.4% of subjects had persistently elevated blood eosinophils ≥2% at all visits over the period of 3 years, however, exacerbations were not examined prospectively in this cohort.[195] In a previous asthma study a 90% rule was applied to identify persistent eosinophilic inflammation in sputum.[310] However, due to the limited number of samples available (maximum 5 samples) in our cohort this rule was not applied. A limitation of the longitudinal phenotype method was that individuals with 3 visits could not be classified into intermittently eosinophilic group (n=14/80 and 20/99 for sputum and blood respectively).

The relationship between steroid responsiveness and eosinophilic inflammation has been previously reported.[175, 177, 254] Specifically the association between airway eosinophilia and response to FEV1, quality of life and exacerbation rate in response to the withdrawal of the inhaled corticosteroid treatment in patients with higher blood eosinophils.[175, 177, 207] In AERIS study it is important to note that the use of ICS was high in all patients and at enrolment there was no difference across the groups. This suggests that eosinophilic inflammation persisted despite of the high use of inhaled corticosteroids.

In summary, there are similarities and differences between airway and systemic eosinophils inflammation. Groups with predominantly raised airway and systemic eosinophils over time had similarities in the systemic inflammatory profile, higher rate of exacerbations associated with raised eosinophils and in the airway bacterial prevalence that was higher in the Winter compared to Summer. Predominantly eosinophilic inflammation in airway and systemic compartments is predicted by the eosinophils in the appropriate compartments at enrolment. Likewise,

exacerbations associated with raised eosinophils in airway and systemic compartments were predicted by the eosinophils at enrolment. Furthermore, I demonstrated that exacerbations with raised eosinophils in airway and systemic compartments were associated with lower likelihood of airway bacterial presence. There were also the differences, namely, there was no seasonality impact on the exacerbations associated with raised airway eosinophils whereas the likelihood of exacerbations associated with raised blood eosinophils was more dominant in the Summer than Winter. Moreover, airway bacterial prevalence was higher in the group with rarely blood eosinophils≥2% but no such observation was found in the group with rarely sputum eosinophils>3%. Both differences could be potentially explained by the much smaller sample size in the airway analyses as similar trends were present in these analyses for the airway groups.

Limitations

It is important to recognise that these results are representative of a cohort of moderate to very severe COPD patients with frequent exacerbations receiving a high level of clinical intervention, including ICS, as part of the intensive study. This might have had an impact on the severity of exacerbations and recovery, as reported previously that early therapy improves the outcome at exacerbations. [144] Thus the number of potential severe exacerbations might be smaller. This is an observation and I am not in the position to prove or disprove this hypothesis. Secondly, AERIS study was not originally designed and therefore was not powered to investigate longitudinal eosinophilic inflammation and this limited the numbers that were included in each eosinophilic group. This is reflected in the fact that the results are post hoc, that multiplicity of analyses has not been taken into account, and in the presence of wide confidence intervals, which result in borderline statistical significance despite sizeable differences.

To conclude, this is the first study to report that longitudinal eosinophilic inflammation is a stable phenotype in COPD which predicts the occurrence of eosinophil-associated exacerbations. These events were seasonal in nature and relate to bacterial aetiology. These data suggest that stratifying COPD patients into eosinophilic groups to potentially aid management is clinically relevant and potentially important, as is the consideration of season in management of exacerbations. Whether oral corticosteroids administered during exacerbations of COPD to patients with predominant eosinophilic inflammation patterns, in particular, outside Winter season, requires further investigation along with other stratification paradigms through well designed intervention studies.

Chapter 7 Blood markers of infection and inflammation in COPD

7.1 Introduction

Abu Ali Ibn Sina (known as Avicenna) was a prominent Persian scholar and polymath and has also been described as a father of early modern medicine.[311, 312] The Canon of Medicine is based on the Hippocratic theory of "four humours" but Avicenna refined and referred to humours as "that fluid, moist 'body' into which our aliment is transformed".[313] The sanguineous humour section of the Canon describes blood in its healthy and unhealthy states. Historically, blood letting was the treatment of choice for many ailments. Avicenna advocated blood letting and stated the general indications for blood letting were to remove superabundant blood, to remove unhealthy blood, or both.[313] Modern medicine has come a long way away from the practice of blood letting as a treatment for most conditions and instead we study the changes that take place within the blood (biomarkers) in different conditions. Contemporary "blood letting" therefore remains common, but in the form of venepuncture to obtain samples to test for specific blood biomarkers. Blood sampling is generally well tolerated by patients and most systemic biomarkers are easily accessible clinically. Although blood biomarkers are not a substitution for a clinical diagnosis based on the patients' history these markers can be a useful complementary tool to confirm the suspicion of an exacerbation and its aetiology, thus allowing the targeted management of an individual patient accordingly.

There is an increased interest in the use of blood biomarkers in the COPD field. The significance of blood biomarkers was previously investigated both during stable disease and during exacerbations of COPD.[47, 191, 223, 314] Raised cellular and cytokine markers were previously found to be associated with exacerbation events.[191, 315] Blood biomarkers are easily accessible and some were postulated to be of a significant prognostic value in COPD.[220, 316 243]

The most commonly studied blood markers in COPD are C-reactive protein (CRP) and fibrinogen. CRP and fibrinogen are markers of systemic inflammation.[208] Their production in hepatocytes is triggered by the initiation of the acute phase reaction via the cytokine IL-6.[209] In the context of COPD exacerbations these markers were found to be associated with increased exacerbations and mortality.[218, 219, 220] Specifically, CRP was found to be useful in confirming an exacerbation visit when associated with a major exacerbation symptom.[218] Furthermore, fibrinogen was studied in the context of predicting future exacerbation events and, high levels of fibrinogen were previously reported to be associated with an increased risk of severe exacerbations over the following

twelve months.[221] In another study of severe COPD exacerbations, raised CRP levels along with body temperature were associated with viral and mixed viral/bacterial presence.[222] Increased numbers of white blood cells (WBC) are cellular markers of inflammation and were also reported to be mildly elevated in patients during exacerbations.[191] Furthermore, Thomsen et al reported that simultaneously raised leukocytes along with CRP and fibrinogen were associated with an increased risk of future exacerbations.[192]

Procalcitonin (PCT) is also a marker of systemic inflammation thought to be of a bacterial aetiology.[224] PCT was previously studied in a COPD population and was proposed to be a good guide for antibiotic treatment in patients with exacerbations of COPD.[226] Interferon-inducible protein of 10 kDa (IP-10) is secreted in response to interferon-γ and TNF- α and was found to be elevated in COPD patients in response to viral infection.[234, 235] Troponin-T (TNT) and B-type natriuretic peptide (BNP) are markers of myocardial injury and were previously examined in a COPD population, particularly during acute exacerbations.[242 243, 244]TNT and BNP are markers of myocardial injury and when measured during exacerbations of COPD were reported to be associated with higher morbidity (namely, elevated BNP was associated with worsening cardiac diastolic/sys function) and mortality.[243 248]

However, the role of clinically available blood markers in predicting an exacerbation event and in particular its aetiology remains unclear. In the previous chapter I presented evidence for the utility of blood eosinophils for phenotyping COPD patients and the different type of exacerbation events experienced by this group of patients. Furthermore, I demonstrated that patients with lower blood eosinophils and higher sputum colour had a significantly higher systemic inflammatory profile specifically at exacerbations.

The aim of this chapter was to see if any of the blood biomarkers analysed either alone or in combination could detect an exacerbation event and more importantly to identify its aetiology, with a view to aiding management of the specific event. Furthermore, the additional value of the change in the biomarkers' concentration in detecting an exacerbation event was also examined.

7.2 Results and comments

7.2.1 Blood markers during clinical stability

I first assessed the blood biomarkers during clinical stability over the first year of the study. I examined the stability of the blood biomarkers analysed over 12 months using intra-class correlations (ICC). In accordance with previously suggested classifications of ICC results[317], BNP, PCT and CRP appear to have poor reliability. WBC, neutrophils, fibrinogen, SPD, TNT and IP-10

were moderately reliable, whereas SPD was good and IL-6 had excellent reliability over the 12 month period. (**Table 7.1**)

Table 7.1 Intraclass correlation coefficient (ICC) for blood biomarkers at stable state over 12 months

	N	Median(IQR)	ICC(95% CI)
WBC*10^9/L	460	7.6(2.30)	0.61(0.53; 0.70)
Neutrophils*10^9/L	460	4.8(1.90)	0.50(0.40; 0.59)
Fibrinogen g/L	441	4.6(1.10)	0.56(0.47; 0.65)
CRP mg/L	464	4.0(7.00)	0.25(0.15; 0.35)
PCT ng/ml	464	0.07(0.03)	0.35(0.25; 0.46)
IL-6 pg/mL	339	3.52(2.48)	0.94(0.92; 0.96)
IP-10 pg/mL	453	137.0(93.50)	0.63(0.55; 0.71)
SPD ng/mL	455	161.0(104.0)	0.82(0.77; 0.87)
hsTNT-T μg/L	449	0.01(0.01)	0.66(0.58; 0.74)
BNP pg/mL	449	101.0(139.8)	0.45(0.35; 0.55)

All markers are non-parametric and presented as median and IQR. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

I then investigated if there was a significant difference between clinically-stable disease and acute exacerbations, by comparing levels of the biomarkers at enrolment and at first exacerbation. () All blood biomarkers except Troponin-T (TNT) and SPD were increased at 1st exacerbation compared to the enrolment visit. Intriguingly, SPD levels at exacerbations were lower than at enrolment visits, contrary to previously published reports.[240, 241]

Table 7.2 Differences in blood markers between enrolment and 1st exacerbation with data available.

	N*	Enrolment Median(IQR)	1st exacerbation** Median(IQR)	P value
WBC*10^9/L	103	7.60(2.30)	8.00(3.50)	<0.001
Neutrophils*10^9/L	103	4.80(1.60)	5.30(3.20)	<0.001
Fibrinogen g/L	94	4.85(1.13)	5.30(1.43)	<0.001
CRP mg/L	104	5.00(7.50)	8.50(15.50)	<0.001
PCT ng/ml	105	0.06(0.03)	0.07(0.04)	<0.001
IL-6 pg/mL	71	4.04(2.63)	5.33(7.80)	0.004
IP-10 pg/mL	102	148.00(91.25)	174.00(173.75)	0.014
SP-D ng/mL	102	177.00(102.00)	144.00(130.15)	<0.001
TNTμg/L	101	0.01(0.01)	0.01(0.01)	0.375
BNP pg/mL	101	98.10(161.00)	120.00(205.10	<0.001

* number of available pairs of the blood marker of interest at enrolment and first exacerbation ** first exacerbation with valid data available; Wilcoxon signed ranks test used for significance as non-parametric data. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

Given the significant difference in the blood markers between stable and exacerbation visits, I conducted a ROC curve analysis seeking to establish if these biomarkers could identify an exacerbation visit out of a combined set of stable and exacerbation visits.

7.2.2 Blood markers in identifying a visit being an exacerbation

The ROC curve analysis revealed a significant association between exacerbations and all biomarkers, except SPD and TNT. (**Table 7.3**, **Figure 7.1**) Only WBC, neutrophils, fibrinogen, CRP, IL-6 and PCT were found to have AUC >0.6. I therefore calculated the sensitivity and specificity for WBC, Neutrophils, fibrinogen and CRP applying a clinically accepted upper limit of normal as a cut off . This analysis revealed that these markers were highly specific (>70%) but only fibrinogen and CRP were sensitive (57.5% and 57.7% respectively) in identifying exacerbations at the specified cut off.

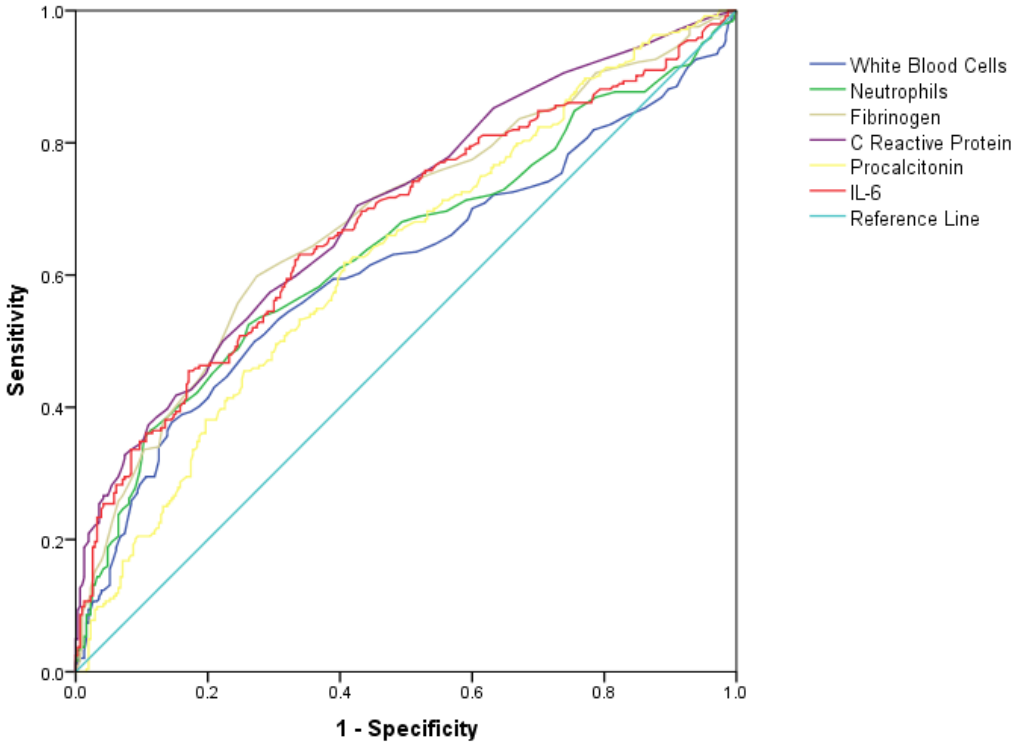


Figure 7.1. Receiver operating characteristics curve for blood biomarkers positively predicting exacerbation visits with an area under the curve >0.60 (95% confidence interval). ROC curve represents data from visits when all markers were available at the time of the visit (n= 554).

