Investigating the Symptoms of Airways Disease

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Abstract

Background

Airways diseases are increasingly recognised to be poorly defined phenomena with overlapping pathophysiology and symptoms. They are a significant and growing cause of morbidity, with increasing numbers of people affected globally and no improvement in key outcomes in the UK for the last decade despite ever increasing expenditure. The classification of airway diseases has changed little in the last 50 years, and may no longer be fit for purpose due to the growing appreciation of the complexity and heterogeneity of airways disease and the advent of molecular-based diagnostic techniques to target specific treatment.

Aim

To investigate whether strategies based on the measurement of selected phenotypic and biological characteristics of airways disease can help to improve the understanding of their pathogenesis and targeting of treatment.

Methods

Three characteristics of airways disease, namely (1) exhaled nitric oxide, (2) chronic productive cough of unknown cause and (3) the airway microbiota were described/measured in selected cohorts of patients in three clinical studies. Measurement of each of these characteristics was used to answer focused clinical questions regarding the pathogenesis and treatment of aspects of airways disease.

Results

(1) The baseline measurement of FE_{NO} in steroid naïve subjects with symptoms suggestive of asthma had a low diagnostic value for asthma but was an excellent predictor of inhaled steroid treatment response. (2) A cohort of subjects with chronic productive cough of unknown cause was described. These subjects tended to have radiological evidence of airway dilatation and chronic inflammatory changes but not significant bronchiectasis. Their cough responded well to treatment with azithromycin, with ongoing neutrophilic airway inflammation a particularly strong predictor of treatment response. (3) There were no significant differences in the abundance or community structure of the bacterial communities in the airways between subjects with mild (BTS 2) or severe (BTS 4) asthma or between severe (BTS 4) asthma patients taking inhaled fluticasone or budesonide. However a number of differences in relative abundance of certain species (including enrichment of *Haemophilus parainfluenzae* in the fluticasone group) were noted on comparison of the groups.

Conclusions

This thesis provides support for a new approach to the classification and treatment of airways disease. The recognition of pathologically important processes (treatable traits) which can be used to predict response to targeted treatment has the potential to revolutionise the management of airways disease and result in improved patient outcomes.

Thesis: Aim and Outline

Airways diseases are increasingly recognised to be poorly defined phenomena with overlapping pathophysiology and symptoms. They are a significant and growing cause of morbidity, with increasing numbers of people affected globally and no improvement in key outcomes in the UK for the last decade despite ever increasing expenditure. The classification of airway diseases has changed little in the last 50 years, and may no longer be fit for purpose due to the growing appreciation of the complexity and heterogeneity of airways disease and the advent of molecular-based diagnostic techniques to target specific treatment. Recognition of specific phenotypic and biological markers underlying patterns of disease which will respond to targeted treatments has the potential to revolutionise the management of airways disease and result in improved patient outcomes.

The overall aim of this thesis is to investigate whether selected phenotypic and biological characteristics of airways disease can be used to improve targeting of treatment. An outline of the structure of the thesis with individual study aims is as follows:

Firstly, the current definitions of airways disease and the existing healthcare burden of these conditions will be determined. Existing literature regarding the causes and specificity of symptoms of airways disease will be reviewed. The pathophysiological processes underlying airways disease and main phenotypic groups in which these characteristics predominate will then be considered along with review of the main treatments targeting these processes.

Chapter 2 titled ‘The utility of exhaled nitric oxide in patients with suspected asthma’ explores the importance of making a diagnosis of asthma in order to institute timely and effective treatment to control symptoms, the features and diagnostic accuracy of tests used to ‘diagnose’ asthma and the most recent guidelines for asthma diagnosis. The aim of the study is to investigate the utility of measuring exhaled nitric oxide for diagnosing asthma or predicting response to inhaled steroid treatment.

Chapter 3 ‘Chronic Productive Cough and the use of Macrolides in Airways Disease’ reviews the current literature regarding the symptom of chronic productive cough and its causes as well as the mechanisms of action and use to date of macrolides in respiratory disease. The aim of the study is to describe in detail the underlying pathophysiology of a cohort of patients with the symptom of chronic productive cough who have had the usual underlying causes for this symptom excluded whilst simultaneously assessing the effectiveness of an open label trial of low dose azithromycin in treating this symptom.

Chapter 4 ‘The microbiota in asthma’ examines the small but rapidly growing body of evidence in the emergent field of respiratory bacterial microbiota analysis. These techniques use DNA based sequencing to examine the bacterial communities of the airways in unprecedented detail. A systematic study of the airways microbiota of subjects with different severities of asthma using different doses and types of inhaled steroids is described. The aim of this study is to examine the effect of inhaled steroid dose and type on

the bacterial content of the airways, providing information that may be relevant to the targeting of inhaled steroid treatment.

Declaration of work performed personally

I was an academic clinical fellow attached to the Nottingham Respiratory Research Unit (NRRU) from August 2010 until June 2013 and was subsequently employed as a research fellow at the NRRU from June 2013 until August 2016.

The study protocol and ethics approvals for the 'Reducing Costs in Asthma Management (RCAM)' study had already been written and obtained when I started my PhD. Once I had started my PhD I project managed the study and screened, recruited and took consent from the majority of the participants (45) in the study, from subject RCAM025 onwards. I also performed all of the clinical measurements for these study subjects. Eight of the participants from the study had informed consent taken by a research nurse who also performed the clinical measurements for these study subjects. The few sputum samples that were obtained in the study were processed by one of the NRRU clinical scientists. I entered the majority of the data into a clinical database designed by the NRRU database manager who checked the data for errors. I performed all statistical analysis of the study data with some guidance by a medical statistician who also checked the results.

The idea for the 'Microbiota in Asthma (MIA)' study was partly mine. I wrote the study protocols and all other study documents for the studies 'An open label trial of azithromycin in chronic productive cough (AZCC)' and MIA and obtained ethical approval for these studies from Research Ethics Committees, the MHRA and the Nottingham University Hospitals NHS Trust Research and Innovation Department.

I was the co-ordinating project manager for both of these studies and designed the study specific documents (including the case report form) for these studies and submitted all necessary amendments to the appropriate regulatory bodies.

I screened, recruited and took informed consent from all participants in the AZCC study and was present at all study visits including bronchoscopy visits, performing all bronchoscopies myself under the supervision of a named consultant. I performed all clinical measurements for the study and processed some of the sputum samples for the study myself, although the majority were processed by the clinical scientists of the NRRU who also processed all of the bronchoscopy samples. The sputum and bronchial wash supernatant cytokine bioplex assays were also performed by the NRRU clinical scientists. I contacted the consultant histopathologist and radiologist who reviewed the bronchial biopsies and CT scans from the study and recorded the results of their reviews. I entered the majority of the data into a clinical database designed by the NRRU database manager who checked the data for errors. I performed all statistical analysis of the data with assistance provided by a medical statistician.

I screened all the participants of the MIA study and recruited and took informed consent from 50 of the 72 participants. I also performed all clinical measurements for the study on these subjects. The other 22 participants were recruited and had informed consent taken by one of the department research nurses. The clinical measurements for these subjects were mostly performed by the same research nurse although I supervised sputum induction for

all participants. I processed 20 of the sputum samples for the study myself, and the rest were processed by the NRRU clinical scientists. The samples for microbiota analysis were transferred to King's College London, where DNA extraction was performed and the resultant DNA was sent to HPA Colindale for sequencing and post sequencing clean up. I entered the majority of the data into a clinical database designed by the NRRU database manager who checked the data for errors. I performed statistical analysis of the clinical data from the study but the qPCR and sequencing data was analysed by PhD/postdoctoral fellows at King's College London.

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Firstly I would like to thank my supervisor Professor Tim Harrison for giving me the opportunity to do this research and for his expert guidance and advice along the way. I hope to continue to be involved in research in future and this chance to obtain a grounding in research and work towards achieving my PhD will always be very much appreciated.

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Special thanks are also reserved for Dr Dominick Shaw who acted as my second supervisor and was usually the first to spot the many and varied mistakes made whilst writing up this thesis and accompanying papers!

I owe a big debt of gratitude to Dr Ken Bruce at King's College London for his extra-supervisory duties and for helping to demystify the often complex world of the microbiota via his collection of analogies and metaphors which are second to none! Thanks also to Drs Damien Rivett and Masirah Zain from KCL who helped with the microbiota analysis.

Thank you to my ever supportive family, especially my father Gary, whose years of examining theses had not prepared him for the Herculean task of proof reading mine!

Finally, the biggest thanks of all are extended to my incredible wife Carla for her unwavering encouragement and support throughout all of my endeavours. I dedicate this thesis to her and my son Alex, who was born whilst this research was ongoing and who will always be one of my proudest achievements.

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Abstracts arising from this thesis

Primary Care Respiratory Society UK 2015: Oral presentation

Martin MJ, Wilson E, Gerrard-Tarpey W, Shaw DE and Harrison TW. Is exhaled nitric oxide useful in patients with suspected asthma? Primary Respiratory Care Society Meeting 2015, 16th October 2015

npj Primary Care Respiratory Medicine (2015) 25, 15060; **DOI:**10.1038/ npjpcrm.2015.60; published online 13 October 2015

Thematic Poster ERS London 2016

Martin MJ, Wilson E, Shaw DE and Harrison TW. The predictive value of symptoms for asthma diagnosis and inhaled steroid treatment response. London 2016

European Respiratory Journal (2016) 48: PA836; **DOI:** 10.1183/ 13993003.congress-2016.PA836

Publications arising from this thesis

Martin MJ, Harrison TW. Causes of chronic productive cough: An approach to management. Respir Med. 2015 Sep;109(9):1105-13. doi:10.1016/j.rmed.2015.05.020. Epub 2015 Jul 6. (review)

Martin MJ, Wilson E, Gerrard-Tarpey W, Meakin G, Hearson G, McKeever TM, et al. The utility of exhaled nitric oxide in patients with suspected asthma. Thorax. 2016 Jun;71(6):562-4. doi: 10.1136/thoraxjnl-2015-208014. Epub 2016 Feb 22.

Other publications arising from this research period (not included in thesis)

Martin MJ, Lee H, Meakin G, Green A, Simms RL, Reynolds C, et al. Assessment of a rapid liquid-based cytology method for measuring sputum cell counts. Thorax. 2016 Aug 8. pii: thoraxjnl-2016-208817. doi: 10.1136/thoraxjnl-2016-208817. [Epub ahead of print]

Chapter 1: Background

1.1 Introduction

"Airways diseases" can be broadly defined as diseases affecting the transmitting structures (airways) that allow the passage of gases into and out of the lungs. They affect the airways by causing inflammation of the airway walls, which can result in tissue damage, narrowing or obstruction of the airways. Many different 'types' of airway disease including asthma, chronic obstructive pulmonary disease (COPD),'bronchitis' and bronchiectasis have been described which affect over 400 million people worldwide (1).

Despite the increasingly high global burden of airways disease and many advances in the understanding of the causes, progression and management of these conditions they remain poorly defined with few changes in their classification in the last 50 years. A change in the approach to classifying airways diseases may be required owing to an increasing recognition of their underlying complexity and heterogeneity and the promise of forthcoming novel biological agents that may be targeted to specific patients using certain biomarkers.

Airways diseases lead to the development of various symptoms including cough, wheeze and shortness of breath. The accuracy of diagnosing airways diseases based upon eliciting these symptoms in a clinical history is uncertain. However, previous work has indicated that a significant proportion of primary care physicians often make diagnoses of airways diseases on features from clinical history alone so it is important to establish the discriminatory value of these symptoms.

The diagnosis and management of airways disease may be improved using objective tests capable of measuring and quantifying the pathophysiological processes that underlie them. For example, establishing the underlying pattern of airway inflammation in subjects with airways disease has been determined to be of great value in guiding treatment. Exhaled nitric oxide (FE_{NO}) is an easily measured biomarker of ongoing Th2 inflammation in the airways, but its role in the diagnosis and management of airways disease remains unclear.

The current classification of airways diseases means that some subjects presenting with symptoms of airways disease are not easily categorised with one of the existing disease labels. One such cohort of subjects present with the symptom of chronic productive cough ("chronic bronchitis") which is not explained by any of the recognised causes for this symptom even after thorough investigation. Initial indications suggest that treatment with long term low dose macrolides may improve the symptom burden in this group of patients. However, the underlying pathophysiology of this cohort of patients and their response to macrolide treatment are still to be determined.

Finally, a hitherto under-explored component of the pathophysiology of airways disease is the contribution of bacteria extant within the airways to features of disease. New DNA-based detection techniques have revealed that communities of bacteria (microbiota) in the airways of subjects with airways disease are different to those from healthy subjects and include potentially pathogenic species. Whether or not the composition of the microbiota

differs between subjects with different severities of airways disease using different doses and types of inhaled steroids is yet to be established.

It is clear from the preceding background that there are a number of important factors in the treatment and management of airways disease that require further consideration and investigation. Accordingly it would be appropriate to review in greater detail: the epidemiology, symptoms, and pathophysiology of airways disease, the use of investigations in the diagnosis of airways disease, causes of chronic productive cough and the use of macrolides in airways disease and the existing knowledge regarding the composition of the microbiota in airways disease.