Table 7.3 Table of biomarkers identifying exacerbation visit. Cut off for sensitivity and specificity

Biomarker	n**	AUC	95% CI	p value	Cut-off*	Sensitivity	Specificity
WBC*10^9/L	787	0.615	0.574; 0656	<0.001	11.0	20.1%	93.3%
Neutrophils*10^9/L	787	0.641	0.601; 0.681	<0.001	7.5	24.5%	92.6%
Fibrinogen g/L	756	0.681	0.642; 0.721	<0.001	5.0	57.5%	71.4%
CRP mg/L	800	0.695	0.657; 0.732	<0.001	7.5	57.7%	69.4%
PCT ng/ml	788	0.611	0.571; 0.651	<0.001			
IL-6 pg/mL	617	0.679	0.636; 0.722	<0.001			
IP-10 pg/mL	787	0.591	0.550; 0.632	<0.001			
SP-D ng/mL	790	0.470	0.429; 0.511	0.148			
hs-TNT µg/L	782	0.524	0.483; 0.566	0.242			
BNP pg/mL	781	0.585	0.545; 0.626	<0.001			

Cut off represents as a clinically acceptable upper limit of normal. n=of exacerbation and stable visits.* For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

However, the ROC curve analysis did not account for subjects' repeated measurements. I thus further sought to answer if a set of clinically available blood markers could identify exacerbation visits and if the odds were further enhanced in the winter than in the summer when accounting for subjects' effect.

To account for subjects repeated measurements as a result of multiple exacerbations, I used STATA software to test the odds of identifying an exacerbation visit. This analysis revealed that for every 1 unit increase in WBC the odds of a visit being an exacerbation was 21%; for neutrophils 32%; for fibrinogen 102% and for CRP 6% higher. The odds of a visit being an exacerbation was 1% higher for every 1 unit increase in IL-6. IP-10, SPD, TNT and BNP values were rescaled for reasons described earlier in the Methods (Section 3.1.7). For every 10 units increase in IP-10 the odds of the visit being an exacerbation was increased by 4% and for every 100 units increase in BNP the odds were increased by 5%. (**Table 7.4**)

Table 7.4 Odd ratios for INDIVIDUAL blood markers identifying exacerbation visit.

	n	OR(95%CI, p value)
WBC*10^9/L	787	1.21(1.13;1.30, <0.001)
Neutrophils*10^9/L	787	1.32(1.21;1.43, <0.001)
Fibrinogen g/L	756	2.02(1.70; 2.42, <0.001)
CRP mg/L	800	1.06(1.04; 1.07; <0.001)
PCT ng/ml	788	1.12(0.86; 1.44, 0.403)
IL-6 pg/mL	617	1.01(1.00; 1.04, 0.031)
IP-10 pg/mL*	787	1.04(1.02; 1.06, <0.001)
SPD ng/mL *	790	0.99(0.97; 1.01, 0.338)
hs-TNT μg/L *	782	1.06(0.94; 1.20, 0.350)
BNP pg/mL*	781	1.05(1.02; 1.08, 0.004)

Presented as OR (95% CI, p value) accounting for subjects multiple measurements.

** These markers were rescaled as the OR per 1 unit increase was not informative. SPD and IP-10 were downscaled by 10, BNP was downscaled by 100 and TNT was upscaled by 100.*
For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

Based on these analyses PCT, SPD and TNT did not appear to demonstrate any significant value in identifying exacerbation visits and therefore they were excluded from further exacerbation analysis in this section.

Having examined how the biomarkers' absolute concentration contributed towards identification of an exacerbation, I then explored if the change in biomarker's concentration between the exacerbation and stable visits had any additional value in identifying exacerbation visits.

7.2.3 Identification of exacerbation visit based on biomarkers change from enrolment.

It is not uncommon practice for a physician to compare blood tests from previous visits when encountering a COPD patient with a suspected exacerbation. Such practice helps to establish if the inflammatory markers have risen and if this increase could signify an exacerbation event. I therefore investigated if the change in the biomarkers concentration was associated with the visit being an exacerbation. Additionally, I investigated if the change in the biomarkers concentration had any additional value in identifying an exacerbation visit.

Firstly, I studied the scale of change in blood biomarkers from any stable visit to an enrolment visit and from exacerbation visit to enrolment visit. The changes in the blood markers were considerably higher between exacerbation and enrolment visits compared to the change between any stable and enrolment visit in neutrophils, fibrinogen, CRP, IL-6, IP-10 and BNP. (**Table 7.5**)

Table 7.5 Description of change in individual blood markers for stable and exacerbation visits.*

	Change from stable to enrolment$^{\alpha}$			**Change from exacerbation to enrolment$^{\beta}$**		
	n	**Median(IQR)**	**Min; Max**	**n**	**Median(IQR)**	**Min; Max**
WBC*10^9/L	330	0.30 (1.70)	-4.80; 14.00	325	0.80(2.60)	-3.60; 13.00
Neutrophils*10^9/L	330	0.40(1.60)	-4.30; 14.80	325	0.70(2.35)	-3.70; 14.40
Fibrinogen g/L	293	-0.20(1.00)	-3.40; 3.40	285	0.30(1.45)	-3.10; 4.90
CRP mg/L	337	0.00(3.01)	-79.00; 122.00	336	2.55(17.00)	-56.00; 314.00
IL-6 pg/mL	188	-0.25(2.21)	-48.30; 21.20	217	0.71(6.47)	-41.50; 349.50
IP-10 pg/mL	322	-9.00(55.65)	-534.00; 449.00	321	3.00(98.00)	-441.00; 1356.00
BNP pg/mL	316	6.30(57.53)	-9790.00; 2776.00	316	19.55(82.33)	-9732.00; 10602.00

*Significance of differences could not be tested in this analysis due to multiple and different contributions towards stable and exacerbation visits per individual; $^{\alpha}$ Change in biomarkers calculated as difference between any stable visit (excluding enrolment) and enrolment visit; $^{\beta}$ Change in biomarker calculated as difference between exacerbation visit and enrolment visit. For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

The likelihood of the visit of interest being an exacerbation was positively associated with the changes in the concentrations of WBC, neutrophils, fibrinogen, CRP, IL-6 and IP-10. However, when comparing the biomarkers' change in concentrations with the absolute concentrations at

the time of the visit the odds of identifying an exacerbation visit were similar across biomarkers apart from BNP. (**Table 7.6**) The odds of the visit being an exacerbations was 5% higher for every unit increase in BNP absolute concentration, however when comparing the BNP change in concentrations and the absolute concentrations these were similar and with a minor difference in the confidence interval.

Table 7.6 Odd ratios to identify if the visit is an exacerbation for change* in individual blood markers and for the absolute concentrations.

	Change in biomarker*		**Absolute concentration** $^{\alpha}$	
	N	**OR(95% CI, p)**	**N****	**OR(95% CI, p)**
WBC*10^9/L	655	1.21(1.11; 1.33, <0.001)	655	1.18(1.09; 1.27, <0.001)
Neutrophils*10^9/L	655	1.26(1.14; 1.39, <0.001)	655	1.27(1.16; 1.38, <0.001
Fibrinogen g/L	578	2.22(1.75; 2.81, <0.001)	578	2.26(1.81; 2.81, <0.001)
CRP mg/L	673	1.05(1.03; 1.07, <0.001)	673	1.06(1.04; 1.07, <0.001)
IL-6 pg/mL	405	1.10(1.05; 1.15, <0.001)	405	1.14(1.08; 1.20, <0.001)
IP-10 pg/mL**	643	1.04(1.02; 1.06, <0.001)	643	1.04(1.03; 1.06, <0.001)
BNP pg/mL**	632	1.01(0.99; 1.03, 0.305)	632	1.05(1.02; 1.09, 0.003)

**Change in blood marker was calculated as difference between biomarker at visit of interest – biomarker at enrolment*

*N - denotes the number of visits; N** - the number of visits limited to match the visits when the change in biomarker was available and calculated.*

$^{\alpha}$ *Actual absolute concentration of a biomarker at the time of the visit*

*** These markers were rescaled as the OR per 1 unit increase was not informative. IP-10 were downscaled by 10, BNP was downscaled by 100.*

For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

Thus, even though the change between exacerbation and enrolment visits was considerable, the change in concentration did not appear to have any additional value in identifying an exacerbation visit. In conclusion, both absolute concentrations and the degree of change in concentrations of WBC, neutrophils, fibrinogen, CRP, IL-6 and IP-10 are useful in identification of an exacerbation visit. The question that remains is can these biomarkers, when measured at enrolment, aid in predicting those who are likely to experience an exacerbation visit?

7.2.4 Prediction and risk of an exacerbation event

To answer this question I studied if blood biomarkers at enrolment carried a hazard of exacerbation by conducting a Cox proportional analysis on each biomarker individually. Having applied this analysis I revealed no significant increase in hazard ratios for any blood marker and

therefore I did not proceed to a multivariate modelling analysis. (**Table 7.7**) However, if there was no increased hazard of individual biomarkers based on the unit change, could it be that the risk of exacerbations are only observed among those subjects with the highest blood biomarkers?

Table 7.7. Hazard ratio for individual biomarkers at enrolment to predict hazard of exacerbation.

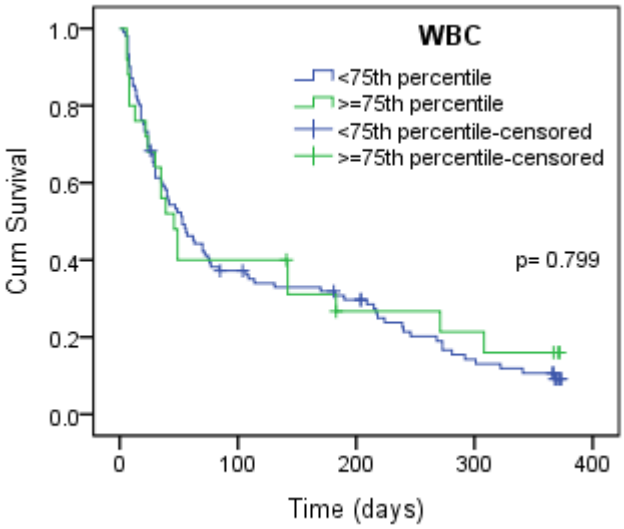
	HR	**95%CI**	**p-value**
WBC*10^9/L	0.99	0.89; 1.10	0.852
Neutrophils*10^9/L	1.00	0.878; 1.14	0.992
Fibrinogen g/L	1.02	0.810; 1.294	0.843
CRP mg/L	1.00	0.98; 1.02	0.846
IL-6 pg/mL	1.000	1.00; 1.01	0.506
IP-10 pg/mL *****	0.997	0.984; 1.011	0.671
BNP pg/mL *****	1.007	0.992; 1.022	0.344

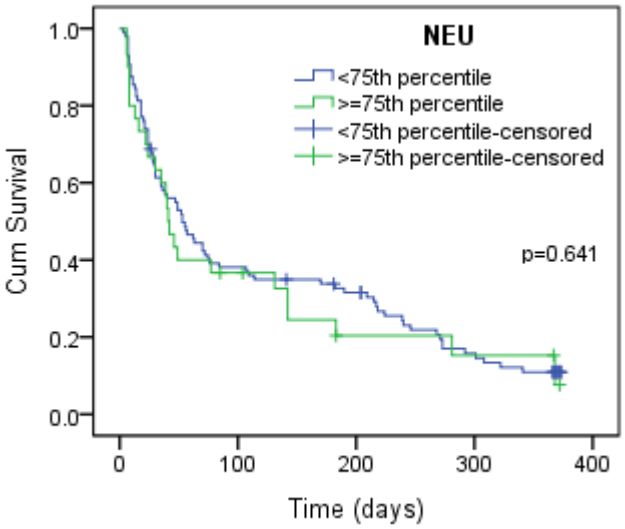
Hazard ratio derived from Cox proportional hazard analyses

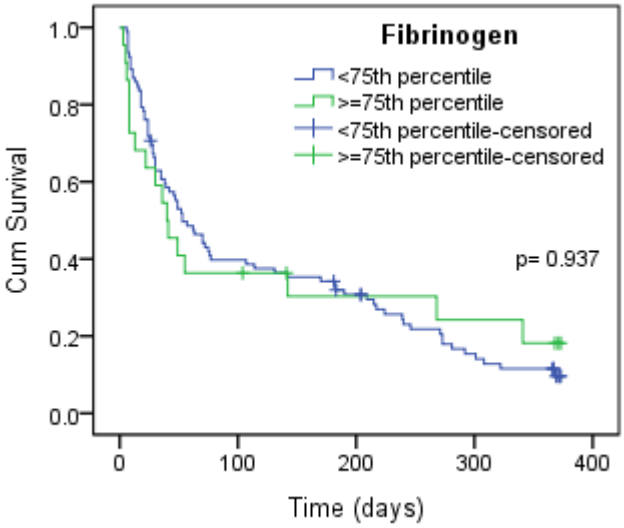
**These markers were rescaled as the OR per 1 unit increase was not informative. IP-10 were downscaled by 10, BNP was downscaled by 100.*

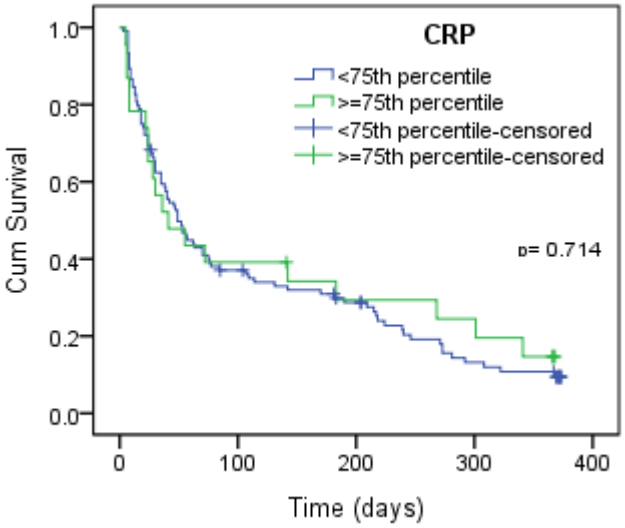
For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

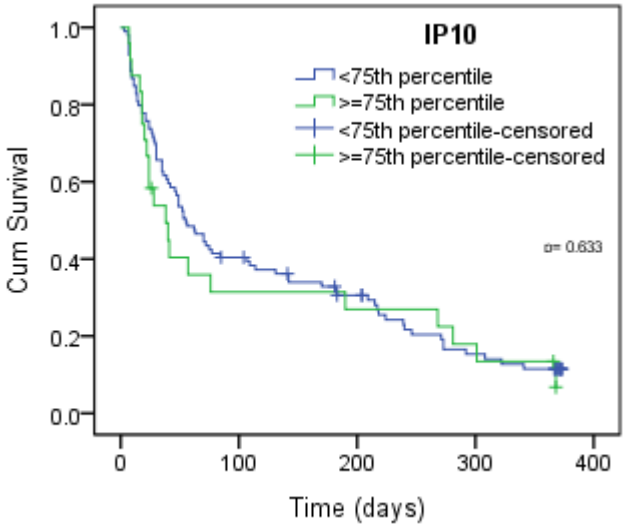
I therefore stratified the cohort into two groups for each biomarker measured at enrolment: greater and equal to the 75th percentile for a whole group cut off and lower than 75th percentile similar to previous studies.[318-320] The group with higher IL-6 at enrolment (≥75th percentile) appeared to have a shorter time to first exacerbation but this pattern was not significant. All other biomarkers demonstrated no significant hazard of exacerbations. (**Figure 7.2**)

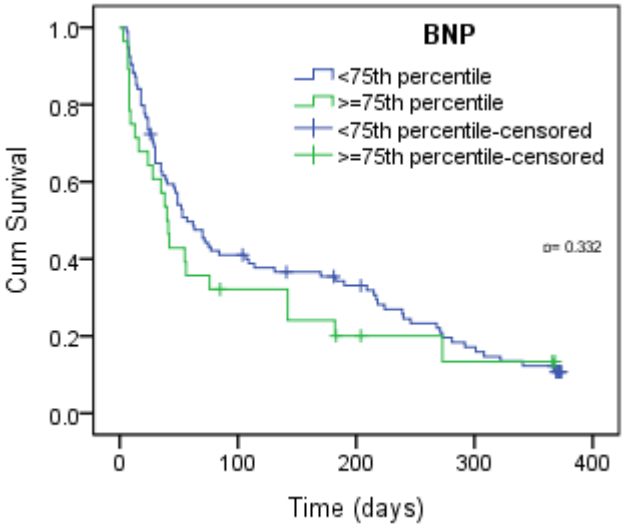

WBC
<75th percentile
>=75th percentile
<75th percentile-censored
>=75th percentile-censored
p= 0.799
Cum Survival
Time (days)


NEU
<75th percentile
>=75th percentile
<75th percentile-censored
>=75th percentile-censored
p=0.641
Cum Survival
Time (days)


Fibrinogen
<75th percentile
>=75th percentile
<75th percentile-censored
>=75th percentile-censored
p= 0.937
Cum Survival
Time (days)


CRP
<75th percentile
>=75th percentile
<75th percentile-censored
>=75th percentile-censored
p= 0.714
Cum Survival
Time (days)


IP10
<75th percentile
>=75th percentile
<75th percentile-censored
>=75th percentile-censored
p= 0.633
Cum Survival
Time (days)


BNP
<75th percentile
>=75th percentile
<75th percentile-censored
>=75th percentile-censored
p= 0.332
Cum Survival
Time (days)

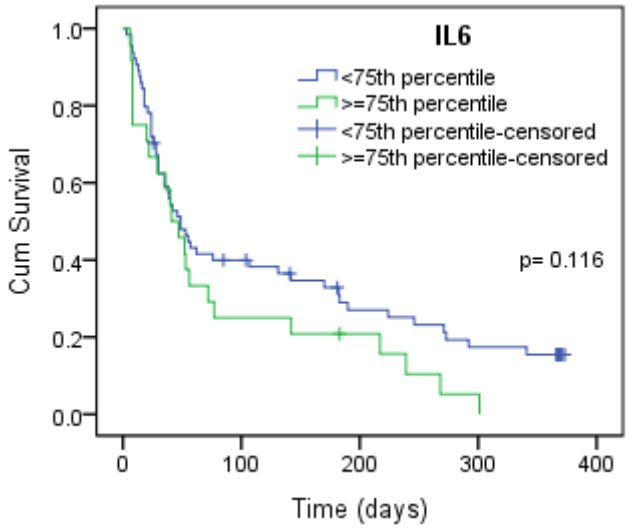


Figure 7.2 Kaplan Meier survival curves, illustrating time to 1st exacerbation for individual biomarkers stratified into subgroups: ≥75th percentile and <75th percentile. P value derived from log rank test.

I further examined if blood markers at enrolment could be useful in predicting frequent (≥2 a year) and non-frequent (<2 a year) exacerbators and again found no such association. (**Table 7.8**)

Table 7.8 The value of individual blood markers at enrolment in predicting frequent and non-frequent exacerbators over the 1st year in study.

	n	AUC	95% CI	P value
WBC*10^9/L	126	0.473	0.371; 0.574	0.600
Neu*10^9/L	126	0.499	0.397; 0.602	0.990
Fibrinogen g/L	114	0.530	0.424; 0.637	0.578
CRP mg/L	127	0.544	0.443; 0.645	0.395
IL-6 pg/mL	88	0.509	0.387; 0.631	0.880
IP-10 pg/mL	123	0.499	0.396; 0.602	0.980
BNP pg/mL	122	0.572	0.470; 0.674	0.171

For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

7.2.5 Airway infection and systemic markers

As discussed in the previous chapter, the causes of exacerbations are multifactorial and therefore the character of an exacerbation may change dependent on the type of exacerbation experienced. I therefore analysed the biomarker data to identify if there was an association of each marker with bacterial or viral presence. PCT is a systemic marker and is advocated for use in secondary care to guide the management of bacterial infections.[227] I have, therefore, re-introduced PCT into the analyses of airway infection and systemic markers. Those exacerbations associated with the presence of airway bacterial presence had significantly raised fibrinogen and CRP compared to the group without airway bacteria at 1st exacerbation. There was a trend towards higher median Procalcitonin level in PPM+ve patients compared to PPM-ve patients (0.72 and 0.07 respectively) but this difference did not attain statistical significance (p=0.436). In contrast, patients with airway viral presence had higher IL-6 and IP-10 compared to individuals without viral presence at 1st exacerbations. (**Table 7.9**)

Table 7.9 Blood markers (absolute concentrations) in airway infection groups at 1st exacerbation.

	PPM					**VIRUS**				
	-ve		**+ve**			**-ve**		**+ve**		
	n	**Median(IQR)**	**n**	**Median(IQR)**	**p-value**	**n**	**Median(IQR)**	**n**	**Median(IQR)**	**p-value**
WBC* 10^9/L	36	7.95(3.53)	58	8.35(3.43)	0.331	49	8.30(3.35)	36	8.15(4.40)	0.887
Neutrophils* 10^9/L	36	5.05(2.98)	58	5.50(3.48)	0.122	49	5.30(3.15)	36	5.40(4.23)	0.862
Fibrinogen g/L	36	4.80(1.28)	58	5.40(1.50)	0.004	48	5.05(1.45)	37	5.30(1.05)	0.205
CRP mg/L	36	7.50(9.75)	58	10.50(27.75)	0.021	49	9.00(14.50)	36	9.50(11.75)	0.548
PCT ng/ml	33	0.07(0.05)	57	0.72(0.04)	0.436	46	0.07(0.04)	36	0.07(0.03)	0.918
IL-6 pg/mL	27	4.47(5.58)	53	5.08(7.35)	0.891	39	3.50(7.10)	34	5.66(4.90)	0.015
IP-10 pg/mL	35	188.00(158.00)	59	156.00(144.00)	0.100	48	136.00(72.75)	38	216.50(225.50)	<0.001
BNP pg/mL	34	121.50(294.50)	59	110.00(143.70)	0.833	48	144.50(179.30)	38	98.05(58.45)	0.295

For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

The comparison of biomarkers' absolute concentrations at exacerbation does not take into account subjects' baseline level, i.e. the degree of change in biomarker's concentration between stable and exacerbation states. Therefore, I studied if the change in blood biomarkers from 1st exacerbation to enrolment was different between groups without and with airway infection

present. I found that there was significantly more positive change in fibrinogen and CRP in PPM+ve compared to PPM-ve groups. (**Table 7.10**) I also revealed that there was a positive change in IP-10 in VIRUS+ve compared to VIRUS-ve groups. These findings in biomarkers' change confirmed that fibrinogen and CRP biomarkers were significantly different between PPM groups as well as IP-10 was different between VIRUS groups.

Table 7.10. Blood markers (change in concentration[α]) in airway infection groups at 1st exacerbation.

	PPM					**VIRUS**				
	-ve		**+ve**			**-ve**		**+ve**		
	n	**Median(IQR)**	**n**	**Median(IQR)**	**p-value**	**n**	**Median(IQR)**	**n**	**Median(IQR)**	**p-value**
WBC*10^9/L	35	0.60(1.80)	57	0.70(3.20)	0.169	48	0.80(2.18)	36	0.30(3.03)	0.083
Neutrophils*10^9/L	35	0.50(1.60)	57	0.60(3.05)	0.146	48	0.60(2.95)	36	0.45(2.55)	0.254
Fibrinogen g/L	30	0.00(0.90)	52	0.40(1.58)	0.007	41	0.10(1.10)	35	0.20(1.00)	0.395
CRP mg/L	35	0.10(7.01)	58	3.00(27.75)	0.020	49	1.00(10.00)	36	3.00(5.75)	0.260
PCT ng/ml	33	0.00(0.03)	57	0.01(0.03)	0.187	46	0.01(0.03)	36	0.01(0.03)	0.937
IL-6 pg/mL	22	0.79(4.73)	38	0.48(7.64)	0.830	28	-0.06(4.39)	25	1.76(9.29)	0.066
IP-10 pg/mL	32	29.00(176.6)	58	8.00(58.50)	0.186	44	-8.35(51.25)	38	42.00(161.83)	<0.001
BNP pg/mL	31	16.30(48.90)	57	30.00(63.65)	0.612	44	28.10(80.98)	37	27.20(61.15)	0.319

[α]*Change in blood marker was calculated as difference between biomarker at exacerbation – biomarker at enrolment.* For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

However, the analysis of a biomarker's absolute concentration and change between biomarkers at enrolment and exacerbations included only 1st exacerbations with microbiological data available, thus leaving a low sample size for analysis. I therefore investigated if any of the blood markers could be indicative of airway infection when all data were applied. I first applied a ROC analysis using all exacerbation data available and then used the more robust OR test that allowed adjustment for subjects' repeated measures. I also asked how the biomarker change could be useful in identifying bacterial presence. I discovered that WBC, blood Neutrophils, Fibrinogen, CRP, and IL-6 had a positive association with airway bacterial presence with AUC ranging between 0.5 and 0.6. Although all biomarkers but blood Neutrophils (p=0.041) had a trend towards significance. IP-10 demonstrated a significant but weak association in predicting bacterial infection (AUC 0.393, p=0.0002)) but strong positive association with respiratory viral presence with AUC 0.685. In addition, IL-6 and fibrinogen were moderately associated with viral infection at exacerbations. (**Table 7.11**)

Table 7.11 Area under the curve for individual biomarkers to identify bacterial and viral presence at all exacerbations.