1.2 Airways Disease: Definitions and Epidemiology

The terminology used to describe airways disease has an interesting history and has long been a subject of great debate. Arguably the modern roots of current definitions of airways disease arise from a meeting of the CIBA Guest Symposium in 1959 (2), when at that time the terms “asthma”, “emphysema” and “chronic bronchitis” were often used interchangeably. The recommendations of the symposium were that these conditions should collectively be known as “chronic non-specific lung disease” (CNSLD). However, this phrase was considered too “cumbersome” for clinical practice and it was suggested therefore that patients should be classified with the following diagnostic labels: (1) “Chronic bronchitis” which “refers to the condition of subjects with chronic or recurrent excessive mucous secretion in the bronchial tree” and/or (2) “Generalised obstructive lung disease” which “refers to the condition of subjects with widespread narrowing of the bronchial airways, at least on expiration, causing an increase above the normal in resistance to air flow”. The latter category could be further subdivided into groups comprising (i) “intermittent or reversible” i.e. asthma or (ii) “irreversible or persistent” which would later come to be known as chronic obstructive pulmonary disease (COPD).

The idea that these described diagnostic labels were distinct conditions and arose from different underlying pathophysiological processes would become known as the “British hypothesis” (3). This was in contrast to the so called “Dutch hypothesis”, first put forward by Orie and de Vries in 1961 (4). This alternative hypothesis stated that “asthma, chronic bronchitis and emphysema should be considered as different expressions of the same disease entity, in which both endogenous (host) and exogenous (environmental) factors play a role in the pathogenesis”. The Dutch hypothesis therefore opposed the use of distinct diagnostic labels, and recommended instead the original collective term of CNSLD. Debate still continues as to the pros and cons of these different hypotheses of airways disease (5, 6) with both protagonists seemingly accumulating growing bodies of evidence to endorse their respective positions (7, 8) and some studies also providing support for both hypotheses (9). Current international guidelines regard asthma and COPD as distinct and epidemiological studies also define them differently, although in recent years the ‘crossover’ diagnostic label of Asthma-COPD Overlap Syndrome has also been formally recognised (10).

The current definitions of asthma, COPD and bronchiectasis with estimates of their prevalence, morbidity and mortality are discussed in more detail below.

1.2.1 Asthma

1.2.1.1 Definition

Asthma is defined by the Global Initiative for Asthma (GINA) principally as "a heterogeneous disease, usually characterized by chronic airway inflammation" which "is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation" (11).

This definition, which is notable in its lack of specificity, describes asthma as a *heterogeneous* disease, i.e. a disease that is diverse in its characteristics and identifies the key features of the condition as being airway inflammation, variable respiratory symptoms and intermittent airflow obstruction. It does not attempt to objectively define asthma based on physiological or biological parameters.

1.2.1.2 Epidemiology

Asthma is one of the most common chronic diseases in the Western world, with around 10% of the UK population affected (12) and with an estimated 300 million cases worldwide. However, national prevalence figures are difficult to compare owing to the lack of a universal standardised definition (13). After increasing for many decades the prevalence of asthma in Western industrialised countries now seems to be falling (14). Nevertheless, the increasing incidence in developing countries such as China and India means that global prevalence of the condition is rising (15).

1.2.1.3 Morbidity/Mortality

Asthma is a significant cause of morbidity globally, accounting for an estimated 22.2 million disability adjusted life years (DALYs - i.e. the sum of years lost due to premature mortality and years of life lived with disability, adjusted for the severity of disability). This figure represented ~1% of the global disease burden in 2013 (16). The condition is estimated to cause 489,000 deaths worldwide per year (0.9% of all causes globally) (17) and was responsible for 1216 deaths in the UK in 2014 (18).

Both the morbidity and mortality from asthma improved markedly from the 1950s to the 2000s (19, 20). However, over the last decade the rates of asthma exacerbations (21), hospitalisation from asthma (22), asthma control measures (23) and mortality from asthma in the UK (24, 25) have not significantly changed.

The social and economic costs of asthma are substantial in developed and developing countries (26, 27) and the overall cost of asthma to the UK economy is estimated to be around £1 billion per year (18, 28). This increasing cost burden is mostly due to the increasing *direct* costs of asthma from treatment and hospitalisation due to asthma exacerbations (26, 29) but also significant *indirect* costs due to absenteeism from work and loss of productivity (30).

1.2.2 Chronic Obstructive Pulmonary Disease (COPD)

1.2.2.1 Definition

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) defines COPD as follows (31):

"COPD, a common preventable and treatable disease, is characterized by airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and comorbidities contribute to the overall severity in individual patients"

Again it is evident that this definition is more a general description of features usually associated with COPD than an attempt to define the condition based on objective physiological or biological parameters.

1.2.2.2 Epidemiology

The prevalence of COPD is difficult to estimate accurately due to the lack of a standardised definition. Different studies have used varying case definition criteria, including symptomatology as assessed by questionnaire, doctor diagnosis or lung function criteria (32). Indeed, differences in the lung function testing criteria for assessing limitation between the two main published guidelines in this area led to a significant difference in the number of subjects classified as having airflow obstruction, and hence COPD (33).

A meta-analysis of population based COPD prevalence data published between 1990 and 2004 generated a pooled prevalence of 7.6%, but this was largely based on data from Europe and North America with limited reports from elsewhere (34). A more recent meta-analysis suggested a global increase in the prevalence of COPD, with prevalence increasing in all regions, but particularly in the Eastern Mediterranean and African regions (albeit based on limited data) (35).

1.2.2.3 Morbidity/Mortality

Morbidity and mortality from COPD is substantial and increasing globally. In 2013 an estimated 72 million DALYs (2.9% of global disease burden) were lost to COPD (16) representing an increase of 8.2% since 2005. COPD caused an estimated 2,931,000 deaths worldwide in 2013 (5.3% of all causes globally) (17); an increase of 21% since 1990. In the UK, COPD caused 29,776 deaths in 2012 (36).

Premature mortality from COPD seems to be improving in developed countries although figures from the World Health Organisation (WHO) showed that early mortality from COPD was twice as high in the UK than the rest of Europe in 2012 (37).

The number of hospitalisations and emergency admissions of patients with COPD increased in the USA between 2001-2012 (38) and the rate of emergency admissions for the condition in the UK did not change significantly between 2003 and 2013 (39). The overall cost of COPD to the UK economy is estimated at £1.2 billion (40) and both direct and indirect costs of COPD are expected to continue increasing globally over coming years (41).

1.2.3 Bronchiectasis

1.2.3.1 Definition

Bronchiectasis is generally defined as the “irreversible abnormal dilatation of the bronchi”. This structural pathological change is usually accompanied by clinical symptoms, the most common of which is a chronic productive cough (42). The condition is diagnosed by a high resolution computed tomography (HRCT) scan demonstrating a bronchus with an internal diameter wider than its adjacent pulmonary artery which fails to taper. Bronchi which can be visualised 1-2 cm from the pleural surface is an additional radiological feature (43).

1.2.3.2 Epidemiology

Limited current data regarding international prevalence rates for bronchiectasis are available, although studies from the UK and USA suggest the prevalence of the condition is increasing (44-46). The total prevalence in the UK was estimated at 301/100,000 men and 351/100,000 women in 2004, rising to 486/100,000 in men and 566/100,000 in women in 2013 (46). It is unclear if this reflects a true increase in the number of cases or increased recognition of the condition due to more widespread HRCT scanning (45, 47). Bronchiectasis is more prevalent in women and the prevalence generally rises with age, being highest in those aged ≥70 years (46).

1.2.3.3 Morbidity/Mortality

A valid estimate of the morbidity from bronchiectasis is difficult owing to a lack of data in comparison to other chronic respiratory diseases. Subjects with bronchiectasis have a significantly increased risk of mortality in comparison with the general population. In the UK the age adjusted mortality rate is 1438/100,000 for women with bronchiectasis as compared to 636/100,000 in the general population, whilst for men these mortality rates are 1915/100,000 vs. 895/100,000 respectively (46). Mortality due to bronchiectasis apparently increased in the UK from 797 recorded deaths in 2001 to 908 in 2007, although this increase was driven by increasing mortality rates in the two oldest age groups which were simultaneously falling in the three youngest age groups (48).

The limited data available regarding the cost of inpatient episodes of bronchiectasis suggest the direct costs of managing the condition are considerable (49, 50). These direct costs are likely to continue to rise, with increased numbers of hospitalisations and emergency admissions secondary to the condition reported in both the USA (51) and Germany (52); as well as increasing numbers of ICU admissions secondary to bronchiectasis in the UK (53). The indirect costs of bronchiectasis are unclear but these are also likely to be significant.

1.2.4 Airways Disease: The scale of the problem

Ambiguity in the definitions of distinct 'conditions' of airways disease above may reflect the growing recognition of the complexity and heterogeneity of airways disease. A lack of specificity has almost become necessary when "defining" asthma and COPD so that these disease labels might encompass the increasingly diverse spectrum of airway pathology and resultant patterns of disease recognised in a growing number of people worldwide.

The massive, and increasing, healthcare and socioeconomic burden secondary to airways disease is readily apparent. The growing numbers of people affected are likely to lead to increasing direct and indirect healthcare costs in the coming decades and treatment costs are also likely to increase due to growing availability of a number of novel therapies. Unfortunately, the improvements seen from the 1950s to the 2000s in key outcomes such as exacerbations of airways disease, hospitalisations for airways disease and asthma control measures seem to have stalled over the last decade despite an increased expenditure (23).

A new approach to the characterisation and management of airways disease may be required to reflect their complex and heterogeneous nature and to improve treatment outcomes. The purpose of this thesis was 1) to examine the phenotypic and pathophysiological characteristics of certain airways diseases and 2) to investigate novel strategies based on the identification of these characteristics that might improve the treatment of disease.

The first step in this process is to consider the importance and discriminatory value of the most obvious and direct phenotypic characteristics expressed by individuals with airways disease: their symptoms.

1.3 Symptoms of Airways Disease

Airways disease usually leads to the development of various symptoms in an affected subject. These symptoms include cough, wheeze, shortness of breath (or *dyspnoea*) and a sensation of 'tightness' of the chest.

The accuracy of diagnosing airways disease based upon eliciting these symptoms in a clinical history is unclear. However, previous work has indicated that a significant proportion of primary care physicians often make diagnoses of airways disease based on history alone (54-56). It is important therefore, to establish the discriminatory value of these symptoms.

Accordingly, the prevalence of certain respiratory symptoms and their predictive value for the diagnosis of airways disease will be discussed along with a brief overview of other pulmonary and extrapulmonary conditions that can also cause these symptoms.

1.3.1 Cough

Causes of the symptom of cough are often classified by duration, for example the categories used by the American College of Chest Physicians (ACCP), are "acute" (<3 weeks), "sub-acute" (3-8 weeks), or "chronic" cough (>8 weeks) (57). As the majority of cases of acute and sub-acute cough are secondary to infection these will not be discussed further in this section, and only cough more likely to be secondary to airways disease, i.e. "chronic" cough with a duration of >8 weeks, will be considered.

The causes of a predominantly productive cough are discussed later (Section 1.6.2) and so this section describes cough in general terms.

1.3.1.1 Prevalence

A recently completed meta-analysis found that the regional prevalence of chronic cough varied between ~2% in Africa to ~18% in Oceania, with a prevalence in Europe of around 13% (58). However, this analysis was subject to several limitations including significant heterogeneity in the definition of chronic cough and a relative lack of data from non-European countries.

Other factors or conditions that have recognised associations with chronic cough include respiratory wheezing (59), symptoms of gastro-oesophageal reflux disease (60), smoking (61) and exposure to airborne environmental pollutants (62, 63).

1.3.1.2 Specificity as a symptom of airways disease

a) Asthma

Several investigators have attempted to determine the sensitivity and specificity of symptoms of airways disease for the diagnosis of asthma. These studies have differed slightly in their use of a 'gold standard' signifier of asthma diagnosis. In some studies this 'standard' has been a physician diagnosis of asthma based on symptoms alone, whilst others have used a physician diagnosis of asthma based on symptoms plus an objective test. A comparison of the values of sensitivity and specificity of each of these symptoms for asthma diagnosis is shown in Table 1.1.

Sistek *et al.* in the SAPALDIA study (64) attempted to predict the diagnostic value of 11 different respiratory symptoms to diagnose asthma in 9651 subjects who completed a symptom questionnaire. 'Doctor diagnosed asthma' (DDA) was defined as a positive answer to each of the questions: "Have you ever had asthma?" "Was this asthma confirmed by a doctor?" and "Have you had an attack of asthma in the last 12 months?". Two-hundred and twenty-five subjects (2.3%) had DDA. The symptom of chronic cough was defined as a positive answer to the question: "Do you usually cough during the day or at night, on most days for as much as 3 months each year over at least 2 years?" and the symptom of nocturnal cough defined as a positive answer to the question: "Have you been woken up by an attack of coughing at any time in the last 12 months?". Chronic cough had a sensitivity of 21.5% with a specificity of 95.2% for DDA, whilst nocturnal cough had a sensitivity of 49.3% and a specificity of 72.3%.

A similar study by Sistek *et al.* (65) used the same symptom questionnaire in 784 patients who also underwent objective testing for asthma in the form of the methacholine bronchial challenge test. This test assesses the response of the airways to a nebulised agent that is known to cause airway constriction (methacholine) and a positive result is often interpreted as objective evidence of a diagnosis of asthma (discussed in greater detail in Section 2.2.2.4). The same definitions of DDA, chronic cough and nocturnal cough were used and one-hundred and four subjects (8.3%) had DDA. In this population the sensitivity and specificity for the symptom of chronic cough were found to be 43.1% and 83.9% respectively for DDA whilst the symptom of nocturnal cough had a sensitivity of 60% and a specificity of 66.1%. Bronchial challenge testing by comparison had a sensitivity of 84.6% and specificity of 80.5% for DDA.

Choi *et al.* (66) assessed the use of a questionnaire containing five questions regarding the symptoms of asthma to discriminate between 210 subjects with asthma, as diagnosed by a positive methacholine challenge or bronchodilator reversibility testing, and 92 without asthma. These authors found that paroxysmal coughing was less common in asthmatics than in non-asthmatics and had only a 16% and 42% sensitivity and specificity respectively for diagnosing asthma.

Schleich *et al.* (67) used a similar symptom questionnaire to interrogate the symptoms experienced by 174 corticosteroid naïve subjects with respiratory symptoms who were diagnosed as having asthma (n=82) or not having asthma (n=92). Subjects were assigned to the two groups on the basis of methacholine challenge testing. The symptoms of diurnal cough (sensitivity 66% and specificity 26%) and nocturnal cough (sensitivity 37% and specificity 65%) were again found to be lacking in both sensitivity and specificity for the diagnosis of asthma.

Schneider *et al.* (68) attempted to determine the diagnostic accuracy of certain respiratory symptoms listed on a structured questionnaire for asthma and COPD in subjects from GP (n=219), inpatient (n=300) and outpatient (n=259) settings. Asthma was diagnosed by a respiratory physician on the basis of results of whole body plethysmography as well as bronchodilator reversibility testing (in those with airways obstruction) or methacholine challenge (in those without obstruction). The sensitivity and specificity for the symptom of

cough for asthma diagnosis varied from 43-53% and 32-64% respectively in the different healthcare settings.

Finally Lim *et al.* (69) investigated whether five questions on symptoms of asthma as recommended by the GINA guidelines could accurately diagnose asthma, as defined by a positive methacholine challenge test, in a group of 680 subjects. Methacholine challenge testing was positive in 164 patients and negative in 516 and the symptom of nocturnal cough had a sensitivity of 62% and a specificity of 44% for the identification of methacholine test positive patients.

b) COPD

A number of studies have observed significant associations between symptoms of cough and productive cough and a diagnosis of COPD or "airflow limitation". An increased likelihood of COPD diagnosis in subjects with the symptom of dry cough or "cough" (unspecified if productive or not) has been observed in studies by Lamprecht (70), Freeman (71), Hanania (72), Van Schayck (73), Albers (74), Minas (75) and Ohar (76) (See Table 1.2). Increased odds of COPD in subjects specifically with a cough productive of sputum were noted in studies by Lamprecht (70), Medbo (77) and Ohar (76).

Few studies have attempted to assess the predictive value of symptoms for diagnosing COPD. Ohar *et al.* (76) investigated the accuracy of respiratory symptoms to diagnose airflow obstruction in 3955 subjects undergoing work-related medical evaluations. COPD was diagnosed in subjects with airflow obstruction ≥40 years who had a smoking pack year history of ≥20 years. Subjects with cough had increased likelihood of airflow obstruction, and this was further increased in smoking subjects with a cough (n=2917). The sensitivity and specificity of cough for COPD (airflow obstruction and specified smoking history) in the whole population were 69% and 48% respectively and were very similar in the smoking subjects alone at 71% and 44% (Table 1.2).

Murgia *et al.* (78) investigated the sensitivity and specificity of chronic bronchitis symptoms (cough with sputum production for ≥3 months within 1 year for 2 consecutive years) for the diagnosis of COPD (actually airways obstruction as defined by pulmonary function measures) in a sample of 3892 subjects from the general Swedish population. For the whole population chronic bronchitis symptoms were found to have a sensitivity of ~5% and a specificity of ~98% for airways obstruction. These values were similar when groups were sub-classified by gender and smoking status.

The definitions of 'COPD' in both of these studies (76, 78) are questionable and both studies could more accurately be described as assessing the value of symptoms to predict airways obstruction (both of which may include a significant percentage of subjects with asthma) but their findings are summarised here for completeness.

c) Bronchiectasis

Published data for the predictive value of symptoms for diagnosing radiologically significant bronchiectasis is scarce.

It is clear that the symptom of productive cough is present in a high proportion of subjects with bronchiectasis. For example, King *et al.* (42) found that 99/103 (96%) subjects in a cross-sectional study of patients with new diagnoses of bronchiectasis had the symptom of productive cough

A few authors have previously investigated the degree of correlation between the symptom of sputum production/productive cough and the diagnosis and extent of bronchiectasis as demonstrated on HRCT scan. Smith *et al.* (79) studied 40 subjects with chronic sputum production and found the continual production of purulent sputum (as described on clinical history taking) had a significant predictive value for the presence of bronchiectasis. This value is not listed in the paper, but by calculation using a 2x2 table the odds of a subject having bronchiectasis with the symptom of continuous purulent sputum were around 7 times those of subjects who never produced purulent sputum or only did so during exacerbations (odds ratio (OR) 6.9; 95% confidence interval (CI) 1.3-37.2). The calculated sensitivity and specificity of this symptom for bronchiectasis were 55.6% and 84.6% respectively. Lynch *et al.* (80) assessed the relationship between the clinical and HRCT findings of 261 subjects with radiologically diagnosed bronchiectasis. They found a weak but significant positive correlation between daily sputum volume and scores of bronchiectasis severity on CT ($r=0.2$; $p<0.01$) but no correlation between dyspnoea and bronchiectasis score.

Finally, Kamath *et al.* (81) investigated the association between clinical features and a radiological diagnosis of bronchiectasis in 46 subjects with clinical features suggestive of bronchiectasis (results only available as conference abstract). Cough, cough productive of sputum, and haemoptysis were not found to be significant predictors of bronchiectasis (See Table 1.3).

1.3.1.3 Other conditions that cause cough

As well as asthma there are a number of other conditions that can cause chronic cough.

a) Cough variant asthma/atopic cough/eosinophilic bronchitis

These three related conditions have all been described as causes of chronic cough.

Cough variant asthma (CVA) presents as a dry cough without other symptoms of asthma such as dyspnoea or wheeze (82, 83). Diagnosis is based on the clinical history plus demonstration of airways hyperreactivity (84) and eosinophilic inflammation in sputum or broncho-alveolar lavage (BAL) (85). The pathophysiological mechanism for cough in CVA is not fully understood (86).

Eosinophilic bronchitis (EB) describes a group of patients with cough secondary to eosinophilic airway inflammation but with no bronchial reactivity (87). Diagnosis of the condition is made in patients with an appropriate clinical history, negative bronchial challenge and significant sputum eosinophilia (88). EB shares many similar pathological features to asthma but unlike in asthma the airway submucosa does not demonstrate mast cells within airway smooth muscle (89), which may explain why subjects with EB do not have hyperreactive airways like many of those with asthma.

Atopic cough (or eosinophilic tracheobronchitis) was described by Fujimura *et al.* (90) as an isolated chronic cough in atopic individuals with cough hypersensitivity and normal bronchial responsiveness who demonstrate eosinophilic inflammation in sputum (91). These authors have suggested that atopic cough is a distinct entity to CVA and EB with a differing clinical course (92) although this view is controversial (93). Many of the features of atopic cough and EB overlap, although a few differentiating features have been described including a) a lack of eosinophils in BAL fluid of subjects with atopic cough (94), b) a lower risk of progression to asthma in atopic cough (95) than in EB patients and c) a significant treatment response to H1 receptor antagonists in atopic cough patients (94) that is not usually seen in those presenting with EB.

The reported percentage of cases of cough seen in the specialist respiratory clinic owing to asthma or one of these three conditions (which are difficult to separate owing to the definitions used) have varied between 10-35% in the UK and USA and ~50% in Japan (96). EB alone has previously been estimated to account for around 15% of cases of cough referred to secondary care in the UK (97).

b) Gastro oesophageal reflux disease (GORD)

GORD is defined as "symptoms or complications resulting from the reflux of gastric contents into the oesophagus or beyond, into the oral cavity (including larynx) or lung" (98). The condition has a prevalence of 10-30% in both Europe and the USA which is thought to be increasing (99).

Typical symptoms of GORD include heartburn, chest pain and regurgitation but the condition may also cause chronic cough. Several mechanisms for GORD-associated cough have been proposed but the two most commonly accepted of these are the "reflux" and "reflex" theories (100). The *reflux* theory posits that reflux (which may be acidic or non-acidic in pH) that rises above the oesophagus is aspirated into the larynx and pulmonary tree and stimulates cough as a protective mechanism (101). The *reflex* theory states that due to the shared embryological origin of the oesophagus and trachea reflux into the oesophagus can trigger an oesophageobronchial reflex which manifests as cough (102). There is some evidence that cough itself can trigger reflux, which may in turn lead to more cough and the establishment of a so-called "cough-reflux-cough" cycle (102).

Estimates of the contribution of GORD to the overall burden of chronic cough vary greatly. Figures from various studies included in a review of the literature of causes of chronic cough range between 5-40% of cases of cough secondary to GORD in the specialist respiratory clinic in the UK and USA (96). GORD associated cough would appear to account for far fewer cases of chronic cough in Japan (96).

c) Post-nasal drip syndrome (PNDS)

PNDS refers to the drainage of nasal secretions into the pharynx. It is a clinical diagnosis based on patient reported symptoms of a 'drip' sensation at the back of the throat, accompanied by the need for frequent throat clearing and nasal stuffiness or nasal discharge. Other clinical features that support the diagnosis include the presence of nasopharyngeal or oropharyngeal secretions and/or mucosal 'cobblestoning' on

examination, radiological findings and an improvement in cough symptoms with appropriate treatment (103).

The pathophysiological mechanism of cough associated with PNDS was classically thought to be due to stimulation of cough receptors in the larynx from the 'drip' down of secretions from the nose and sinuses (104), although there appears to be little direct evidence to support this. Alternative mechanisms such as increased sensitivity of cough receptors in the upper airway (105) or increased central sensitisation to nasal sensory afferent input (106) may be involved. Owing to the unclear mechanism of cough the 2006 ACCP guidelines recommended the term 'Upper Airway Cough Syndrome' be used in preference to PNDS (103).

The percentage of cases of cough seen in the specialist respiratory clinic caused by PNDS is difficult to determine owing to the lack of pathognomonic features of the condition and different diagnostic criteria and estimates have ranged from 6% to 87% in the UK and USA (96).

1.3.2 Wheeze

A wheeze is a continuous musical sound produced on expiration that is often thought to be a "classic" sign or symptom of asthma although it can result from a spectrum of respiratory disorders that cause airflow obstruction (107, 108). Wheezing results from the passage of air through narrowed or obstructed airways from the larynx down to the small bronchi and is thought to be due to oscillations of opposing walls of the narrowed airway (109). Airway narrowing may occur due to bronchoconstriction which can be a feature of asthma but can also be caused by airway wall oedema as well as extrinsic or intrinsic compression of the airways (110).

The causes of wheeze in children and adults differ greatly. Wheeze is extremely common in children and by the age of six up to 50% will have had at least one episode of wheeze (111). Small children commonly develop wheeze acutely secondary to upper and lower airway infections and sometimes may develop more serious conditions such as laryngotracheobronchitis (croup) or bronchiolitis. Recurrent childhood wheeze is often secondary to asthma, allergy and GORD (112).

In adults, asthma and COPD are two of the most common causes of recurrent wheeze although there are various other extra- (e.g. vocal cord dysfunction) and intra-thoracic (e.g. bronchiectasis, cardiac failure) causes of chronic wheeze (113).

1.3.2.1 Prevalence

The prevalence of wheeze in adults has previously been assessed in three large scale population studies that were used to estimate the prevalence of asthma.

The first of these, the National Health And Nutrition Examination Survey (NHANES) included nearly 19,000 US adults aged ≥20 surveyed between 1988-1994 (114). The mean prevalence of wheeze (defined as any episode of wheeze in the last 12 months), including all age and ethnic groups was 16.4% and this figure was significantly higher (17.7%) in non-Hispanic

whites than in any other ethnic group. In addition to ethnicity, poverty, smoking status, hay fever and obesity were all found to be significantly associated with wheezing.

The European Community Respiratory Health Survey (ECRHS) I included nearly 140,000 adults aged 20-44 surveyed between 1990 and 1993 from 22 different countries including a few non-European countries such as India, USA and New Zealand (115). The median prevalence of wheeze (any episode of wheeze in the last 12 months) was 20.7% and this figure varied greatly between the countries surveyed from 4.1% in India to 32% in Ireland (115). Factors found to be significantly associated with wheeze considering the data as a whole included the use of gas cookers and occupational exposure. Independent analysis of regional data demonstrated significant associations between wheeze and age, allergic sensitisation, smoking status, living in damp dwellings and number of siblings (116).