	PPM			Virus		
	n	**AUC (95% CI)**	**P value**	**n**	**AUC (95% CI)**	**P value**
WBC*10^9/L	176	0.560(0.495; 0.626)	0.075	118	0.508(0.437; 0.578)	0.826
Neu*10^9/L	176	0.569(0.504; 0635)	0.041	118	0.504(0.434; 0.574)	0.910
Fibrinogen g/L	170	0.559(0.491; 0.627)	0.090	116	0.575(0.507; 0.643)	0.034
CRP mg/L	179	0.558(0.493; 0.622)	0.085	120	0.563(0.497; 0.628)	0.069
PCT ng/ml	175	0.502(0.433; 0.570)	0.964	118	0.539(0.471; 0.607)	0.264
IL-6 pg/mL	153	0.556(0.484; 0.627)	0.135	109	0.581(0.510; 0.653)	0.029
IP-10 pg/mL	181	0.393(0.329; 0.457)	0.002	123	0.685(0.621; 0.749)	<0.001
BNP pg/mL	180	0.466(0.398; 0.533)	0.315	123	0.459(0.393; 0.526)	0.239

For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

I then tested the odds of airway infection present both for an absolute biomarker concentration at exacerbation and for a change in concentration in biomarker between an exacerbation visit and enrolment using all exacerbations and adjusting for a subjects repeated measures. I found that the absolute concentrations of blood biomarkers measured at exacerbation were useful in identifying airway bacterial presence at exacerbations for a whole cohort. For every 1 unit increase in WBC the odds of bacterial presence was 27% higher; for neutrophils 27% higher; for fibrinogen 54% higher and for CRP 1% higher. These findings of biomarkers absolute concentrations were similar when the biomarkers' change in concentrations was tested with the only exception being fibrinogen. The odds of airway bacterial presence was 65% higher for every unit increase in change of fibrinogen from the exacerbation to enrolment. (**Table 7.12**) Interestingly, Procalcitonin was not significantly associated with bacterial presence unlike in a previously reported meta-analysis.[321] In identifying airway viral presence at exacerbations IP-10 appeared to be useful: for every 10 units increase in IP-10 the odds of viral presence was 7% higher. Odd ratios for IP-10 change in concentrations were significant but similar to the absolute concentrations. (**Table 7.12**)

Table 7.12 Odd ratios of blood markers identifying the presence of airway infection at all exacerbations over 12 month period.

	PPM absolute concentration [α]		**PPM change in biomarker**[β]	
	n	**OR(95%CI, p value)**	**n***	**OR(95%CI, p value)**
WBC*10^9/L	298	1.27(1.09; 1.48, 0.003)	298	1.29(1.09; 1.52, 0.003)
Neu*10^9/L	298	1.27(1.08; 1.49, 0.004)	298	1.28(1.08; 1.52, 0.004)
Fibrinogen g/L	260	1.54(1.06; 2.22, 0.022)	260	1.65(1.14; 2.40, 0.008)
CRP mg/L	306	1.01(1.00; 1.02, 0.026)	306	1.01(1.00; 1.02, 0.024)
PCT ng/ml	295	23.11(0.00; 133061, 0.477)	295	15.99(0.00; 73781.46, 0.520)
IL-6 pg/mL	204	1.00(0.99; 1.01, 0.769)	204	1.00(0.99; 1.02, 0.418)
IP-10 pg/mL **	290	0.98(0.96; 1.00, 0.135)	290	0.98(0.96; 1.00, 0.090)
BNP pg/mL **	284	1.00(0.96; 1.05, 0.874)	284	1.00(0.97; 1.04, 0.977)
	Viruses absolute concentration [α]		**Virus change in biomarker**[β]	
	n	**OR(95%CI, p value)**	**n**	**OR(95%CI, p value)**
WBC*10^9/L	284	1.00(0.90; 1.10, 0.932)	284	0.92(0.82; 1.04, 0.188)
Neu*10^9/L	284	0.99(0.89; 1.11, 0.870)	284	0.96(0.86; 1.08, 0.526)
Fibrinogen g/L	249	1.23(0.96; 1.57, 0.107)	249	1.17(0.91; 1.49, 0.216)
CRP mg/L	292	1.00(0.99; 1.01, 0.948)	292	1.00(0.99; 1.01, 0.983)
PCT ng/ml	281	1.24(0.58; 2.65, 0.575)	281	1.25(0.56; 2.81, 0.587)
IL-6 pg/mL	192	1.00(0.99; 1.00, 0.217)	192	1.00(1.00; 1.01, 0.452)
IP-10 pg/mL **	276	1.07(1.04; 1.11, <0.001)	276	1.08(1.04; 1.12, <0.001)
BNP pg/mL **	271	0.99(0.96; 1.02, 0.436)	271	0.99(0.97; 1.01, 0.501)

[α]*Change in blood marker was calculated as biomarker at exacerbation – biomarker at enrolment*

*n - denotes the number of visits; n** - the number of visits limited to match the visits when the change in biomarker was available and calculated.*

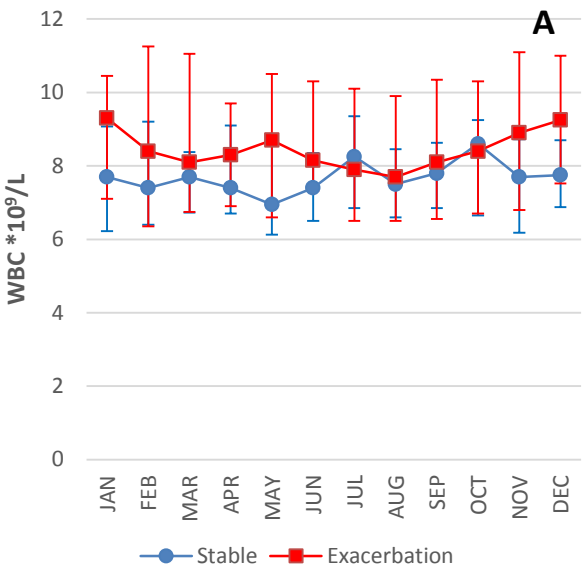
[β] *Actual absolute concentration of a biomarker at the time of the visit*

***These markers were rescaled as the OR per 1 unit increase was not informative. IP-10 was downscaled by 10, BNP was downscaled by 100.*

For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

7.2.6 Seasonality and blood biomarkers

As previously discussed, there is a marked association between season and exacerbations, specifically, with the type of exacerbation experienced i.e. bacterial, viral or eosinophilic. In this section I sought to establish if the blood biomarkers differed between the Winter and Summer seasons. There were 217 exacerbations in the Winter and 138 in the Summer season, respectively. Biomarkers concentration during clinical stability and exacerbations over twelve months is presented in **Table 7.13** and graphically in **Figure 7.3**. Overall WBC concentration (**Figure 7.3**, **A**) appeared to be higher at exacerbations than stable visits per month apart from the Summer months with median (IQR) 8.8(4.08) and 8.0(3.05) in the Winter and Summer accordingly. There was a similar pattern in blood Neutrophils concentration (6.0(3.65) and 5.4(2.80) respectively). (**Figure 7.3**, **B**) Fibrinogen concentration (**Figure 7.3**, **C**) was persistently higher throughout the year compared to the stable visits with no overt seasonal variation over the Winter and Summer months (5.4(1.80) and 5.3(1.70) respectively). Similar pattern was observed in the CRP (10.0(27.50) and 10.0(21.00) respectively) and IL-6 (5.79(9.16) and 5.03(5.38) respectively) concentrations over the year.(**Figure 7.3**, **D and E**) Overall IP-10 concentration (**Figure 7.3**, **F**) appeared to be higher at exacerbations compared to stable and particularly higher in the Winter than Summer (190.0(208.00) and 149.50(145.00) respectively). Apart from the peak in December and February BNP concentration appeared to be not dissimilar from stable. (**Figure 7.3**, **G**) There was a minor rise in BNP over Winter compared to Summer months (138.0(327.00) and 119.0(186.80) respectively). This analysis however included all exacerbations and no adjustment for subjects multiple measures were accounted for. I thus next used a more robust test to check the likelihood of exacerbations in the Winter and Summer.


A
WBC *10⁹/L
12
10
8
6
4
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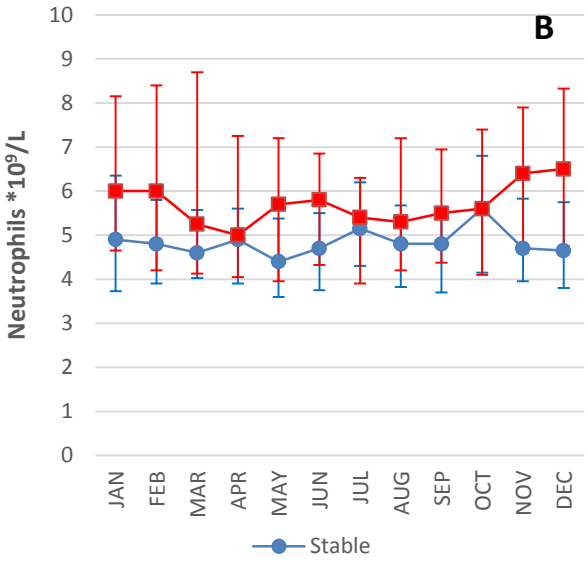
Stable
Exacerbation


B
Neutrophils *10⁹/L
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7
6
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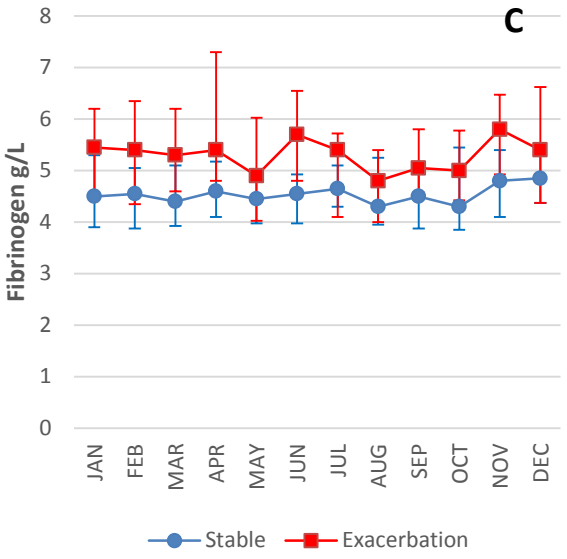
C
Fibrinogen g/L
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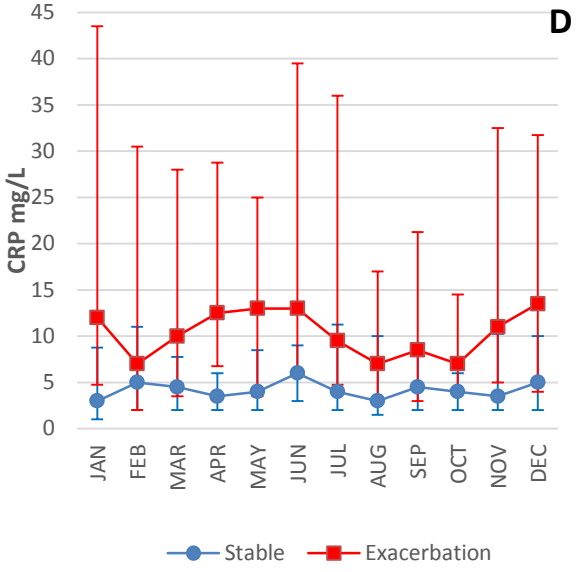
D
CRP mg/L
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30
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20
15
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Stable
Exacerbation

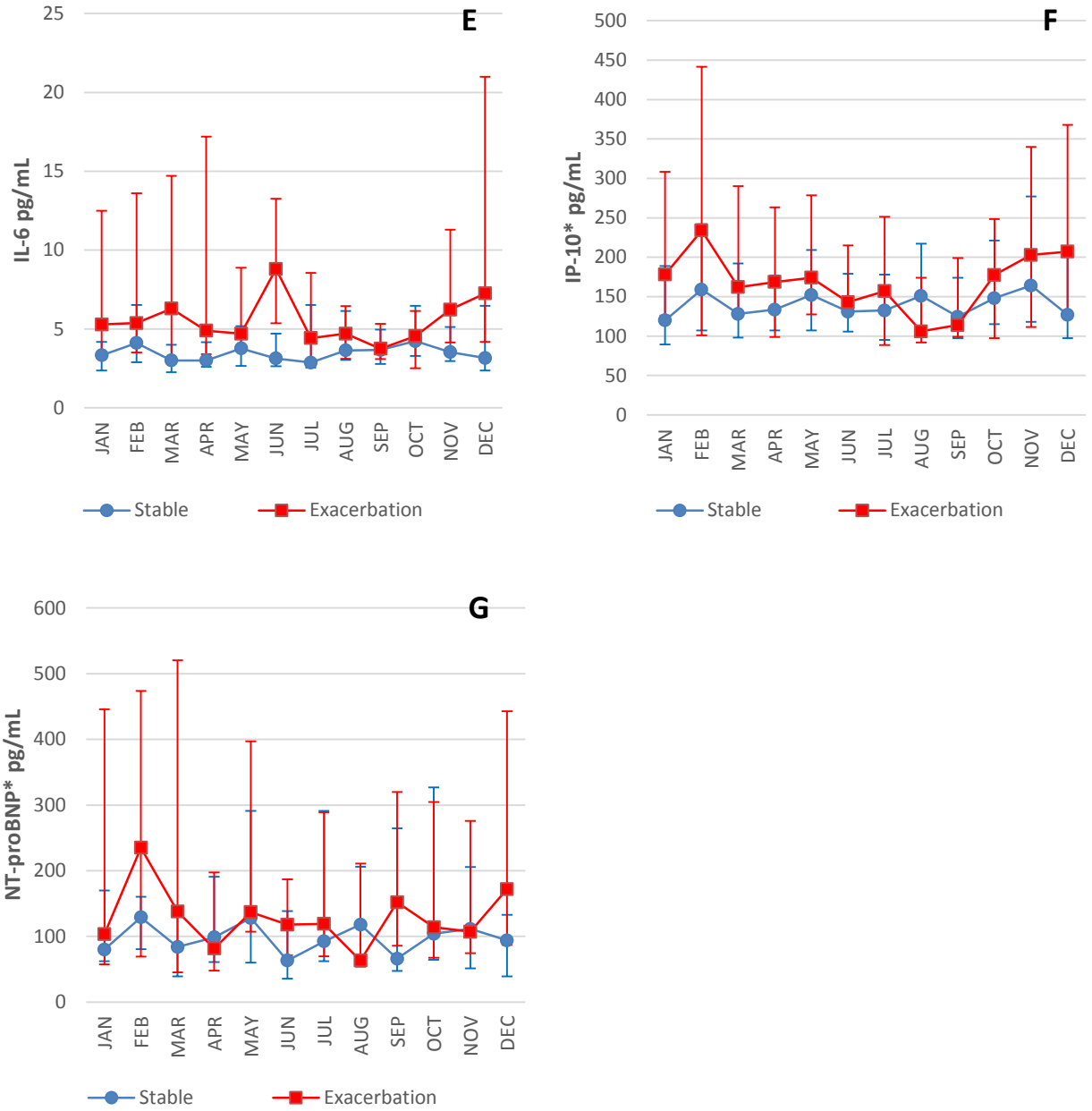

Figure 7.3 Seasonality of individual biomarkers at stable visits and exacerbations over 12 months and odd ratios of an individual biomarker to identify an exacerbation visit. * These markers were rescaled as the OR per 1 unit increase was not informative. IP-10 were downscaled by 10, BNP was downscaled by 100.