Finally the RHINE study included nearly 15,000 adults aged 30-54 from Northern Europe surveyed between 1999-2001 who had previously taken part in the first ECRHS survey (117). The prevalence of wheeze (any episode of wheeze in the last 12 months) was 17.3% and wheeze was found to be strongly associated with increased risk of new onset asthma.

1.3.2.2 Specificity as a symptom of airways disease

a) Asthma

The SAPALDIA study reported wheeze (i.e. a "wheezing or whistling in your chest at any time in the last 12 months") to be the most sensitive symptom for diagnosing asthma with a sensitivity of 75% and specificity of 87% (Table 1.1) (64). When subjects were asked additionally if they had experienced shortness of breath in combination with wheeze in the last 12 months the sensitivity was reduced to 65% but with an increase in specificity to 95%. Subjects were also asked if they had experienced wheeze *without* having a cold at the time and this question was also less sensitive (60%) but more specific (94%) than asking about wheeze alone. The authors calculated a measure of diagnostic efficacy which combines the values of sensitivity and specificity (a 'Youden index') for each individual symptom and for combinations of all the different symptoms. Wheeze had the highest Youden index (with 0 being the minimum value and 1 the maximum) of 0.62 of the individual symptoms. The combination of symptoms with the highest Youden index was wheezing in addition to two or more nocturnal symptoms (from cough, chest tightness and dyspnoea) with an index of 0.66.

The study by Sistek *et al.* (65) of adult New Zealanders also found wheeze to be the most sensitive symptom for asthma diagnosis with a sensitivity of 93.9%, although the specificity of wheeze was lower than that determined in the SAPALDIA study at 76.4%. The symptom and combination of symptoms with the highest Youden indices were wheeze alone (0.7) and wheeze plus dyspnoea (0.72).

Tomita *et al.* (118) attempted to derive a 'scoring algorithm' to use for predicting asthma in adult patients by collecting clinical data from 566 adult patients with non-specific respiratory symptoms. Asthma was diagnosed by respiratory physicians on the basis of symptoms and signs with either bronchodilator reversibility and/or bronchial hyperresponsiveness. The only symptom enquired about was wheeze which was found to

have a sensitivity of 30% and a specificity of 87% for asthma diagnosis and this was not used in the final algorithm where instead a "wheeze sound" on examination was used owing to its higher sensitivity (90%) and specificity (95%).

Sensitivities and specificities for wheeze as a diagnostic symptom of asthma in the studies by Choi *et al.* (66), Schleich *et al.* (67), Schneider *et al.* (68), and Lim *et al.* (69) are summarised in Table 1.1.

b) COPD

Wheeze has been demonstrated to have a significant association with a diagnosis of COPD. Odds ratios from studies by Medbo *et al.* (77), Lamprecht *et al*. (70), Hanania *et al.* (72), Van Schayck *et al. (73)*, Kotz *et al.* (119), Minas *et al.* (75) and Vandervoorde *et al.* (120) are listed in Table 1.2. Ohar *et al.* (76) also found a significant association between wheeze and COPD diagnosis and calculated wheeze to have a sensitivity of 68% and specificity of 55% for diagnosing COPD (airflow obstruction).

c) Bronchiectasis

It is unclear how useful the symptom of wheeze is for identifying underlying bronchiectasis although it is likely to have a low predictive value. Previous studies have reported low incidence of reported wheeze as a symptom in populations of bronchiectatic patients. Li *et al.* (121) found that 14/136 subjects had wheeze on presentation, in comparison to 47/136 with cough. Also, King *et al.* (42) noted wheeze *on examination* in only 22 out of 103 (21%) subjects with newly diagnosed bronchiectasis. Kamath *et al.* (81) did not find wheeze to be a significant predictor of radiological bronchiectasis (Table 1.3).

1.3.2.3 Other conditions that cause wheeze

The symptom of wheeze, owing to its underlying pathophysiology, might be expected to have a degree of specificity for airways disease.

However, as mentioned above, some conditions that are not primarily diseases of the lower airways may present as wheeze and be mistaken for cases of airways disease, perhaps most notably vocal cord dysfunction and 'cardiac asthma' (secondary to congestive heart failure) and these will be discussed briefly here.

a) Vocal Cord dysfunction (VCD)

VCD or "paradoxical vocal fold motion" is a syndrome characterised by abnormal vocal cord adduction leading to partial airway obstruction at the level of the larynx (122). The vocal cords may adduct in a paroxysmal fashion during the inspiratory or expiratory phases of the respiratory cycle resulting in symptoms such as inspiratory stridor (often mischaracterised as wheezing), cough and a feeling of tightness in the chest or throat (123).

The three main criteria used to establish a diagnosis of VCD are (1) clinical symptoms e.g. dyspnoea, noisy breathing or stridor (2) visualisation of vocal cord adduction on laryngoscopy and (3) consistent pulmonary function tests. Pulmonary function testing usually reveals normal spirometry with no significant reversibility, normal airway reactivity as assessed by bronchial challenge testing and an abnormal flow volume loop, normally with

a flattened inspiratory limb which can be reproduced with a challenge test such as histamine (124, 125).

The proportion of cases of wheeze (or more correctly, stridor) secondary to VCD is unclear. The condition is frequently misdiagnosed as asthma (126) and the two conditions commonly co-exist with up to 50% of VCD patients also having a diagnosis of asthma based on objective measures (123, 127). This may make it difficult to determine which of these two conditions is the primary cause of a subject's symptoms. Wheeze is a common symptom reported by VCD patients, with a prevalence of 36% (124) and 51% (128) being reported in two previous reviews of the literature.

b) Cardiac asthma

The term "cardiac asthma" has been used to describe airflow obstruction and resultant wheezing secondary to congestive heart failure (129). The observation that airflow obstruction occurs during times of cardiogenic pulmonary oedema was first made over a century ago (130) and various mechanisms including reflex bronchoconstriction due to increased pulmonary vascular pressure (131), airway obstruction due to intraluminal oedema (131) or bronchial mucosal hypertrophy (132) and increased airway hyperresponsiveness (AHR) (133, 134) have been postulated.

Cardiac asthma is prevalent in elderly patients (>65 years old) with congestive heart failure and may be present in up to a third of these cases (135). However, other signs and symptoms of congestive heart failure would usually be present in these patients, making primary airways disease less likely as the cause of wheeze.

1.3.3 Dyspnoea

Dyspnoea has been defined as "a subjective experience of breathing discomfort that consists of qualitatively distinct sensations that vary in intensity" (136).

Three of the most well described of these sensations include "air hunger", increased work or effort of breathing and chest tightness. Accumulating evidence suggests that distinct pathophysiological mechanisms may underlie these sensations, which may be experienced separately or in combination by a subject whose perception of these sensations is influenced by myriad non-sensory factors including emotional state (137) and attention (138).

"Air hunger" or "unsatisfied inspiration" is the perception of not being able to "take in" enough air. This seems to arise when increased ventilatory demand (e.g. due to exercise or when a subject is hypercapnic or hypoxic) cannot be met by a subject's ventilatory capacity (139). This creates an imbalance between the respiratory motor drive of the brainstem (which is relayed to the cerebral cortex via a so-called 'corollary discharge') and the afferent feedback from mechanoreceptors in the lungs, airways and chest wall leading to the development of the unpleasant air hunger sensation (140).

An unpleasant sensation of increased "work" or "effort" of breathing is often reported by patients with obstructive lung diseases or respiratory muscle weakness (141). This sensation has been reproduced in research volunteers by increasing external resistance to breathing

(142), weakening respiratory muscles through fatigue (143) or neuromuscular blockade (144). This unpleasant sensation is probably due in some way to the decreased ability of respiratory muscles to meet ventilatory demand when required (owing to deranged ventilatory mechanics). This phenomenon leads, in turn, to an increased central respiratory motor drive and heightened perception by the subject of increased respiratory effort (142, 145).

The cause of the sensation of 'chest tightness' in subjects with asthma has not been fully explained although this is likely to be related to bronchoconstriction (146). The chest tightness sensation may not result from the increased work of breathing during bronchoconstriction but instead be due to the stimulation of airway receptors such as rapidly adapting receptors (RARs) or C-fibre receptors (147).

1.3.3.1 Prevalence

Prevalence rates of dyspnoea symptoms are difficult to estimate owing to the subjective description of this sensation (148, 149) as well as linguistic differences that may lead to either conflation or separation of dyspnoea or 'shortness of breath' with a chest tightness sensation (150). Also, some subjects may be more likely to perceive they are dyspnoeic in the absence of an organic cause (151) and activity induced dyspnoea may sometimes be mistakenly perceived as pathological when in fact it is appropriate for an individual at a certain level of fitness (152).

Figures from the ECHRS I survey suggested a median prevalence of nocturnal dyspnoea (any episode of being woken by shortness of breath in the last 12 months) of 7.3% with a range of 1.5% in Iceland to 11.4% in Australia. The same survey also found a median prevalence of nocturnal chest tightness (any episode of being woken by chest tightness in the last 12 months) of 13.5% with a range of 6.2% in Italy to 20.5% in Australia (115).

The RHINE study found a prevalence of nocturnal dyspnoea (any episode of being woken by shortness of breath in the last 12 months) of 3.5% with no association between this symptom and increased risk of new onset asthma. The prevalence of nocturnal chest tightness (any episode of being woken by chest tightness in the last 12 months) was 7.3% and there was no association with this symptom and increased risk of new onset asthma (117).

1.3.3.2 Specificity as a symptom of airways disease

a) Asthma

Despite the subjectivity in the definitions of dyspnoea and chest tightness, sensitivity and specificity values for their potential for asthma diagnosis have been determined. However the values obtained for both sensitivity and specificity of dyspnoea and chest tightness as symptoms of airway disease vary markedly from study to study (Table 1.1). Generally the specificity values of dyspnoea at rest from these studies are high for asthma, whereas values for sensitivity and specificity of dyspnoea on exertion are moderately good.

b) COPD

Dyspnoea has been demonstrated to have a significant association with a diagnosis of COPD. Odds ratios from a number of studies are listed in Table 1.2. Ohar *et al.* also found a

significant association between dyspnoea and COPD diagnosis and calculated dyspnoea to have a sensitivity of 75% but a specificity of only 37% for diagnosing COPD.

c) Bronchiectasis

The predictive value of dyspnoea for bronchiectasis is again unclear, although this is likely to be low. King *et al.* (42) found 62/103 (60%) subjects to be dyspnoeic at the time of diagnosis of bronchiectasis. Lynch *et al.* (80) found no correlation between dyspnoea score and extent of bronchiectasis on HRCT, although the study by Smith *et al.* (79) found a weak positive correlation between these two variables.

Kamath *et al.* (81) did not find "breathlessness" to be a useful predictor of radiological bronchiectasis (Table 1.3).

	Sistek et al. (2001)	*Sistek et al. (2006)*	*Choi et al.*	*Schleich et al.*	*Schneider et al.*	*Lim et al.*	*Tomita et al.*
Cough:							
Diurnal							
Sensitivity	21.5	43.1	16	66	43-53		
Specificity	95.2	83.9	42	26	32-64		
Nocturnal							
Sensitivity	49.3	93.9				62	
Specificity	72.3	76.4				45	
Wheeze:							
Diurnal							
Sensitivity	75	65	9	57	52-76	51	30
Specificity	87	95	79	62	34-66	66	87
Nocturnal							
Sensitivity				56			
Specificity				79			
Chest tightness:							
Diurnal							
Sensitivity				73	31-44		
Specificity				60	54-83		
Nocturnal							
Sensitivity	49	20					
Specificity	86	75					
Dyspnoea:							
At rest							
Sensitivity	47.1	43.1	11	73	9-40		
Specificity	94.9	92.9	71	55	78-88		
On exercise					***On walking***		
Sensitivity	69.3	75.4			5-36	70	
Specificity	75.7	76.5			32-93	49	
					With minimal exercise		
Sensitivity					3-32		
Specificity					43-94		
Nocturnal							
Sensitivity	46.2	41.5					
Specificity	96	95.8					

Table 1.1: The sensitivities (%) and specificities (%) of different symptoms of airways disease for the diagnosis of asthma, as reported in different studies

	Lamprecht et al.	*Medbo et al. (a)*	*Freeman et al.*	*Hanania et al. (b)*	*Van Schayck et al. (c)*	*Kotz et al.*	*Albers et al.*	*Minas et al.*	*Vandevoorde et al.*	*Ohar et al. (d)*	*Murgia et al.*
Cough:											
Odds Ratio (95% CI)	**2.3* (1.7-3.1)**	1.1† (0.8-1.5)	**2.4 (1.2-4.7)**	**2 (1.3-3.0)**	1.2		**2.3 (1.1-4.6)**	**2.5 (1.7-3.6)**	NS	**2.0* (1.7-2.3)**	
Sensitivity										69	
Specificity										48	
Cough with sputum:											
Odds Ratio (95% CI)	**2.6* (1.9-3.4)**	**1.6† (1.1-2.4)**			1.3	1.5 (0.7-2.2)	2.2 (0.8-5.9)	NS	NS	**1.7 (1.4-2.1)**	
Sensitivity										56	0.05
Specificity										60	0.98
Wheeze:											
Odds Ratio (95% CI)	**3.4* (2.6-4.5)**	**1.5† (1.2-1.8)**	2.2 (0.9-5.5)	**1.8 (1.1-2.8)**	**1.6 (p<0.001)**	**1.7 (1.1-2.7)**		**1.5 (1-2.3)**	**4.7 (2.1-10.4)**	**1.9 (1.6-2.3)**	
Sensitivity										68	
Specificity										55	
Dyspnoea:											
Odds Ratio (95% CI)	**2.4* (1.9-3.2)**	**1.8† (1.2-2.6)**	**3 (1.5-5.9)**	0.9 (0.5-1.4)	**1.3 (p<0.001)**		0.9 (0.4-1.9)	**2.4 (1.6-3.5)**	NS	**1.2 (1-1.5)**	
Sensitivity										75	
Specificity										37	