This subsequent analysis was conducted using STATA software that accounted for a subject's repeated measurements. I found that the odds of identifying an exacerbation visit were similar at Winter and Summer seasons. (**Table 7.13**) In particular, there did not appear to be a seasonal impact on the OR individually in WBC, neutrophils, fibrinogen, CRP, IP-10. Although the BNP ORs were similar in both seasons, the OR for BNP in identifying exacerbation was only significantly

different in the Winter ; no significant effect was demonstrated in the Summer possibly attributable to the lower number of exacerbations.

Table 7.13. Odd ratios for individual blood markers in identifying exacerbation visit

	Year-round	Winter	Summer
WBC*10^9/L	1.21(1.13;1.30, <0.001)	1.22(1.12; 1.33, <0.001)	1.13(1.03; 1.25, 0.010)
Neutrophils*10^9/L	1.32(1.21;1.43, <0.001)	1.34(1.21; 1.49, <0.001)	1.20(1.07; 1.34, 0.002)
Fibrinogen g/L	2.02(1.70; 2.42, <0.001)	1.77(1.44; 2.18, <0.001)	2.00(1.56; 2.56, <0.001)
CRP mg/L	1.06(1.04; 1.07; <0.001)	1.06(1.04; 1.09, <0.001)	1.04(1.02; 1.06, <0.001)
IL-6 pg/mL	1.01(1.00; 1.04, 0.031)	1.01(1.00; 1.03, 0.134)	1.03(1.00; 1.07, 0.075)
IP-10 pg/mL *****	1.04(1.02; 1.06, <0.001)	1.03(1.02; 1.05, <0.001)	1.03(1.01; 1.06, 0.010)
BNP pg/mL *****	1.05(1.02; 1.08, 0.004)	1.05(1.01; 1.09, 0.013)	1.03(0.99; 1.07, 0.186)

Presented as OR (95% CI, p value) accounting for subjects multiple measurements.

These markers were rescaled as the OR per 1 unit increase was not informative. SPD and IP-10 were downscaled by 10, BNP was downscaled by 100 and TNT was upscaled by 100.

For the interpretation of abbreviations in this table please refer to Definitions and Abbreviations section on pages xxi-xxiii

7.2.7 Prediction of airway bacteria at exacerbations - "To treat or not to treat?"

Having established the markers that were useful in identifying an exacerbation visit, specifically, the ones that signalled towards bacterial presence I next asked how these findings could potentially guide clinical practice when the decision on an exacerbating patient with COPD is being made. A conventional microbiology approach takes days to confirm the presence of airway bacteria thus "To treat or not to treat?" still remains an open question at the encounter with unwell COPD patient. I earlier demonstrated that blood neutrophils, fibrinogen and CRP were useful markers in identifying airway bacterial presence. Using all exacerbations I examined how accurate the above markers are in identifying airway bacterial presence when the biomarker's clinically accepted upper limit of normal is applied as a cut off. I found that blood neutrophils>7.5*10^9/L were 67.5% sensitive and 44.4% specific, fibrinogen>5g/L - 64.5% and 51.7%, CRP>7.5mg/L - 59.1% and 42.4% respectively. As demonstrated in Chapter 5 sputum colour was also strongly associated with airway bacterial presence. I thus tested the accuracy of Sputum colour≥3 in predicting airway bacterial presence and discovered that Sputum colour≥3 was 69.0% sensitive and 49.7% specific. I also previously demonstrated a strong negative association between blood eosinophils≥2% and bacterial presence (Chapter 6). I therefore examined the accuracy of blood eosinophils<2% to predict airway bacterial presence and revealed

that blood eosinophils<2% were 66.2% sensitive and 51.9% specific in identifying airway bacterial presence.

Having evaluated the accuracy of each individual marker I then sought to examine if there is an additional value of a combined biomarker approach in detecting a presence of airway bacteria at exacerbations. As demonstrated earlier CRP, fibrinogen, blood neutrophils, blood eosinophils and sputum colour demonstrated to be useful biomarkers in identifying airway bacterial presence at exacerbations. The pathway of the CRP and fibrinogen synthesis is common, namely, the production of CRP and fibrinogen is triggered by IL-6 in hepatocytes. Similarly, neutrophils and eosinophils have common pathway. Both neutrophils and eosinophils are granulocytes produced in the bone marrow by the haematopoietic cells and differentiated from the common myeloid progenitor cell. Therefore, applying biological plausibility and common pathway of biomarkers (CRP and fibrinogen; systemic neutrophils and eosinophils) two separate models were evaluated using machine learning classifiers. The first model included CRP, blood eosinophils and sputum colour. The second model comprised of CRP, blood neutrophils and sputum colour. CRP was preferred to fibrinogen in the combined predictive models as it is a more widely tested biomarker in clinical practice. To perform these predictions a logistic regression classifier was trained and evaluated on the complete dataset using a leave tenth out cross validation (LTOCV) method to obtain an estimation of the classifiers performance. This was represented in a receiver Operating Characteristic Curve (ROC), where the Area Under the Curve (AUC) is a measure of a classifiers performance. The combined approach using model 1 or model 2 demonstrated no additional value compared to the individual biomarker approach in detecting airway bacterial presence at exacerbations (AUC 0.61 and 0.61 respectively). (**Figure 7.4**,**Figure 7.5**)

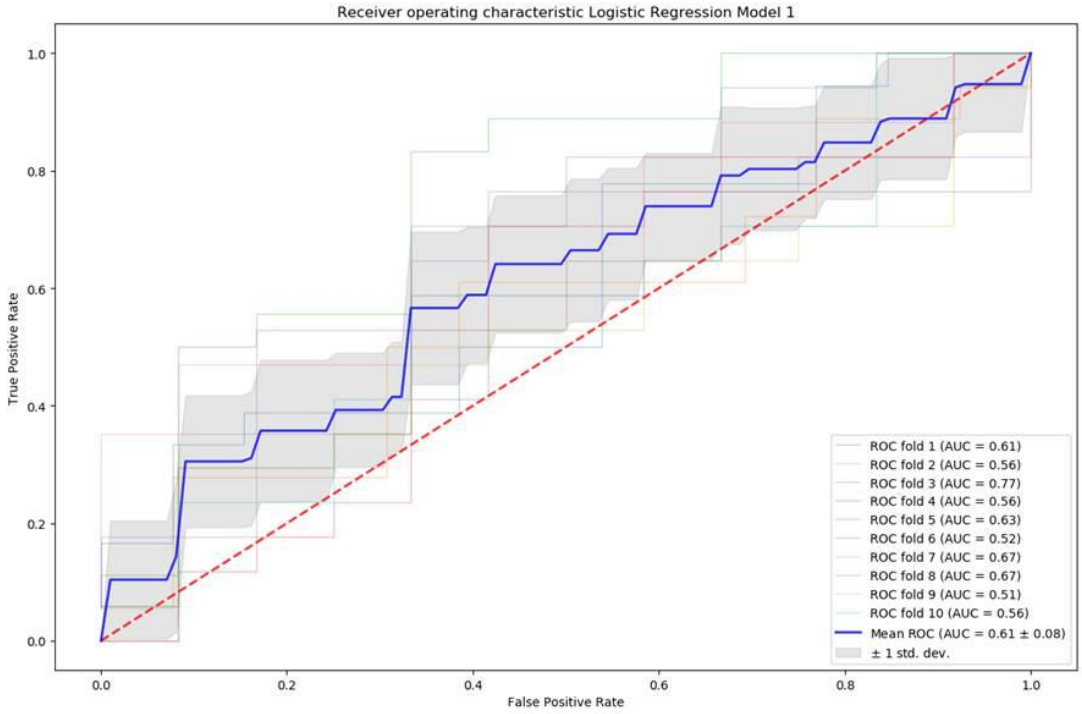

Figure 7.4 Receiver operating characteristic curve illustrating prediction of bacterial infection using combined markers (Model 1 – CRP, blood eosinophils and sputum colour)

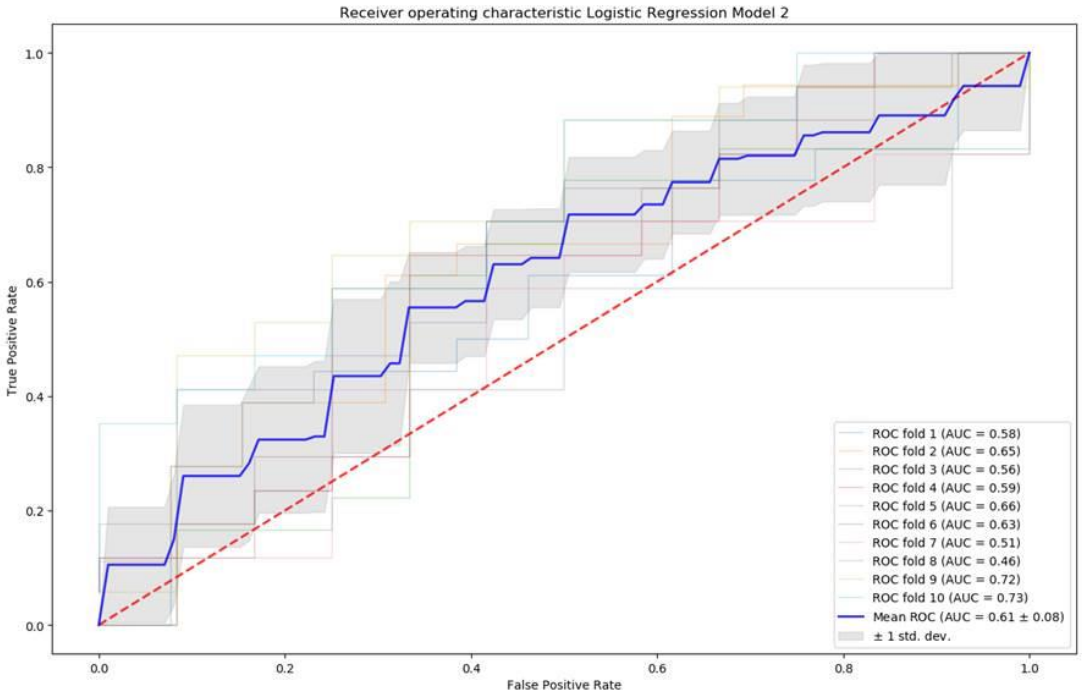

Figure 7.5 Receiver operating characteristic curve illustrating prediction of bacterial infection using combined markers (Model 2 – CRP, blood neutrophils and sputum colour)

7.3 Discussion

In this chapter I demonstrated that WBC, neutrophils, fibrinogen, CRP, PCT, IL-6, IP-10, SPD and BNP significantly differed between the stable and exacerbation clinical states. Furthermore, WBC, neutrophils, fibrinogen, CRP, PCT, IL-6, IP-10, and BNP could identify an exacerbation visit from stable visit. In addition, the change in concentration of the biomarker from the visit of interest to enrolment is a useful marker in identifying exacerbation from stable visit. The absolute concentration of WBC, Neutrophils, fibrinogen and CRP at exacerbation and the change in these biomarkers' concentrations between stable and exacerbation visits were found to be equally useful in identifying airway bacterial presence at exacerbation whereas IP-10 appeared to be useful in identifying airway viral presence. Blood biomarkers at enrolment did not predict the hazard of exacerbation in our cohort.

Blood biomarkers in identifying and predicting an exacerbation visit

Previous studies reported an enhanced systemic inflammatory profile during exacerbations compared to the stable visits but these reports were based on a blood biomarkers' absolute concentrations.[47, 220, 283] To my knowledge this is the first study to report both the significance of the blood markers' absolute concentration alongside the change in biomarkers concentration in successfully identifying an exacerbation visit.