Table 1.2: The odds ratios, sensitivities (%) and specificities (%) of different symptoms of airways disease for the diagnosis of COPD (statistically significant odds ratios highlighted in bold)

All OR calculated using multivariate logistic regression except *univariate analysis and †binary logistic regression

COPD case definition unless otherwise noted defined as post-bronchodilator FEV_1/FVC ratio <0.70

(a) COPD case definition: pre BD FEV_1/FVC ratio <0.7 if ≤69 years; pre BD FEV_1/FVC ratio <0.65 if ≥70 years

(b) COPD case definition: pre BD FEV_1/FVC ratio <0.7

(c) COPD case definition: pre BD FEV_1/FVC ratio < lower limit of normal as per ATS-ERS guidelines

(d) COPD case definition: pre BD FEV_1/FVC ratio <0.7 and aged ≥40 years and ≥20 pack year smoking history

	Kamath et al.	*Smith et al.*
Cough:		
Odds Ratio (95% CI)	1.7 (0.3-11)	
Sensitivity	91.3	
Specificity	13.6	
Cough with sputum:		
Odds Ratio (95% CI)	0.32 (0-3.3)	**6.9 (1.3-37.2)**
Sensitivity	87	55.6
Specificity	4.6	84.6
Haemoptysis:		
Odds Ratio (95% CI)	1.2 (0.3-4.3)	
Sensitivity	30.4	
Specificity	72.7	
Wheeze:		
Odds Ratio (95% CI)	2.3 (0.7-7.5)	
Sensitivity	65.2	
Specificity	54.5	
Dyspnoea:		
Odds Ratio (95% CI)	1.31 (0.4-4.5)	
Sensitivity	69.6	
Specificity	36.4	

Table 1.3: The odds ratios, sensitivities (%) and specificities (%) of different symptoms of airways disease for the diagnosis of bronchiectasis

1.3.4 Summary

In summary the predictive value of cough, wheeze and dyspnoea have been evaluated in large population studies.

Evidence suggests that none of these symptoms are particularly sensitive or specific for diagnosing airways disease when used alone. Wheeze is likely to be the most useful symptom in identifying disease with a significant component of airway obstruction such as classical descriptions of asthma or COPD, whilst productive cough is likely to be most useful for diagnosing bronchiectasis. The presence of certain combinations of symptoms that vary in time and intensity may be more specific for an underlying diagnosis of asthma.

Such a lack of discriminatory value for symptoms in diagnosing airways disease is likely due to the fact that multiple types of airways disease and many other conditions not related to the airways cause the same symptoms. Accordingly, although eliciting an accurate description of symptoms will always be an important starting point in the description of airways disease, superior ways of characterising patients disease are required. This process should begin with an understanding of the underlying pathophysiology of airways disease.

1.4 Pathophysiology of Airways Disease

1.4.1 Introduction

There is some controversy in the field of airways disease regarding the extent to which airways disorders can be classified as distinct conditions or whether these should be considered as a spectrum of disease with different pathological components. Key concepts that require definition at this point are the *phenotype* and *endotype* of an individual. The *phenotype* of an individual refers to the observable disease characteristics of that individual resulting from the interaction of its genetic material with the environment. The term *endotype* describes a subtype of a disease defined by a distinct pathological mechanism (153).

Clearly there are many patients who display the 'classical' asthma phenotype with features of allergic asthma starting in childhood, just as there are many of those with the 'classical' COPD phenotypes of smoking-induced neutrophilic chronic bronchitis or emphysema. However, there are also a significant number of individuals who exhibit features classically attributed to both of these conditions (154) or who may additionally display features of disease more commonly associated with chronic infective states such as bronchiectasis (155).

The recognition of different phenotypes of airways disease and the different pathological components that contribute to this disease is a research area of growing interest. This has led to the development of biological therapies targeting specific endotypes. There is much further work to be done to elucidate the distinctive patterns of disease underlying particular phenotypes. Such an understanding should ultimately lead to more targeted therapies required for successful treatment of a spectrum of disease and this will enable clinicians to progress from the current 'one size fits all' approach to the management of airways disease.

1.4.2 Previous definitions and divisions of airways disease components

The characterisation of airways disease as a combination of clearly defined pathological components is not a new concept, having first been proposed in the 1960s as part of the "Dutch Hypothesis" (4) outlined in Section 1.2. This hypothesis emphasised the description of different pathological components in each individual patient on an overall spectrum of "chronic non-specific lung disease".

The separation of different pathological mechanisms into "components" of lung disease is known as a *nominalist* approach to the definition of disease (156), and this approach has been advocated by various authors (5, 157, 158) in contrast to the *essentialist* approach that considers asthma, COPD and bronchiectasis as being distinct disease entities with little or no overlap.

A system devised to characterise airways disease based on the assessment of five relatively independent pathophysiological abnormalities, "the A to E of airways disease" has previously been described (159). The aim of this approach is to provide a framework by which to assess the relative contribution of each of these disease components to an individual patient's disease and use this to guide phenotype-directed treatment. Even more recently a new paradigm based on the identification of phenotypic or endotypic

characteristics ('treatable traits') to guide individualised treatment in airways disease irrespective of a disease label has been proposed (160). It is therefore important to discuss some of the most well characterised pathological components of airways disease and explore the relationships between these different factors. In addition, the relative contributions of these factors to common phenotypes of airways disease and targeted treatments for each of these components is also assessed.

1.4.3 Airway Hyperresponsiveness (AHR)

1.4.3.1 Definition

AHR refers to the exaggerated narrowing of airways through airway smooth muscle mediated bronchoconstriction following exposure to a direct or indirect stimulus (161).

This is detected clinically using bronchial challenge testing, which exposes subjects to a stepwise increase in the dose of a recognised bronchoconstriction stimulus such as methacholine whilst measuring their lung function, as in the so-called methacholine challenge test (MCT). The measure used to quantify AHR is the provocative concentration or dose of the agent that induces a 20% fall in FEV_1 (PC_{20} or PD_{20}). This test will be discussed further in the 'Diagnosis of Asthma' section (Section 1.5.3.4).

1.4.3.2 Affected Phenotypes

AHR is a particularly characteristic feature of the classical asthma syndrome, and is thought to be responsible for the symptoms of short term, sudden onset shortness of breath, wheeze and chest tightness, sometimes in response to a recognised allergen. It has been suggested that the presence of AHR in asthmatic patients may be an independent risk factor for a reduced FEV_1 later in life (162, 163) and fixed airflow obstruction (164).

AHR is also commonly noted in patients with a diagnosis of COPD (165) with up to 50% of COPD patients having some degree of AHR (166). Its presence in patients diagnosed with this condition is associated with an increased risk of mortality (167).

Healthy individuals with no recognised respiratory disease can also demonstrate AHR on bronchial challenge testing as well as smokers and those with conditions including allergic rhinitis and respiratory infections (168). Data from a cohort study suggest that in asymptomatic individuals, AHR is a risk factor for the later development of airways disease (169).

1.4.3.3 Pathological Mechanism

It has been observed that some patients with asthma lack a protective bronchodilator effect that occurs on deep inspiration which is present in healthy individuals (170). The loss of this protective mechanism appears to be critical in the development of AHR (171) and may be due to increased inflammation and mast cell infiltration of airway smooth muscle as noted in a group of asthma patients (172). Mast cell infiltration of the smooth muscle bundle seems to be one of the key pathological changes in AHR (173), and previous work has demonstrated a linear relationship between these two variables (174).

A series of *in vitro* studies have implicated abnormalities in airway smooth muscle structure and function in the development of AHR. These include increased airway smooth muscle

mass (175), which was thought to lead to AHR by generating excessive force when contracting (175, 176). However, more recent work suggests that increased dynamic muscle stiffness (due to the failure of the airway smooth muscle to relax and lengthen during deep inspiration) may be a more important mechanism (177). Other factors that have been proposed to contribute to AHR in asthma include increased vagal tone (178) and increases in free intracellular calcium (179) or activation of the Rho kinase pathway (180) leading to increased smooth muscle contractility.

1.4.3.4 Treatment

a) β-agonists

Short and long acting β_2-agonists are the most effective established treatments for AHR, although β_2-adrenergic receptors may become downregulated with over frequent exposure to inhaled β_2-agonist (181) and lead to patient tolerance to β_2-agonist therapy (182, 183). New ultra-long-acting forms of β_2-agonists (indacaterol) have recently been developed.

b) Long acting anti muscarinic receptor antagonists (LAMAs)

LAMAs have been demonstrated to have a significant and sustained bronchodilator effect in patients with COPD (184) and a modest sustained improvement in bronchodilation using LAMAs has also been observed in subjects with asthma poorly controlled by standard treatment (185). The bronchodilation effect of LAMAs occurs via competition with acetylcholine at muscarinic receptors on airway smooth muscle (186). This established bronchodilator effect of LAMAs might be expected to mitigate against AHR although the direct evidence that LAMAs reduce AHR ("*bronchoprotective*" effect) is mixed as some studies have demonstrated a bronchoprotective effect of LAMAs (187, 188) whilst others have not (189). It has been suggested that LAMAs may exert a bronchoprotective effect via indirect mechanisms (i.e. by effects on the levels of inflammatory mediators) rather than by a direct effect on airway smooth muscle (190). Therefore any future studies aiming to further elucidate the effect of LAMAs on AHR may need to utilise different types of ('indirect') bronchial challenge testing that do not assess bronchoconstriction to direct challenge agents such as methacholine or histamine.

c) Inhaled steroids

It is well established that inhaled steroids improve AHR (191, 192), possibly as early as 3 hours after the first dose (193, 194). This effect is dose dependent (195) and AHR may continue to improve for weeks to months after starting treatment (191, 196, 197) . The improvement is usually at least one doubling dose shift in PC_{20}/PD_{20} with a low-medium dose inhaled corticosteroid (ICS) (<1000 µg BDP equivalent/day) and two doubling dose shifts with high dose ICS (≥1000 µg BDP equivalent/day) (192). The mechanisms by which ICS improve AHR are not fully delineated but these may include a decrease in airway vascular permeability (198) or inhibition of the overexpression and activation of CPI-17 (199), an inhibitor protein which inhibits phosphorylation of myosin phosphatase and ultimately leads to smooth muscle contraction.

d) Anti TNFα agents

TNFα is a mast cell produced mediator that has been strongly linked to the development of AHR making it a promising therapeutic target in refractory asthma (200, 201). Use of the

TNFα blocker etanercept was initially evaluated in a small randomised controlled trial (RCT) conducted by Berry *et al.* (202) which compared this treatment against placebo in a double-blind, crossover pilot study of 10 patients with refractory asthma. Etanercept treatment significantly improved bronchial reactivity, as assessed by MCT, and quality of life scores in refractory asthma patients.

Unfortunately, a multicentre RCT (203) assessing the effect of TNFα blockade using golimumab in 309 patients with severe persistent asthma had to be discontinued 24 weeks into treatment due to the significantly increased risk of serious infection and cases of malignancy seen in the group treated with golimumab. No significant differences were seen in the endpoints of pre-bronchodilator FEV_1 and number of severe exacerbations between the active and placebo groups at the point the trial was stopped. However, post-hoc analysis of the data demonstrated a significantly reduced number of severe exacerbations in a sub-group of patients with significant reversibility (>12% at baseline).

e) Anti TSLP antibodies

TSLP is a cytokine derived from epithelial cells which is an important mediator in allergic inflammatory responses and acts directly on mast cells and eosinophils (204). The expression of TLSP has been demonstrated to be increased in subjects with asthma (205) and a genetic variant of the TSLP gene is associated with increased risk of asthma and AHR (206).

An RCT of 31 subjects with mild allergic asthma demonstrated reduced AHR to specific allergens and to methacholine as well as reductions in indices of airway inflammation following anti TSLP treatment (207). Further evaluation of this intervention in subjects with severe poorly controlled asthma is ongoing (208).

f) Bronchial thermoplasty

Bronchial thermoplasty is a technique that delivers radiofrequency (RF) energy to airway tissue causing heating of the airway tissue. RF energy is delivered via a catheter during bronchoscopy to proximal conducting airways with the intention of heating tissue and reducing airway smooth muscle mass (209, 210). To date, three RCTs of bronchial thermoplasty have been conducted.

The multicentre AIR1 trial (211) compared bronchial thermoplasty (n=56) against usual care (n=56) in asthmatic patients requiring inhaled corticosteroids and long acting β-agonists (LABAs) to control asthma. The intervention group experienced significantly fewer asthma exacerbations 3 and 12 months post treatment, as well as improved morning peak flows, asthma quality of life and asthma control 12 months after treatment. There was no significant difference in lung function or, interestingly, in airway responsiveness following treatment however, and adverse events requiring hospitalisation (including asthma exacerbations and partial left lower lobe collapse in one patient) were more common in the intervention group.