Specifically, I demonstrated that IL-6, CRP and fibrinogen were significantly higher at exacerbation compared to the stable state. Furthermore, these markers were able to identify an exacerbation visit, consistent with previous findings.[218, 322] A clear increase in CRP levels during exacerbations was previously reported.[191, 220] Andelid et al examined systemic neutrophil mobilisation in patients with COPD during clinical stability and at exacerbations and compared with healthy smokers/never-smokers controls.[191] They found evidence for neutrophil mobilisation and activation during clinical stability (neutrophil elastase and neutrophil concentration) and further increases during exacerbations (neutrophil concentration and myeloperoxidase). Andelid et al examined a relative exacerbation value ("percent change from baseline") of systemic markers (MPO, NE and CRP) but did not examine how these could be useful in identifying an exacerbation visit.[191] In addition, other authors reported that IL-6 and fibrinogen levels increased at exacerbation[220], however, they did not address how the change in biomarkers concentrations was associated with the identification of an exacerbation visit.[220] Hurst et al reported that CRP concentration, in addition to major symptoms reported by a patient, had added value in confirming AECOPD.[218] In another study of severe to very severe COPD patients Gallego et al reported that higher CRP was associated with exacerbations requiring hospitalization (>100mg/L). However, in their study Gallego et al had a larger proportion of severe COPD exacerbation (40%) compared to the AERIS study.[322]

Another marker of systemic inflammation white blood cell count, specifically the neutrophil fraction, in the AERIS study demonstrated that WBC and neutrophils were mildly but significantly elevated during exacerbation compared to the stable state. Furthermore, neutrophils were demonstrated to be useful in identifying an exacerbation visit from a stable visit. My findings are in line with previous studies that reported an increase in blood neutrophils at exacerbations[47, 191] Neutrophils are granulocytes that are known for their antibacterial role.[168, 188] I demonstrated that both WBC and blood neutrophils were associated and predictive of airway bacterial presence. Some authors demonstrated that systemic neutrophil counts were positively associated with airway bacterial presence at exacerbations but the group did not evaluate the predictive value of the blood neutrophil in identifying airway bacterial presence.[191]

Furthermore, I showed a significant increase in IP-10 levels at exacerbations compared to clinical stability. IP-10 is a chemokine secreted in response to viral infection.[142] Thus my finding could be explained by the fact that the proportion of exacerbations with at least one virus present increased from 13.6% at stable state to 41.3% at exacerbation.

Intriguingly, SPD level at exacerbations was lower than at enrolment visits, contrary to previously published reports.[240, 241] While the maximum SPD concentrations were lower at enrolment than at 1st exacerbation (min and max at enrolment 45.00 and 413.00 respectively; at 1st exacerbations 40.90 and 477.00 respectively), both median and mean SPD were lower at 1st exacerbations. SPD is made by type II alveolar cells; this may explain the fact that patients with severe emphysema at exacerbations have no systemic influx of SPD from the lungs during an acute airway inflammation. However, the AERIS cohort does not represent a severe emphysema cohort at enrolment as suggested by the densitometric parameter (median Hounsfield Units -877(46)) assessed by HRCT.[323 324] Furthermore, there was no association between Hounsfield units and SPD level at enrolment (rho 0.024, p=0.815), which was in accordance with a previously reported finding[239]. The difference between the AERIS finding and previous reports could be in the methodology. Ju et al reported an increase in SPD during exacerbations in a cohort of patients with only severe exacerbations[240]. Whereas Shakoori et al reported their finding on a group of exacerbating COPD patients with hospitalised and less severe exacerbations, although, the exact proportion of severe, moderate and mild exacerbations was not disclosed.[241] Moreover, Shakoori et al sampled less severe patients on the day of presentation to the hospital whereas severe patients were sampled at day 7-10.

Previous reports on cardiac enzymes, Troponin and BNP, claimed that the rise in the cardiac enzymes' level accompanied exacerbation events and was associated with worse prognosis.[242, 243, 248] In the AERIS cohort I found no significant difference in cardiac Troponin level and mild but significant differences in BNP level between stable and exacerbation states. This could be explained by the fact that previous studies were performed on clinically more unwell patients requiring hospital admission.[243, 246] In particular, Vallabhajosyula et al reported only exacerbating patients requiring ICU admission, whereas the vast majority of exacerbations in the AERIS cohort were of a moderate severity.[248]

When examining if baseline blood markers could be used in predicting future exacerbations or frequent exacerbators group I found no such association unlike a previous study. [192] Thomsen et al reported that a simultaneously elevated baseline CRP, fibrinogen and leukocyte levels were associated with a higher risk of frequent exacerbations. It is noteworthy that Thomsen et al conducted their analysis on the much larger Copenhagen City Heart Study and Copenhagen

General Population Study databases. Furthermore, the Copenhagen cohorts were a general population where the COPD patients with GOLD A-to-D were enrolled and retrospective information on only moderate and severe exacerbations were included into analysis. In another study the odds of an increased exacerbation frequency was associated with a higher level of white blood cells.[118] When the analysis was conducted for the COPD stage 2 group only a higher level of fibrinogen was associated with higher exacerbation rate. However, this study was again performed on a much larger sample.

Airway infection and usefulness of blood biomarkers

In the AERIS study I demonstrated that airway bacterial presence at first exacerbation was associated with a higher inflammatory profile (neutrophils, CRP and fibrinogen levels) and no such association was found between the groups with and without the respiratory viral presence. Furthermore, I demonstrated that both blood markers absolute concentrations during acute exacerbations as well as the change in concentration (WBC, neutrophils, CRP and fibrinogen) could identify airway bacterial presence. The presence of airway bacteria was previously reported to be associated with higher systemic inflammatory profiles by some studies[59, 283, 325] although in the study by Chang et al the difference failed to reach significance at exacerbations[325]. Moreover, Gallego et al in their study of severe COPD patients a higher CRP was found to be associated with airway bacterial presence (58.3 mg/L, IQR 21.0–128.2) compared to when respiratory viruses were detected at exacerbation (37.3 mg/L, IQR 18.6–79.1).[322] Specifically, higher CRP was associated when *S. pneumoniae* and *H. influenzae* were identified (74.1 and 74.5mg/L respectively). In contrast to the previous reports and my own findings, Clark et al reported that airway bacterial presence in patients with an acute exacerbation of COPD was not associated with either raised CRP or temperature.[158, 222, 322] Furthermore, Clark et al reported that it was only viral or viral/bacterial presence that was associated with the raised CRP. The difference in the findings by Clark et al could be due to various factors. Firstly, they investigated a cohort of patients with COPD diagnosis confirmed from the hospital and GP notes with no additional investigations performed to confirm the accuracy of the COPD diagnosis. Clark et al also only examined severe exacerbations and the bacterial detection in this cohort was only 44%. Additionally, one third of patients were already treated with antibiotics at the time of the sputum sampling in the Clark et al study. Moreover, it is unclear how long these patients were treated with antibiotic therapy prior to the sampling in this study.

Respiratory viruses are though to play a significant role in triggering exacerbations of COPD.[326] IP-10 plays an important role in a primary response to viral infection in COPD exacerbations.[142] Thus having examined this marker I demonstrated that IP-10 was associated with respiratory viral

presence. This finding is consistent with previous studies.[47, 142, 235] Furthermore, I demonstrated that IP-10>130 pg/mL was 77.2% sensitive and 40.4% specific in identifying viral presence at exacerbations. In contrast, Bafadhel et al reported a lower IP-10 cut off (56 pg/ml) to be of a similar sensitivity (75%) and higher specificity (65%).[47] This could potentially be explained by that fact that Bafadhel et al had twice as many severe exacerbations in their study compared to the AERIS cohort (21/182 and 20/355 respectively). These investigators also reported lower detection of respiratory viruses (29%) compared to the AERIS study (41.3%) at exacerbations.[47, 275]

The use of Procalcitonin is advocated in the management of acute respiratory tract infections.[233, 327] In a meta-analysis of available studies. Simon et al concluded that PCT was more sensitive and specific in differentiating bacterial from non-infective causes of inflammation (not limited to the respiratory causes) in secondary care.[321] The cut off of 0.25 ng/ml and above suggests bacterial presence thus the usefulness of antibiotics is advocated in that group of patients.[225, 227, 257] Bafadhel et al also studied the usefulness of Procalcitonin and CRP in hospitalised patients and reported that Procalcitonin at the threshold of greater than 0.08 ng/mL could be identify useful in identifying patients with pneumonia from severe exacerbation of COPD and asthma patients.[223] In a randomised interventional study by Christ-Crane et al patients admitted to the hospital with suspected lower respiratory tract infection were randomised into Procalcitonin guided or standard therapy groups.[227] Christ-Crane et al found the use of antibiotics was significantly lower in the Procalcitonin group and no significant difference in clinical outcomes was observed between the study arms.[227] In our study I found that although there was a trend towards differences in Procalcitonin levels between the exacerbation and stable states as well as the groups with and without bacterial presence at exacerbations, these differences did not attain statistical significance. Therefore, Procalcitonin was not useful in identifying nor in predicting exacerbations and in particular exacerbations associated with bacterial presence. This could be explained by the fact that PCT is a useful marker of sepsis and in our cohort we had largely patients with mild and moderate exacerbations of COPD treated in the community with very few severe exacerbations (n=20/355) over the twelve months period.[228] Another potential explanation is bacterial colonisation in COPD. In the AERIS cohort 48.7% of all samples obtained at stable visits were positive for airway bacterial presence with 58.9% positive at exacerbations. However, the information on the association between the bacterial colonisation, exacerbations in COPD and Procalcitonin is scarce. In one small observational study PCT level was investigated in patients admitted with AECOPD to ICU.[229] The group reported that PCT level was less than 0.1 µg/L (the level of low probability of bacterial infection) in patients with known bacterial colonisation (n=3) compared to the PCT of less than 0.25 µg/L (the level when the bacterial infection is not strongly suspected) in all patients with detected PA on admission to ICU (n=4).[229]

However, based on the findings of this study it is challenging to make any conclusion on the PCT level at severe exacerbations with the prior bacterial colonisation due to a small sample size.

Prediction of airway bacteria at exacerbations - "To treat or not to treat?"

As demonstrated earlier, higher plasma neutrophils, fibrinogen, CRP, sputum colour and lower blood eosinophils could be a useful tool when encountering an exacerbating patient and, particularly, when the question of to treat or not to treat with antibiotics is posed. I purposefully examined the value of clinical upper limits of normal for blood biomarkers, as cut offs, in detecting the presence of airway infection as this is what, as a clinician, one would view as an abnormal result (above the upper limit of normal range). Thus the sensitivity of plasma neutrophils, fibrinogen and CRP in identifying airway bacterial presence ranged from 59 to 69% and specificity 42 and 52%. Furthermore, the accuracy of sputum colour≥3 and blood eosinophils<2% in detecting airway bacteria at exacerbations was also within the above range. Miravitlles et al reported that most patients with chronic bronchitis had green (29.8%) and yellow (56.7%) sputum during exacerbations with the pooled estimate corresponded with 94% sensitivity and 15% specificity in identifying airway bacteria.[154] It is noteworthy that in our sputum colour assessment (sputum colour ≥3) we allowed for dark yellow and light-dark green colours and only 45.7% of patients were reported to have sputum colour ≥3. However when all yellow (sputum colour 2 and 3) and green (sputum colour 4 and 5) sputum colour grades were adjusted for, the prevalence of sputum colour ≥2 was 89.6% giving sensitivity of 91.9% and specificity of 13.8%, in line with previous studies.[123, 154] Given a modest specificity of sputum colour ≥2 I would therefore suggest we continue using Sputum colour ≥3 as a cut off for identification of airway bacteria. In the light of the above when I see a patient with enhanced COPD symptoms and when the decision needs to be made whether to treat with antibiotics or not these markers (blood neutrophils, fibrinogen, CRP, sputum colour and blood eosinophils) could be applied to support my decision. These could be elevated in isolation or in combination.

A combined biomarker approach has been previously applied in search of a better to tool to identify an exacerbation event or its phenotype.[47, 218, 223] Other authors assessed the value of individual biomarkers in detecting airway bacterial presence at exacerbations.[158] In the present chapter I assessed if there was any additional value of a combined biomarker approach in detecting bacterial infection at exacerbation. Having combined the individual biomarkers, that were earlier identified as being useful in identifying bacterial presence at exacerbations, into two models I found that the combinatory approach did not add an additional value. Therefore, the use of individual clinically available biomarkers at exacerbations is more practical and easy to use in a

busy clinical practice than calculating the combined markers approach as the latter does not boost the accuracy of the diagnosis of bacterial presence at exacerbations.

There are some caveats related to the analysis of blood biomarkers in the AERIS cohort. Previous studies reported inflammatory resolution by measuring systemic markers from exacerbations into recovery.[315] In our study, unlike the monthly sputum sampling, blood samples were collected only quarterly. In addition, blood samples from "not recovered" visits were omitted from the analyses thus affecting the sample size and not allowing the close monitoring of the inflammatory profile dynamics from exacerbations into recovery. Another possible limitation to the blood biomarker analysis is that a large proportion of exacerbations in the AERIS cohort did not require hospitalisation (moderate and mild exacerations, 85.6% and 8.7% respectively) thus not allowing study of a distinct inflammatory profile during exacerbations that may have developed if patients had been left longer without treatment. Nevertheless, there are various advantages to early detection of an exacerbation. Namely, patients were treated early, impacting upon the proportion of severe exacerbations.[144] Early identification also allowed 91.4% of exacerbation visits to be carried out prior to antibiotics administration, which may have had less impact on the bacterial culture results. Furthermore, as only a small proportion of the AERIS COPD exacerbations required admission to hospital, the majority of exacerbations were managed in an outpatient setting. Therefore, I believe that my findings from the AERIS cohort are representative of the bulk of real-world unwell COPD outpatients.[328]

I demonstrated that IP-10 was useful in detecting respiratory viral presence at exacerbations. However, this marker is not currently clinically available thus my findings can not yet be implemented into clinical practice.

In summary, the change in the blood markers' concentrations is a useful tool in identifying an exacerbation visit and, specifically bacterial presence at exacerbation. WBC, Neutrophils, Fibrinogen and CRP appear to be useful as markers associated with airway bacterial but not viral presence. This is an important finding when assessing patients with an acute exacerbation of COPD as in the absence of the real time screening tool for the presence of bacteria these findings may guide the prescription of antibiotics. However, further larger studies to confirm these findings and further search for clinically available markers to aid the management of COPD exacerbations are required.

Chapter 8 Discussion and Future Work

Exacerbations of COPD are the largest single cause of respiratory emergency respiratory admission. Physicians learn how to manage exacerbations of COPD early in their career on acute medical wards. The management of COPD exacerbations that was engrained in us is usually stereotyped and limited to "oxygen, nebulisers, steroids and antibiotics". Furthermore, the diagnosis of COPD exacerbations is mainly based on symptoms, the treatment we learn is generic and does not account for different exacerbation phenotypes. Most first line clinicians are trained to give "the benefit of the doubt" and treat an exacerbation event with the standard approach, particularly in an outpatient setting, as there is the fear that a patient is not monitored closely and may deteriorate. Considering the potential side effects of medications and cost involved, this generic approach to exacerbation treatment without exacerbation phenotyping may not be beneficial and, moreover, hazardous to a patient.

Therefore, the overall aim of my PhD thesis was to determine whether routinely measured biomarkers, measured alone or in combination, can be used in identifying exacerbations of COPD, specifically of a bacterial aetiology. My specific intent was to evaluate the additional value of the routinely measured biomarkers in identifying bacterial exacerbations.

The findings of my work and their implications were discussed in details in relevant chapters. In this final chapter I will summarise the key findings of the present work and discuss their implications on our understanding of COPD exacerbations. I will then discuss their implications for the future research.

The key findings of the present work are:

- In the AERIS cohort 57.58% of patients in the AERIS cohort had predominantly raised blood eosinophils ≥2% during clinical stability over twelve months. This confirms previous reports that eosinophilic inflammation is a stable phenotype in this group of COPD patients.
- The group with predominantly raised eosinophils could be predicted at enrolment: those with blood eosinophils ≥2% at enrolment were more likely to be in the group with the predominantly raised blood eosinophils ≥2% over twelve months.
- For the first time an association between the persistence of raised blood eosinophils and eosinophil associated exacerbations was described. The likelihood of an exacerbation with raised eosinophils (≥2%) was 12.00 times higher in patients with predominantly raised blood eosinophils (≥2%) compared to those with rarely raised blood eosinophils.

- An association between the persistence of the eosinophilic inflammation and seasonality has been reported for the first time whereby a larger proportion of exacerbations had blood eosinophilia in the Summer season compared to the Winter season, although the total number of eosinophilic exacerbations (≥2% in blood) per month remained similar throughout the year. Furthermore, in patients with the predominantly raised blood eosinophils the odds of an exacerbation having eosinophils ≥2% was 3.01 times higher in the Summer than in the Winter season.
- For the first time a seasonal effect on bacterial presence in the group with predominantly raised blood eosinophils was reported. Namely, bacterial infection at exacerbation was higher in the Winter than Summer in patients with predominantly blood eosinophils≥2% at stable visits (OR 4.74; p=0.011). This finding may impact the treatment management of this COPD phenotype at exacerbations in the future. However, further confirmation from larger randomised studies is required.
- The group of patients with rarely raised blood eosinophils over time had higher prevalence of airway bacteria during exacerbations compared to the groups with predominantly and intermittently raised blood eosinophils over time (86.4%, 57.4% and 54.5% respectively, p=0.044)
- A significant proportion of the AERIS cohort demonstrated airway eosinophilia at enrolment (34.8%). One fifth of patients (21%) had a predominantly airway eosinophilia over twelve months during clinical stability.
- There was a higher rate of exacerbations with raised sputum eosinophils in patients with the predominantly raised airway eosinophils over time compared to groups with intermittently and rarely airway eosinophils over time (1.07 ± 1.09, 0.66 ± 0.97 and 0.27 ± 0.79 respectively, p= 0.001).
- The prevalence of bacterial exacerbations was lower among those who had airway eosinophilia at exacerbation, namely, the presence of airway bacteria was 78% lower in those with airway eosinophilia compared to those without.
- For the first time a seasonal variability of the airway bacterial presence in the group with predominantly raised sputum eosinophils>3% is described. Namely, in patients with predominantly raised sputum eosinophils over time airway bacterial presence was more likely in the Winter than Summer season at exacerbations (OR 9.01, p=0.045).
- Sputum colour, as assessed by the five-grade Southampton Sputum colour chart, was significantly associated with and predictive of the underlying airway neutrophilic inflammation during clinical stability and at exacerbations. This finding supports the validity of the Southampton Sputum Colour chart.

- For the first time a value of the numeric change in sputum colour and association with bacterial presence at exacerbations is reported. Specifically, a greater positive change in sputum colour was associated with an increased prevalence of bacterial detection at exacerbations.
- Higher sputum colour, higher blood neutrophils, higher CRP, higher fibrinogen and lower blood eosinophils demonstrated usefulness in identifying airway bacterial presence at exacerbations. This may be useful in boosting clinical confidence when considering a treatment for an unwell COPD patient, particularly, when deciding for appropriateness of antibiotics.

8.1 Sputum Colour change is a useful marker of bacterial presence

The Birmingham group has previously demonstrated the association between the sputum colour and the presence of bacteria.[123, 156] Furthermore, there are previous reports that sputum colour could be used as a guide for antibiotic treatment.[123, 158] However, previous studies on the sputum colour were conducted using different sputum colour charts with different sputum colour grades.[156, 157, 289] In contrast, there is some contradicting evidence that sputum colour using five graded sputum colour chart was not associated with bacterial presence.[161] Furthermore, most of these studies were conducted cross sectionally, at one time point only, and very little is known of the impact of the sputum colour change and the presence of airway bacteria.[154, 157, 158, 160, 293] Firstly, the findings of my work confirm that sputum colour, as assessed by Southampton Sputum Colour (SSC) chart, is associated with the proportion of underlying neutrophils in the sputum. Therefore the SSC is a valid tool for assessment of the degree of the sputum purulence both during clinical stability and at exacerbations. Furthermore, I demonstrated that the change in sputum colour could be used as a surrogate marker of airway bacterial presence, thus guiding the management of antibiotics in exacerbating patients. I believe that sputum colour should be used more widely in the clinical practice to guide antibiotic therapy. However, to validate the use of the sputum colour and, particularly the change in the sputum colour, further work is required. This I present later in the “Future work” section.

8.2 Eosinophilic inflammation is a distinct phenotype in COPD that persists over time and associated with exacerbations with raised eosinophils.

There is mounting evidence that eosinophilic inflammation is a significant and distinct phenotype in COPD. Specifically, raised sputum and blood eosinophils predict a better response to steroid treatment in COPD patients both during clinical stability and at exacerbations.[175, 177, 253, 254] Recent reports further emphasise the importance of distinguishing between the patients with and without eosinophilic inflammation not only for prognosis of a good response to steroid treatment but also due to the possible side effects of these drugs in patients without eosinophilic inflammation. In the retrospective analysis of three randomised controlled trials (comparing ICS, ICS/LABA, LAMA, LABA and placebo) Pavord et al reported a significant reduction in the exacerbation rate of patients with higher blood eosinophils (≥2%) on inhaled corticosteroids compared to patients in a tiotropium only arm.[301] Moreover, lower blood eosinophils <2% in patients on inhaled corticosteroids demonstrated higher rates of pneumonia.[202] However, little is known about the persistence of eosinophilic inflammation in COPD and its association with exacerbations. My work expands our knowledge of this eosinophilic inflammation phenotype in COPD. Specifically, I have demonstrated that there is a significant proportion of patients with raised sputum and blood eosinophils in the AERIS cohort at enrolment. The raised sputum and blood eosinophils were also predictive of future exacerbations with raised eosinophils. I also reported that raised eosinophils in both compartments are not a random finding but a persistent phenomenon in a subgroup of COPD patients. For the first time, I demonstrated that there is a seasonality effect on eosinophilic inflammation, particularly, with a larger proportion of exacerbations with raised blood eosinophils being observed in the Summer but not Winter season, thus, identifying potential grounds for the tailoring of treatment by season in this eosinophilic group of patients. More importantly, the prevalence of airway bacteria was found to be higher in patients with lower blood eosinophils at exacerbation. In the context of bacterial infection and seasonality, I reported that patients who have predominantly higher blood eosinophils over time have an increased likelihood of bacterial infection at exacerbations over Winter but nor Summer months. This is yet another significant finding identifying the group of COPD patients that are more likely to benefit from the administration of antimicrobial agents in the Winter months but this finding requires larger randomised studies to test this.

8.3 The usefulness of biomarkers to detect airway bacteria at exacerbations

8.3.1 Individual biomarkers in identifying airway bacterial presence at exacerbations

The use of systemic markers is commonly used to confirm a severe exacerbation event in clinical practice. CRP and Procalcitonin were previously reported to be good indicators of bacterial presence in patients with acute respiratory illness in some studies.[47, 223] Other authors however demonstrated that CRP not Procalcitonin being useful in identifying bacterial exacerbations of COPD patients.[158] In contrast, other authors demonstrated no predictive value of CRP in the context of airway bacterial presence at exacerbations of COPD.[154] In my work I report that blood neutrophils, fibrinogen, CRP demonstrated usefulness in identifying bacterial presence at exacerbation. I found Procalcitonin was not a useful biomarker, either in identifying an exacerbation event or in identifying bacterial presence at exacerbations in the AERIS cohort.

8.3.2 Combined biomarkers approach in identifying bacterial presence at exacerbations

Srugo et al attempted a combinatorial approach in a form of a novel assay that integrated blood biomarkers to identify bacterial versus viral infections in children admitted to the hospital.[329] There were previous studies attempting to use combined markers to confirm exacerbation of COPD event and to identify different exacerbations phenotypes.[47, 218] Other authors demonstrated the role of individual markers such as the sputum purulence and systemic inflammatory markers being useful in detecting bacterial infection.[123, 158] However, there are no previous reports on a combined markers approach aimed at identifying bacterial presence at exacerbations using routinely available markers from separate biological compartments (i.e. sputum colour as a marker of neutrophilic airway inflammation along with systemic markers). I earlier demonstrated that higher sputum colour, lower blood eosinophils, lower airway eosinophils, higher CRP, higher fibrinogen, higher blood neutrophils were useful in detecting airway bacterial presence at exacerbations.

When I combined these biomarkers into two different models to examine if there was an additional value in using these markers together in identifying bacterial presence at exacerbations, I found that this method did not increase my confidence in confirming a bacterial exacerbation. Thus I propose that higher sputum colour, higher CRP, higher blood neutrophils and lower blood eosinophils should be further assessed in larger scale trials in their accuracy of predicting bacterial presence at exacerbations of COPD.