The RISA study (212) evaluated the use of bronchial thermoplasty in severe asthmatics with persistent symptoms despite high dose ICS (>750 µg of fluticasone a day plus LABA ± any other medicines including oral steroids). Fifteen subjects underwent bronchial thermoplasty

in comparison to 17 who had usual care. All subjects were maintained on their usual steroid dose for 16 weeks after the procedure and then investigators attempted to wean inhaled (or oral) steroids. Following thermoplasty, patients had a transient worsening of asthma symptoms and 4 of the 15 patients were hospitalised with 2 having partial collapse of the lobe that had been treated. However, 22 weeks after treatment patients from the intervention group had significantly reduced use of rescue medication and improved pre-bronchodilator FEV_1 and asthma control questionnaire scores. After 52 weeks, patients from the thermoplasty group had significantly reduced their steroid doses in comparison to the control group and maintained the improvements in reduced rescue medication use and asthma control scores.

Finally, the AIR2 study (213) compared thermoplasty (n=190) to a sham treatment (n=101) in asthmatic patients with symptoms refractory to high dose ICS/LABAs. Patients were randomly allocated to the thermoplasty or sham groups and the primary outcome was the difference in Asthma Quality of Life Questionnaire (AQLQ) scores from baseline at 6, 9, and 12 months. The intervention group demonstrated a significantly higher improvement in AQLQ scores as well as showing a significant reduction in severe exacerbation rate (32%), the number of days lost from work/school/daily activities (66%) and the number of A&E visits (84%).

Follow up studies for the AIR, AIR 2 and RISA trials have suggested that the procedure has a good long term safety profile, with no added clinical complications and no significant deterioration in the measured benefits in the thermoplasty groups compared to the control groups up to 5 years after the procedure (214-216).

1.4.3.5 Summary

AHR is a prominent pathological feature of airways disease most commonly found in patients with classical asthma syndrome, although it may be present independently or in conjunction with other conditions. The pathophysiological processes underlying AHR that have been identified to date include abnormalities of airway smooth muscle structure and function and smooth muscle infiltration by mast cells. AHR is usually effectively treated with β-agonist therapy and ICS, but other options are now available for patients with symptoms refractory to β-agonist treatment including anti-muscarinic agents, and in more severe cases, bronchial thermoplasty.

1.4.4 Fixed Airway Obstruction (FAO)

1.4.4.1 Definition

Airway obstruction (or “limitation”) is defined in the 2005 European Respiratory Society (ERS) guidelines as “a disproportionate reduction of maximal airflow from the lung in relation to the maximal volume that can be displaced from the lung” (217). In terms of spirometry the ERS guidelines define this as an FEV_1/VC ratio “below the 5^{th} percentile of the predicted value” (217) whereas GOLD guidelines define this as an FEV_1/FVC ratio <70% (218). Hence, fixed airway obstruction (FAO) may be defined at its simplest as airway obstruction that does not significantly improve in response to bronchodilators – i.e. the FEV_1

fails to improve by ≥200 mL and ≥12% from baseline (assuming that the baseline FEV_1 is sufficiently low to allow improvement by these parameters, i.e. <~80%).

FAO has also previously been defined by a post bronchodilator FEV_1/FVC ratio <70% on ≥2 occasions (31, 219, 220) although this value has not been universally agreed (221).

1.4.4.2 Affected Phenotypes

The demonstration of FAO is necessary for the diagnosis of COPD and the *fixed* nature of this obstruction (i.e. non-significant reversibility of the airways to bronchodilators) traditionally has been the distinguishing diagnostic feature between COPD and asthma, with the airways in the latter condition classically demonstrating bronchodilator reversibility. However, as mentioned above, a significant proportion of COPD patients demonstrate AHR and some clinical trials have reported up to 50% of subjects with diagnosed COPD had significant reversibility as per American Thoracic Society (ATS) guidelines (222, 223).

FAO also affects a significant proportion of asthmatic patients and seems to be particularly prevalent in subjects with severe or difficult to treat asthma. Long term follow up studies of asthmatic subjects with reversible airflow limitation suggest that around 16-26% of patients eventually develop FAO (220, 224). Studies specifically assessing the characteristics of severe or difficult to treat asthmatics by contrast report prevalence rates of FAO of 50-60% (219, 221).

The 'Asthma-COPD overlap syndrome' (ACOS) is a term used to describe subjects with overlapping diagnoses of asthma and COPD i.e. symptoms relating to increased airflow variability *and* airflow obstruction that is incompletely reversible (10). ACOS is recognised not as a single condition but rather a combination of two or more obstructive airways syndromes such as "asthma", "emphysema" or "chronic bronchitis" (225, 226). Analyses of the prevalence of separate and combined obstructive airways syndromes in large scale population studies estimate 13-19% of subjects with obstructive lung disease in the UK and USA have more than one type of obstructive lung disease (154, 227). Marsh *et al.* (228) categorised around 10% of 469 patients in a study to classify the relative proportions of phenotypes of COPD with ACOS. Patients labelled as having ACOS may experience an increased frequency of more severe exacerbations of airways disease than those who have been diagnosed with either of these conditions in isolation (227, 229).

The presence of FAO in any of the phenotypes described above is a predictor of increased morbidity, including increased decline in FEV_1 and increased frequency of exacerbations (229), as well as mortality (230, 231).

1.4.4.3 Pathological mechanism

Fixed airflow obstruction is thought to result from the pathophysiological process known as airway remodelling. Airway remodelling refers to structural changes observed in the large and small airways of subjects with airway disease including asthma and COPD (232, 233).

Biopsies from patients diagnosed with COPD have revealed many structural changes in the remodelled large and small airways such as increased epithelial thickness and epithelial metaplasia. In addition, changes in the extracellular matrix (ECM) of the airway wall

including fibrosis and goblet cell hyperplasia, and increased thickness of airway smooth muscle (234) are also apparent. Changes of remodelling observed in the small airways and lung parenchyma of subjects with COPD include emphysema with loss of alveolar attachments and collagen deposition (235) and peribronchial fibrosis (236).

Similar pathological features have been noted in the remodelled airways of asthmatics including airway wall thickening, epithelial cell proliferation with increased epithelial cell shedding, subepithelial fibrosis, goblet cell hyperplasia, increased airway smooth muscle mass and bronchial neovascularisation (237, 238).

Despite there being considerable overlap in many of these pathophysiological processes between subjects diagnosed with asthma and COPD, there are also some notable differences.

Firstly, although airway remodelling occurs in both conditions throughout the bronchial tree, there is a higher burden of structural changes associated with COPD in the small airways (236) and lung parenchyma than in asthma. In certain phenotypes of COPD, including alpha 1-antitrypsin deficiency, destruction of the alveolar walls (i.e. emphysematous change) may develop which is not typically a feature of asthma (239). Secondly, airway wall thickening may be less pronounced in COPD (239-241) possibly due to a lesser degree of airway smooth muscle thickening (242), although some studies have shown no difference (243). Thickening of the basement membrane has been well described in asthma and comparative studies assessing basement membrane thickness in bronchial biopsies from asthma and COPD patients have concluded this feature is more prominent in bronchial cells from asthmatic subjects (244, 245). Finally, bronchial neovascularisation/angiogenesis may be a prominent feature of the asthmatic airway (246), particularly in those with severe steroid-dependent asthma (247), whereas this is not a significant finding in COPD (248).

1.4.4.4 Treatment

As the pathogenesis of airway remodelling is not well understood this has understandably hindered the development of therapeutic agents specifically targeted against this process. Despite this, a number of (mostly *in vitro*) studies have sought to assess the effects of conventional asthma therapies on remodelling. The most promising results so far using ICS and bronchial thermoplasty will now be discussed.

a) Inhaled corticosteroids (ICS)

ICS are the most well studied asthma treatment with regards to their effects on airway remodelling. Asthmatic patients with FAO have been demonstrated to show no improvement to LABA monotherapy in terms of lung function and asthma control but may still respond to ICS treatment in terms of these measures (249).

In vitro studies have suggested ICS may reduce airway smooth muscle hyperplasia (250) and improve vascular remodelling (251). A number of clinical investigations have also shown improvements in various parameters related to remodelling with ICS treatment including a reduction in basement membrane thickness (252, 253), epithelial remodelling (254) and

vascular remodelling (255) although these improvements have not been observed in all studies (256, 257).

b) Bronchial thermoplasty

Bronchial thermoplasty (Section 1.4.3.4) has been proposed as a therapy to target airway remodelling owing to its purported mechanism of action in reducing airway smooth muscle (ASM) mass. The evidence of its effect on ASM comes from canine proof of concept studies (209) and a safety study in nine human lung cancer patients (without asthma). Data derived from the human safety study where RF energy was applied to lung segments/lobes which were later resected, indicated that on average a 50% reduction in ASM mass occurred (258).

There are suggestions from a limited case series of 3 patients that bronchial thermoplasty reduces ASM mass in the asthmatic airways (259), and this may be the mechanism leading to an observed clinical benefit. However, this approach requires further evaluation in future clinical trials.

1.4.4.5 Summary

FAO secondary to airway remodelling is undoubtedly an important pathological mechanism in subjects with COPD, severe or difficult to treat asthma and ACOS which leads to increased morbidity and mortality in these groups. Certain pathological features of remodelling have been observed in the airways but the pathogenesis and natural history of this process are not fully understood. This may have led to an under-appreciation of airway remodelling as a significant factor in the phenotyping of airways disease and much further work needs to be done to characterise this phenomenon and develop effective treatment to prevent FAO.

1.4.5 Airway Inflammation

Airway inflammation (bronchitis) is a cardinal feature of airway disease. The importance of recognising the heterogeneity of types of airway inflammation and their underlying pathophysiological mechanisms has only been established relatively recently, in parallel with the introduction and standardisation of investigations that are able to measure and classify types of airways inflammation.

Through the use of one of these techniques, the microscopy and differential cell count of induced sputum, four main subtypes of airway inflammation have been recognised and these are (1) eosinophilic, (2) neutrophilic, (3) mixed granulocytic and (4) paucigranulocytic (260).

Both eosinophilic and neutrophilic airway inflammation will be discussed in further detail below. The mixed granulocytic and paucigranulocytic inflammatory subtypes are of uncertain significance, although it has been suggested that subjects with a mixed granulocytic pattern may represent a ‘transitional’ phenotype between neutrophilic and eosinophilic subtypes or vice versa (261). Patients displaying paucigranulocytic inflammation seem to have relatively normal lung function (262) and display similar gene expression at the RNA level to healthy controls (261), which may suggest this is consistent with a mild inflammatory airways disease phenotype.

1.4.6 Eosinophilic Inflammation

1.4.6.1 Definition

The eosinophilic inflammatory subtype is the most clearly defined with a standard definition of >3% eosinophils/total sputum cell count (263).

1.4.6.2 Affected Phenotypes

Ongoing eosinophilic inflammation is a notable feature of airway disease. Eosinophilic asthma is the best studied asthma 'phenotype' and it is estimated that between 40-50% of people with an asthma diagnosis have underlying eosinophilic airways inflammation (264, 265).

The stability and/or reproducibility of eosinophilic inflammation over time seems to vary with different severities of airways disease. Sputum eosinophilia seems to be persistent in certain sub-phenotypes of asthma with reproducible sputum eosinophil counts obtained over a 5 year period in a cohort of adult patients with severe asthma despite ICS treatment (266). McGrath *et al.* (267) studied a group of 157 patients with mild to moderate asthma and found 35 (22%) to have a persistent eosinophilia whilst not using ICS treatment, whilst 49 (31%) had an intermittent eosinophilia and 73 (47%) were persistently non-eosinophilic. In a comparable group of 167 patients with mild asthma on ICS treatment 12 (7%) were persistently eosinophilic, 34 (20%) had an intermittent eosinophilia and 121 (72%) were non-eosinophilic. This study demonstrates one of the difficulties of determining the underlying type of inflammation, namely that steroid treatment tends to suppress eosinophilic inflammation and may prolong neutrophil survival (268). Sputum inflammatory phenotypes do not seem to be stable in children with asthma. Fleming *et al.* (269) found that of 59 children (42 with severe asthma and 17 with mild to moderate asthma) who had sputum samples processed for differential cell counts every 3 months for a year, 63% displayed 2 or more inflammatory subtypes during this period.

Some investigations have suggested that the degree of eosinophilic inflammation may directly correlate with the severity of disease in patients with an asthma diagnosis (221, 270-273) although others have found no evidence for this (274-276). Other investigators have suggested that it is not asthma severity but asthma control that correlates with the degree of eosinophilic inflammation, with worse control being associated with higher levels of inflammation (277-280). A number of more recent studies in which investigators have attempted to identify sub-groups of asthmatic patients with similar phenotypic characteristics using statistical methods such as cluster analysis have characterised several clusters (groups with shared phenotypic characteristics) of patients exhibiting eosinophilic inflammation with varying severities of disease, and this may explain some of the discrepancies found in previous investigations (281, 282).