8.3.3 Implications for approach to clinical care

The gold standard method to confirm bacterial exacerbation is to examine sputum for the presence of airway pathogen (direct marker). (**Figure 8.1**) The conventional microbiology culture method takes few days and bacterial PCR analysis is not widely available in clinical institutions. Therefore, other clinically available markers (putative markers) are in great need. I have shown that higher sputum colour, higher blood neutrophils, higher CRP, higher fibrinogen, lower blood eosinophils are useful in detecting airway bacterial presence at exacerbations. In the light of my findings I propose to apply these biomarkers in isolation to boost the confidence in diagnosing bacterial presence at exacerbations when encounter with an unwell patient presenting with a suspected exacerbation of COPD. (**Figure 8.1**)

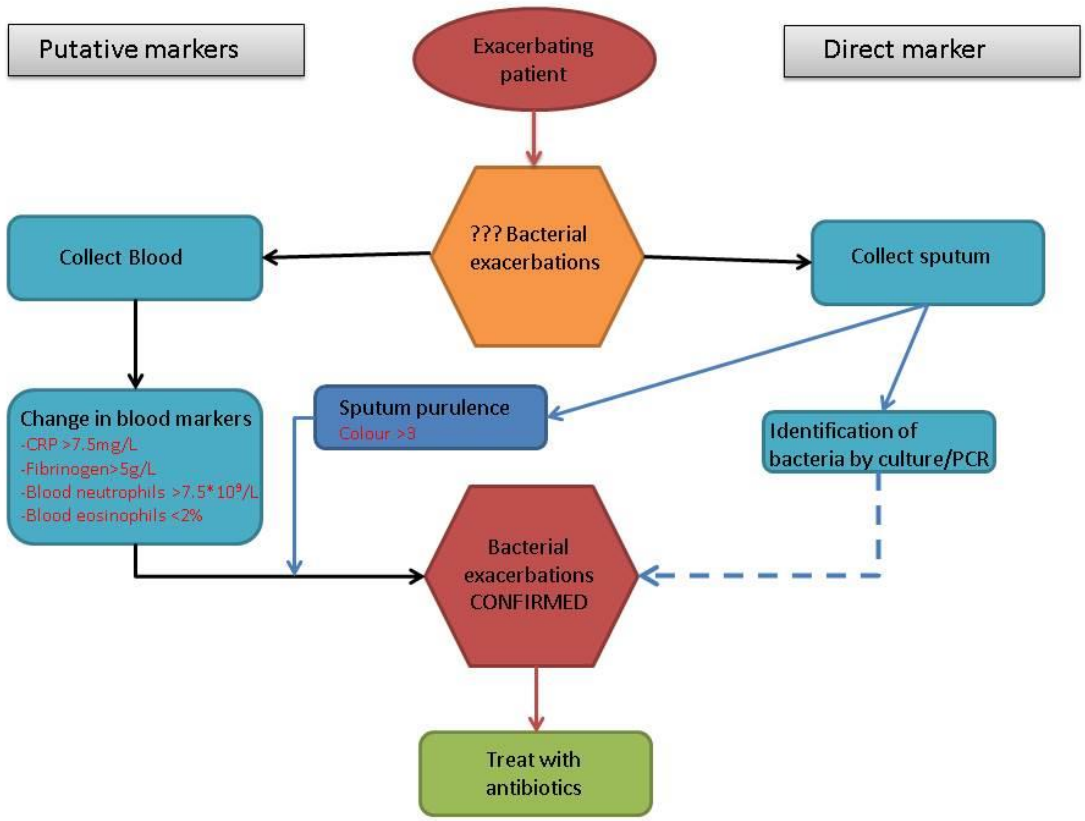

Figure 8.1 illustrating a step wise approach in diagnosis of bacterial exacerbation

8.4 Study caveats impacting the analyses.

Whilst I have previously discussed the limitations specific to each of the biomarkers analysis in each relevant chapter, there are a number of caveats that apply to the whole study

Firstly, the AERIS study was not originally designed, nor powered, to examine the sputum colour, blood biomarkers and eosinophilic inflammation and the relevance of these thus the sample size in each analysis was limited.[261] In addition, there is a significant proportion of mild COPD patients in the community and some individuals with undiagnosed COPD, (i.e. the "missing millions"), that see "their doctor" with "chest infections". Therefore, the general study limitation is that only patients with FEV1<80% and at least one exacerbations requiring treatment were included into the AERIS cohort, thus determining the AERIS cohort population. Hence, our findings are only applicable to the patients with moderate-to-very severe COPD who experience frequent exacerbations. However, as the main aim of the study was to investigate an impact of airway infection at acute exacerbations and generally patients with mild COPD exacerbate less compared to the patients with more severe disease, logically, the recruitment of a mild COPD subgroup would not have fulfilled the study goal.[118]

One more challenge for the present work was that the airway compartment analyses were limited due to the fact that either the sputum sampling was not always successful (i.e. some patients were not able to produce the sample) or that sufficient sputum volume or quality was obtained, despite the efficient sputum induction approach and well trained staff/patients. However, regardless of the challenges of sputum sampling, the sample yield was high during both stable (79.1%) and exacerbation visits (91.3%). Additionally, blood sampling was only quarterly, not monthly as per sputum sampling, and therefore did not allow the close monitoring of the inflammatory picture from exacerbation to recovery.

Another airway marker, sputum purulence, was assessed by technicians using the five grade SSC chart. However, there was no sputum colour chart for patients to describe the numerical change in the sputum purulence and the way our patients recorded sputum purulence was by reporting the "change in sputum colour over the last 24 hours", which was thus limited to "Yes" and "No" answers.

The data availability from the AERIS study has been one of the major limitations for my present work. Although I have data for bacterial and viral presence, I did not have access to the bacterial and viral load data that could have made my analyses more specific. Furthermore, for the PhD analysis I only had access to data from the first twelve months of the study, as the second year data still pending data reconciliation. Publication of this work was and continues to be governed

by strict agreement with the commercial sponsor. The sequence of publications was predetermined to demonstrate adherence to the primary outcomes of the study ahead of any secondary outcomes such as the work described in this thesis.

What should be noted however, is that the AERIS study has been conducted to the highest standards of research governance associated with a clinical trial with a commercial sponsor, with rigorous monitoring, continuous data cleaning and external trials oversight so that the data quality and the study conclusions are sufficiently robust to serve as a platform for future vaccine studies.

8.5 Future work

As discussed earlier in Chapter 5, the findings of the present work on sputum colour were limited due to the AERIS design and applicable predominantly for the moderate exacerbations of COPD. Therefore, to further understand the association of the change in sputum colour and airway bacterial presence it is prudent to design a larger biomarker focused study. Thus I would like to evaluate sputum purulence using the SSC chart assessed by both technicians and by patients. In addition, the majority of AERIS cohort exacerbations were moderate thus in future research I would like to include equal number of moderate and severe exacerbations of COPD cases and randomise these patients to biomarker-guided and current standard treatment algorithm arms. Specifically, I would like to target patients with a positive change in sputum colour at exacerbations. I would collaborate with software engineers that would allow me to design a device or a software application that can assess the degree of purulence using different shades but categorising these into five categories used in SSC (white/clear, light yellow, dark yellow, light green and greenish). Furthermore, ultimately I would like to bundle the sputum purulence assessment along with exacerbation symptoms into the “COPD self management software tool“ where the numerical change in sputum purulence, in addition to other exacerbation symptoms, previous history of exacerbations and other relevant clinical data (e.g. blood eosinophils) can be applied to guide the patient in further management. Namely, the device will provide the output based on the patient’s baseline information and information provided on the day: “stable COPD”, “unlikely exacerbation - continue monitoring”, “symptoms are consistent with exacerbations – inform your healthcare provider and take antibiotics and oral steroids”, “symptoms are consistent with exacerbations – do NOT take antibiotics, take oral steroids” etc.

In Chapter 6 I demonstrated the associations of eosinophilic inflammation with eosinophilic exacerbations, seasonality and airway bacterial presence. In particular, I showed the persistency of the longitudinal eosinophilic inflammation. Given the findings of the present work I would like

to examine biomarker-tailored treatment according to the eosinophil level and the season of an exacerbation event in a longitudinal randomised controlled study of patients with and without raised eosinophils overtime during clinical stability. Previous randomised clinical trials of COPD exacerbation treatment were based on the biomarker measured at time of an exacerbation event.[171, 254] The study by Siva et al attempted to manage airway eosinophilia during clinical stability in order to prevent the rate of exacerbations.[182] To my knowledge there were no trials randomising patients to the biomarker guided or placebo treatment arms at exacerbations based on the persistency of eosinophilic inflammation during clinical stability. Therefore, I would like to compare the treatment failure, antimicrobial and systemic steroids use, health status as the outcomes in the non-inferiority of the biomarker guided at a stable state (based on the persistency of raised blood eosinophils over time at stable state) versus the biomarker-guided at exacerbation event and the standard no-biomarker treatment arms. Alongside the blood eosinophils I would like to examine a less invasive breath biomarker nitric oxide to test if this biomarker can be used in COPD population with persistent eosinophilic inflammation to accurately identify an exacerbation event associated with raised eosinophils and monitor the response to treatment. I would also like to examine Th2 response closely by studying IL-5 alongside the clinically available blood eosinophils. I would design the study with more stringent inclusion criteria with regards to excluding common atopic phenotype using skin prick test, total IgE, RAST to common allergens.

I demonstrated the usefulness of biomarkers in identifying bacterial presence alone and in combination. Considering these findings, I would also like to conduct a randomised controlled study with consideration of biomarker targeted approach: biomarker-alone arm, combined-biomarkers approach and standard treatment approach of COPD exacerbations with outcome measures of failure to treatment management, recovery time, clinician and patient acceptability, utilisation of healthcare resources and additional antibiotic use.

Furthermore, in the light of the rising resistance to antimicrobial agents and utilisation of healthcare resources it is prudent to use objective measurements to establish the nature of COPD exacerbation and direct treatment. Currently, most first line clinicians opt to treat when encounter a patient with known COPD and symptoms that may suggest an exacerbation. In the present work I demonstrated the usefulness of the sputum colour and blood markers in identifying bacterial infection and further showed association of eosinophilic inflammation and bacterial presence at exacerbations. Thus I would like to design and conduct a randomised controlled study in the primary care setting where the use of objective biomarkers is largely limited. I would like to apply the point of care tests in each surgery to examine the use of airway and systemic biomarkers (CRP, fibrinogen, blood neutrophils blood eosinophils and sputum

colour) to guide the treatment of exacerbation of COPD. As the outcomes I would like to assess the rate of treatment failure, recovery rate, cost effectiveness, doctor and patients satisfactory questionnaire.

8.6 **Conclusion**

The work in this thesis supports the hypothesis that routinely measured clinical measurements of airway and systemic compartments appear to be useful in identifying an exacerbation event, particularly, of a bacterial aetiology. I demonstrated that sputum colour, and measures of blood neutrophils, blood eosinophils, CRP, fibrinogen that are available in the clinical practice could be applied to increase our confidence in identifying an exacerbation event associated with airway bacterial presence. Furthermore, I demonstrated for the first time the significance of the persistence of raised eosinophils over time during clinical stability and its association with eosinophilic exacerbations, seasonality and airway bacterial presence. If supported by findings in larger studies, these observations may play an important part in ensuring that the right treatment offered to the right group of patients and at the right season may reduce the number of antimicrobial treatment and systemic corticosteroids at exacerbations.

Epilogue

Returning to the cases of Pete and Jim described in the prologue - would I manage them differently at exacerbations considering the findings of the present work?

Pete is a classic “pink puffer” patient, slim and emphysematous. Given his symptoms at presentation were of an increased breathlessness and cold, he met the criteria for an exacerbation of COPD. In addition, his inflammatory markers on admission were within normal limits. The level of blood eosinophils at exacerbation and during clinical stability would be informative, to see if he had eosinophilic inflammation and, therefore, be in a favoured group benefitting most from the steroids treatment. He did not report a change in either sputum volume or in purulence at that particular exacerbation event. In addition, his inflammatory markers were normal. Given this evidence, I therefore do not think his exacerbation would be driven by airway bacteria, thus, would challenge the use of antibiotics in this case. Furthermore, he has had frequent admissions with COPD exacerbations thus I would also look into the accuracy of the exacerbation diagnosis and perhaps look into psycho-social factors, the latter subject being beyond the scope of my PhD work.

Jim on the other hand is a classic “blue bloater”, chronic bronchitis patient. Given Jim’s reported the symptoms I agree that being on antibiotics was the correct management for this presentation. Perhaps, the question for this patient is if he was on the right type of antibiotic, although clinically relevant, this question is beyond the scope of my thesis. I would investigate this patient further (examine other systemic inflammatory markers during clinical stability and compare with the exacerbation). Specifically, as he produced daily sputum I would educate Jim on the importance of changes in his sputum colour and that this indirectly signifies airway bacterial presence. For instance, I would train Jim in a COPD self-management plan for early recognition of exacerbation symptoms allowing timely treatment and improved outcomes.

Appendix A

Publications

Kim VL, Coombs NA, Staples KJ, Ostridge KK, Williams NP, Wootton SA, Devaster JM, Aris E, Clarke SC, Tuck AC, Bourne SC, Wilkinson TMA, Group AS. Impact and associations of eosinophilic inflammation in COPD: analysis of the AERIS cohort. Eur Respir J. 2017;50(4).

Bourne S, Cohet C, **Kim V**, Barton A, Tuck A, Aris E, Mesia-Vela S, Devaster JM, Ballou WR, Clarke S, Wilkinson T. Acute Exacerbation and Respiratory InfectionS in COPD (AERIS): protocol for a prospective, observational cohort study. BMJ open. 2014;4(3):e004546.

Abstracts

VL Kim, NP Williams, KK Ostridge, MM Naghibi, NA Coombs, JM Devaster, E Aris, SC Clarke, AC Tuck, SA Wootton, SC Bourne, KJ Staples, TM Wilkinson "The persistence of eosinophilic inflammation in COPD over time – AERIS cohort" Abstract accepted for speaking session at Winter BTS 2016.

V.L. Kim, NA Coombs, KJ Staples, NP Williams, KK Ostridge, MM Wojtas, M Peeters, E Aris, JM Devaster, SC Bourne, TM Wilkinson "Eosinophilic inflammation in COPD: during clinical stability and exacerbations. The AERIS study." Late breaking abstract accepted as poster discussion at ERS 2015

V.L. Kim, NA Coombs, KJ Staples, NP Williams, KK Ostridge, MM Wojtas, M Peeters, E Aris, JM Devaster, SC Bourne, TM Wilkinson "Does sputum colour hold the answer? The AERIS study". Late breaking abstract accepted as thematic poster at ERS 2015.

V Kim, N Williams, K Ostridge, A Barton, MM Wojtas, E Aris, M Peeters, JM Devaster, S Bourne, T Wilkinson "Sputum colour in the light of the health related quality of life, airways and systemic biomarkers in exacerbations of COPD." Abstract accepted for speaking session at Winter BTS 2014.

J Jackson, **V Kim** (presented), A Tuck, S Wootton, T Wilkinson "Identification of potentially pathogenic microorganisms by Selected Ion Flow Tube –Mass Spectrometry (SIFT-MS)." Abstract accepted for speaking session at Winter BTS 2014.

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