Eosinophilic inflammation has also been demonstrated in 20-40% of patients with a diagnosis of COPD (283). The relationship between lung function and the degree of eosinophilic inflammation in COPD is unclear. Lams *et al.* (284) demonstrated a significant negative correlation between FEV_1 and the ratio of activated eosinophils to total eosinophils in endobronchial biopsy samples taken from COPD patients and Balzano *et al.* (285) similarly

found a negative correlation between FEV_1 and sputum eosinophil count/eosinophilic cationic protein (ECP) levels. In contrast, Hogg *et al.* (236) found no significant correlation between COPD severity as graded by GOLD score, and level of small airway eosinophilic inflammation.

The diagnostic term 'eosinophilic bronchitis' is generally used to describe a disease state in which eosinophilic inflammation of the airways is the sole pathological feature, without AHR and variable airflow obstruction that might be seen in a classical asthma phenotype (88). EB typically presents as a chronic cough, and although the incidence and prevalence of the condition are unclear, it is thought to be responsible for between 10-30% of cases of chronic cough referred to the specialist respiratory clinic (286).

1.4.6.3 Pathological mechanism

Eosinophilic inflammation has been well studied in asthma, and classically associated with allergic sensitisation and a Th2 cell-dependent, IgE mediated inflammatory response.

The current understanding regarding the immunopathology of eosinophilic inflammation is summarised diagrammatically in Fig 1.1.

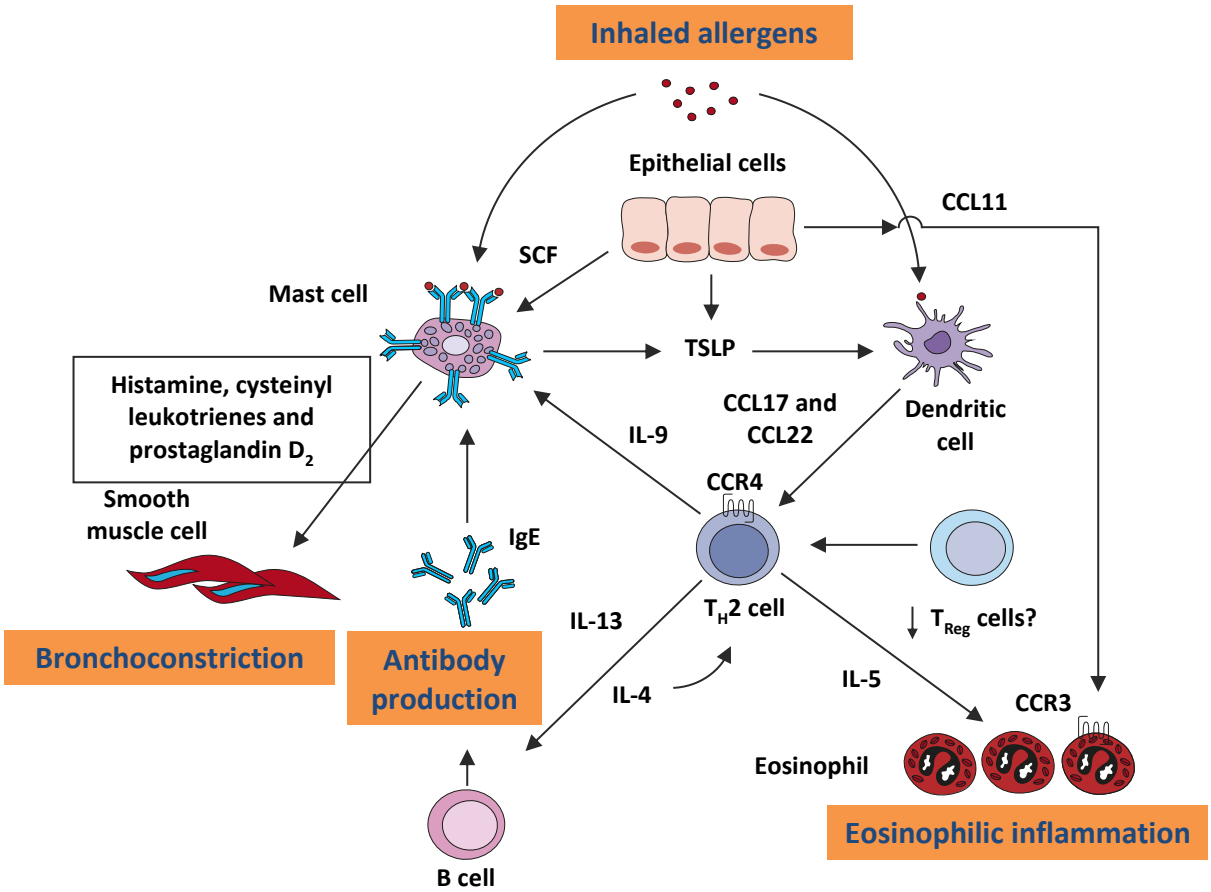


Figure 1.1: Immunopathology of eosinophilic inflammation in asthma (adapted from Barnes P. (287))

Th2 mediated asthma with eosinophilic inflammation can also be triggered by other non-allergenic stimuli such as viral infection and air pollution (288). This may be due to cytokines released from the bronchial epithelium in response to these insults, such as TSLP release in response to viral infections (289) or release of cytokines including IL-8 and GM-CSF in response to airway pollutants (290).

a) Eosinophils

Eosinophils are a key effector cell in the inflammatory response noted in eosinophilic asthma and release a variety of substances including:

- Pre-stored cytotoxic proteins including eosinophil cationic protein, eosinophil peroxidase and major basic protein, all of which may play a role in the epithelial damage observed in the airways of asthmatics (291).
- Th1 and Th2 cytokines and chemokines which contribute to the maintenance of ongoing inflammation (292).
- Fibrogenic cytokines including TGF-β which may contribute to sub-epithelial fibrosis/airway remodelling (293).

Eosinophils can also directly regulate the inflammatory response by influencing Th1 and Th2 cytokine generation from T cells (294) and the pulmonary dendritic cell response to allergen exposure, promoting a Th2 dominated immune response and suppressing Th1/Th17 responses (295).

1.4.6.4 Treatment

a) Corticosteroids

There is substantial evidence that sputum eosinophilia is a strong predictor of response to steroid therapy (268, 296, 297). Various studies have attempted to titrate corticosteroid treatment depending on the degree of eosinophilic inflammation present, as assessed by induced sputum differential cell count or exhaled nitric oxide assessment. A meta-analysis studying the titration of treatment based on sputum eosinophil count based on three studies conducted in patients with asthma concluded that this strategy would be an effective way of minimising asthma exacerbations without a net increase in the dose of ICS (298). A similar study involving patients with COPD (299) found that a sputum based strategy significantly reduced the number of severe exacerbations in the sputum group compared to the control group. A meta-analysis of 6 RCTs including adults, adolescents and children investigating the titration of steroid treatment based on exhaled nitric oxide monitoring (298), which has a positive correlation with sputum eosinophilia (300), failed to show any significant improvement in the exacerbation rate using this approach.

A sub-phenotype of patients with severe refractory asthma has been identified with a characteristically persistent eosinophilia despite standard high dose inhaled steroid treatment (281). This group of patients typically have few daily symptoms, develop features of airways disease later in life (281), and may also have problems with rhinosinusitis (301) or aspirin sensitivity (302). The persistent eosinophilia is associated with persistent airflow limitation (221), an increased rate of asthma exacerbations (303) and a dependence on oral corticosteroid therapy (304). It has been hypothesised that this group of patients may have

a greater degree of small airway inflammation (305), which would explain their relative insensitivity to treatment with standard ICS therapy and need for long courses of systemic steroid treatment to improve their asthma control. Small particle inhaled steroids may be of some benefit in treating patients in this sub-phenotypic group due to their theorised greater penetration into the small airways. Small particle ICS have been shown to reduce the number of eosinophils in small airway biopsies (306) and suppress sputum eosinophil counts in subjects with refractory asthma (307) as well as reducing the number of asthma exacerbations in patients with severe persistent asthma (308). However, many of these patients still require long term systemic steroid treatment and are at risk of the significant side effects of this treatment (309).

Attempts have been made to develop more targeted therapies for refractory asthma, which is frequently associated with ongoing eosinophilic inflammation.

b) Anti IgE

The first biological therapy licensed for asthma treatment in the UK is the recombinant humanised monoclonal anti-IgE antibody omalizumab. Omalizumab attenuates the early and late phase allergic responses to allergen by inhibiting the binding of IgE to the high-affinity IgE receptor (FcεRI) on the surface of mast cells and basophils, resulting in down-regulation of IgE receptors and inhibition of inflammatory mediator release (310).

Although serum IgE levels do not correlate with levels of tissue eosinophils treatment with omalizumab has been shown to reduce airway and blood eosinophil counts (311). This may be because the down-regulation of the FcεRI receptor on basophils and mast cells limits allergic IgE-mediated responses and prevents Th2 cytokine release and eosinophilic airway infiltration (312).

Meta-analyses of multiple clinical trials have confirmed the therapeutic efficacy of omalizumab as an additional treatment to corticosteroids and LABA therapy in patients with severe persistent allergic asthma (313). Data from these trials revealed that baseline total IgE was the only baseline predictor of treatment efficacy, but that there were treatment benefits regardless of IgE levels (314).

c) Anti IL-5

Owing to the importance of the cytokine IL-5 in increasing eosinophil production, recruitment and survival in eosinophilic asthma this is a rational target for therapy in this condition. Animal studies showed significantly reduced levels of airway eosinophils and AHR in response to allergen following anti-IL-5 treatment (315).

Initial clinical trials in humans of anti-IL-5 treatment failed to replicate these findings and despite reducing serum eosinophil counts showed no significant effect on AHR or clinical benefit (316-318). However, these studies did not specify an asthmatic patient cohort with eosinophilic asthma, and the fact that they were undertaken with an unselected asthma cohort may have contributed to their failure to demonstrate any obvious clinical improvement. Later trials that selected patient cohorts with refractory eosinophilic asthma showed improvements in clinical measures with anti-IL-5 treatment (mepolizumab). These included a significant reduction in asthma exacerbations, improved symptom scores and

quality of life and improved FEV_1 (319, 320). Mepolizumab has now been approved by NICE as a treatment for subjects with severe refractory eosinophilic asthma in the UK (321). Further anti-IL5 therapies including reslizumab (322) and benralizumab (323) have also been demonstrated to significantly reduce asthma exacerbation frequency in subjects with severe uncontrolled eosinophilic asthma and are likely to be approved in the near future.

d) Anti IL-13

Another important cytokine in Th2 mediated asthma with a key role in eosinophil recruitment into airways is IL-13 (324). Corren *et al.* (325) conducted a clinical trial of the anti-IL-13 treatment lebrikizumab in 219 adult asthma patients with uncontrolled asthma refractory to corticosteroids and LABA therapy. There was a significant improvement in FEV_1 from baseline in the treatment arm compared to placebo, which was more significant in subjects with high levels of ongoing Th2 inflammation at baseline (as defined by high serum periostin levels). Phase 3 studies however did not consistently show a significant reduction in asthma exacerbations in Th2 biomarker-high patients with lebrikizumab (326).

e) Anti IL-4

IL-4 is an important mediator of allergic asthma, and is responsible for many of the key pathophysiological features of this condition. These include the differentiation of CD4+ T cells into effector Th2 cells, isotype class switching of B cells to produce IgE in the allergen sensitisation stage (327), and promoting goblet cell hyperplasia and mucus production in the early allergic response (328).

IL-4 and IL-13 signal through different receptors, but both receptors share the α subunit of the IL-4 receptor (IL-4Rα) (327). Several anti-IL-4 agents have been investigated in clinical trials thus far. The IL-4 monoclonal antibody pascolizumab was well tolerated in Phase I trials, but failed to show any significant clinical benefit in a Phase II study and further development was discontinued (329). The recombinant human IL-4Rα antagonist altrakincept (330) and the IL-4/IL-13 cytokine heterodimeric receptor antagonist pitinkinra (331) showed some modest benefit in clinical measures in Phase I/II clinical trials but both agents have also been discontinued.

Wenzel *et al.* (332) conducted a double blind placebo-controlled trial of dupilumab, a humanized monoclonal antibody to the IL-4Rα subunit in 104 patients with moderate to severe persistent asthma and eosinophilia. Subjects in the treatment group showed a significant improvement in lung function and a reduced number of exacerbations in comparison to the placebo group after withdrawal of ICS and LABA therapy. Dupilumab also decreased levels of Th2 associated biomarkers from baseline including FE_{NO} and IgE levels. A further large scale RCT (n=769) in patients with uncontrolled persistent asthma also showed significant improvements in lung function and severe exacerbation rate with Dupilumab irrespective of baseline eosinophil count (333). Phase 3 trials are ongoing.

1.4.6.5 Summary

Eosinophilic inflammation is a well-defined, relatively well-characterised process which is a pathological feature of several different airway diseases. It is usually associated with allergic asthma but there appear to be different phenotypes of asthma of which eosinophilic

inflammation is a feature. Eosinophilic inflammation normally responds well to steroids and monitoring sputum eosinophil counts in patients with eosinophilic asthma and COPD has proven to be a successful strategy in titrating steroid doses. However there is a sub-group of patients with severe asthma who may exhibit persistent eosinophilic inflammation refractory to high dose corticosteroid treatment. Newly developed biological agents targeting the chemical mediators of Th2 inflammation are being developed and these may prove effective in subjects with corticosteroid resistant asthma and/or corticosteroid resistant eosinophilic inflammation as part of other airway conditions.

1.4.7 Neutrophilic Inflammation

1.4.7.1 Definition

Diagnostic criteria for neutrophilic inflammation in sputum is less well defined due to a greater variability and an observed increase in differential neutrophil count seen with age (334), although this has previously been defined as either >61% neutrophils/total sputum cell count based on the 95th percentile value in a healthy population (260) or >77.7% based on +2 standard deviations from a healthy population mean (335).

1.4.7.2 Affected Phenotypes

Neutrophilic airways inflammation is well recognised in patients diagnosed with COPD. Elevated neutrophil counts have been detected in sputum and BAL samples from subjects with stable COPD (336) and these have been found to directly correlate with the degree of airflow obstruction (337) and air trapping (338).

Neutrophilic inflammation is also one of the main pathological features of bronchiectasis, in which it is thought to be an ongoing response to bacterial colonisation of the airways. Sputum neutrophilia in bronchiectasis may be particularly prominent, with a percentage of neutrophils of the overall cell count in BAL or sputum of up to 90% (339, 340). Patients with the highest bacterial load in the airways tend to have higher proportions of neutrophils in sputum cell counts (340).

A significant sub-group of 20-30% of patients with diagnosed asthma also have underlying neutrophilic inflammation (260, 296). This is more frequent in older patients (281), obese women (341), smokers (342) and in more severe disease (343, 344), and has also been reported as a significant autopsy finding in cases of fatal asthma (345). The degree of neutrophilia inversely correlates with lung function (346, 347) and has also been demonstrated to relate to the degree of gas trapping (346, 348). The significance of an increased sputum neutrophil count in patients with asthma has however been questioned, owing to the potential confounding effect of steroid treatment in reducing sputum eosinophils and possibly increasing sputum neutrophils (268). However, a neutrophilic inflammatory pattern has been observed in steroid naïve asthmatic individuals (267, 296) as well as asthmatic individuals who have had steroids withdrawn (268), suggesting this is a distinct phenotype.

The stability of the neutrophilic asthma phenotype seems unclear. Studies including those by Green *et al.* (303) and Simpson *et al.* (260) suggest that ‘non-eosinophilic asthma’ is a stable subtype over a period of 1-5 years. This term however includes any patient not

meeting the criteria for eosinophilic asthma (>3% eosinophils in total sputum count) and as such incorporates those with mixed granulocytic and paucigranulocytic inflammatory subtypes as described above as well as individuals displaying a neutrophilic phenotype. Al-Samri *et al.* (349) found a large amount of variability in sputum inflammatory cell types from 61 patients with moderate and severe asthma on corticosteroid therapy over the course of 1 year, with stable phenotypes found in only a third of subjects. A more recent investigation using cluster analysis profiling techniques to try and delineate different phenotypic asthma groups found that sputum inflammatory cell counts are a less stable feature to try and sub-classify disease than physiological variables including lung function, reversibility and age of onset of disease (350).

1.4.7.3 Pathological Mechanism

Current evidence seems to suggest that neutrophilic inflammation may initially occur due to alterations in the innate immune response. The innate immune response is a rapid reaction by elements of the immune system to certain highly conserved structures common to whole classes of micro-organisms known as pathogen associated molecular patterns (PAMPs). PAMPs are quickly recognised by pattern recognition receptors such as toll-like receptors (TLRs) which are expressed by a variety of cells. These include dendritic cells and macrophages, and once recognition has occurred these cells are activated immediately to respond to the detection of a pathogen, mostly through the release of cytokines (351).

In patients with neutrophilic asthma and bronchiectasis increased expression of TLR2, the pro-inflammatory cytokines IL-8 and IL-1β and increased levels of endotoxins have been noted in comparison to patients with eosinophilic inflammation or healthy controls (339). In further support of the role of TLR2 in the development of neutrophilic inflammation Buckland *et al.* (352) observed in a murine model of allergic bronchopulmonary aspergillosis (ABPA) that TLR2 deficiency resulted in decreased levels of airway inflammation, AHR and mucous metaplasia. Increased expression of TLR2 may be secondary to the prolonged presence of bacterial products, pro-inflammatory cytokines or the use of corticosteroids (353). This suggests that corticosteroid use, which is effective at reducing ongoing eosinophilic inflammation, could potentially worsen neutrophilic inflammation (354).

Work by Simpson *et al.* (355) proposed that activation of TLR2 by any of these factors could lead to activation of what the authors describe as the 'Neutrophil Activation Cycle'. This model comprises of positive feedback interactions between three inflammatory mediators, the levels of which are known to be increased in patients with ongoing neutrophilic inflammation, namely IL-8 (CXCL8) (339, 356), and the released neutrophil proteases neutrophil elastase (NE) and matrix metalloproteinase (MMP) -9 (340, 357, 358). These studies suggest that amplification of the original inflammatory response through this cycle may contribute to persistence of bacteria in the airways through the mechanisms of mucus hypersecretion and impaired bacterial phagocytosis. The continuing colonisation of bacteria in the airways only serves to further increase expression of TLR2, resulting in further activation of the Neutrophil Activation Cycle.

Evidence is increasing that Th17 cells, a subset of T helper cells that produce the cytokine IL-17, are also involved in the development of neutrophilic inflammation in asthma (359, 360)

which is resistant to corticosteroid treatment (361). Increased IL-17 levels in sputum from asthmatic patients significantly correlate with sputum neutrophil counts (362) and increased IL-17 expression has been noted in BAL and bronchial biopsies from patients with asthma (363, 364) and COPD (365, 366).

1.4.7.4 Treatment

a) Macrolides

One of the most well studied treatments for neutrophilic inflammation are macrolide antibiotics, which have been shown to significantly improve outcomes in conditions with a component of neutrophilic inflammation including diffuse panbronchiolitis (DPB), COPD, cystic fibrosis (CF) and bronchiectasis (367). Further detail on the trials investigating the use of macrolides to date in airways disease and their postulated mechanisms of action can be found elsewhere (Section 3.2.2).

b) Monoclonal antibodies

Clinical trials of anti CXCL8 and anti CXCR2 (a CXCL8 receptor) therapy have been carried out in patients with COPD. Anti-CXCL8 treatment was demonstrated to be safe and also improved dyspnoea scores in a group of COPD patients over a 3 month period (368). However this made no significant difference to lung function, health scores or 6 minute walking distance. Two separate CXCR2 antagonists have been demonstrated in proof of principle clinical trials to reduce levels of blood (369) and sputum (370) neutrophils in COPD patients, but neither of these resulted in improvement of any clinical measures.

c) Other medications

Other potential agents under investigation for the treatment of neutrophilic inflammation include anti-TNFα therapy (371, 372), statins (373), theophylline (374) and anti-IL-17 monoclonal antibodies (375), but evidence for the significant efficacy of any of these interventions is currently lacking.

1.4.7.5 Summary

Neutrophilic inflammation is an important pathophysiological process in a number of airway diseases. It is less well defined and characterised than eosinophilic inflammation, but elements of its pathobiology are becoming better understood, including the importance of alterations in innate immune mechanisms, the proposed ‘neutrophil activation cycle’ and the role of Th17 cells in its development. Macrolide antibiotics are proving to be an effective treatment in many conditions with underlying neutrophilic inflammation, and other treatment options are currently being investigated.

1.4.8 Summary

Instead of using diagnostic labels airways diseases may instead be described as a combination of relatively independent pathophysiological components. Relative levels of ‘contribution’ of these components in combination with extra-pulmonary pathologies reviewed elsewhere (157) may contribute to the broad spectrum of resultant clinical phenotypes of airways disease. Information derived from quantitative measurement of these pathological components can be used to target treatment specifically against these pathological processes and has been demonstrated to improve patient outcomes.

Further clarification of the relationships between these components is required and a number of investigators are now attempting to define common phenotypes or ‘clusters’ of disease based on the relative contributions of these pathophysiological processes (362).

1.5 The utility of exhaled nitric oxide in patients with suspected asthma

1.5.1 Introduction

As discussed in the previous chapter, asthma is one of the most common chronic diseases in the UK. It was shown there that untreated asthma is associated with a high morbidity and mortality. There is an appreciation that the majority of asthma exacerbations and deaths due to asthma can be avoided with timely recognition. It is therefore essential that the condition is recognised early in order to institute treatment promptly (25).

However, making a diagnosis of "asthma" may be difficult. This arises partly from the imprecise meaning of the term asthma itself and also the non-specific nature of respiratory symptoms as previously discussed.

The importance in recognising and treating asthma combined with the difficulties in making a diagnosis appear to have led to an "over-diagnosis" of asthma and the evidence for this will be discussed below.

Next, the investigations used in patients with suspected asthma which measure different components of the condition such as airflow obstruction, airway inflammation and AHR will be reviewed. Particular emphasis is placed on bronchial challenge testing (a measure of AHR) and exhaled nitric oxide measurement (a measure of airway inflammation).

Guidelines for the diagnosis of asthma have been issued by various organisations to attempt to guide physicians in this subject and the most recent of these are critically appraised.

Finally, a proposed alternative use of exhaled nitric oxide measurement allowing the prediction of a response to a specific treatment (inhaled steroids), rather than attempting to diagnose asthma, will be discussed before outlining the specific aims and objectives of this study.

1.5.2 Over-diagnosis of asthma

With the realisation that asthma morbidity and mortality rates were unacceptably high between the 1980s and early 2000s (376, 377) there were well founded concerns regarding the under-diagnosis of asthma (378-380). An increasing awareness of the condition may have led to much higher rates of diagnosis (14, 381). However, with the current emphasis now being on not 'missing' a diagnosis of asthma and with many diagnoses of asthma being made on clinical assessment alone, which as demonstrated in the previous chapter is unreliable, more recent data suggests that asthma is now over-diagnosed in the community.

LindenSmith *et al.* (382) studied a group of 90 adult asthmatics with physician diagnosed asthma to determine the proportion of these subjects who met the Canadian Thoracic Society (CTS) guidelines for asthma. After subject details were taken and clinical history and a symptom questionnaire were carried out, each of these subjects underwent spirometry with reversibility. Those who did not demonstrate reversibility were asked to keep a 14 day peak flow diary with all subjects undergoing MCT. Asthma was diagnosed in any subject with a suggestive clinical history **and** either significant reversibility, peak flow variability of ≥20%

over a 7 day period or AHR with a PC_{20} of ≤8 mg/mL of methacholine. Of the 90 patients who completed the study only 53 patients (59%) met the CTS guidelines for a diagnosis with asthma. In the group of subjects who were found not to have objective evidence of asthma 23 (62%) were taking medications for asthma. Approximately half of the entire study group had never had any formal pulmonary function testing.

McGrath *et al.* (383) demonstrated in a study of 304 subjects recruited from the community with physician diagnosed asthma that 83 of these (27%) had a negative MCT, and hence a low likelihood of asthma. The subjects with negative MCTs typically had normal lung function, an adult onset of symptoms and no history of exacerbation requiring oral steroids, which in combination with their negative MCTs makes it likely that the majority of these individuals did not have asthma.

Aaron *et al.* (384) conducted a study of 496 individuals (242 obese and 254 non-obese) with physician diagnosed asthma to determine how many had objective evidence of asthma. After clinical histories and symptom/quality of life questionnaires were carried out, spirometry and reversibility tests were performed. Patients with no significant reversibility underwent MCT. Any subject with a negative MCT who was taking inhaled steroids had the dose of these halved and any anti-leukotriene therapy was stopped. A repeat MCT was carried out on the latter subjects 2-3 weeks later. Any of those subjects who had a second negative MCT had all ICS and LABA stopped before undergoing a third MCT 2-3 weeks later. If this third and last MCT was negative all asthma medications were stopped and the subject was followed up 6 months later. Overall asthma was excluded in 77/242 (32%) of obese patients and 73/254 (29%) of non-obese patients with no significant difference in over-diagnosis of asthma between the two groups. Of these 150 patients, 98 (65%) did not require the use of asthma medications or require medical care due to asthma symptoms over a 6 month period. Despite this study finding no difference in the rate of over-diagnosis of asthma between obese and non-obese individuals, epidemiological data suggests that obese subjects are around twice as likely to be diagnosed with asthma as non-obese individuals (385).

Van Huisstede *et al.* (386) attempted to investigate the potential for over- or under-diagnosis of asthma in a study of 86 morbidly obese patients awaiting bariatric surgery. These workers found that of the 32 patients with a physician diagnosis of asthma, 13 (41%) had no objective evidence of asthma, whereas in the 54 subjects who had not previously been investigated for asthma 17 (31%) were newly diagnosed with asthma.

Over-diagnosis of asthma leads to unnecessary over-treatment of subjects with ICS. In addition to the findings of Aaron *et al.* discussed above, other studies have also highlighted the inappropriate over prescription of ICS.

Lucas *et al.* (387) assessed the diagnoses and reasons for using ICS of 2271 patients referred to a primary care diagnostic centre over the course of 6 months for pulmonary function tests. Of these 1171 used ICS and 354 (30%) had no clear indication for using steroids based on their medical history and spirometry results. One-hundred and forty-nine of these patients were asked to stop ICS treatment for 3 months and then re-attend for repeat

spirometry. Of the 71 that did so, only 5 developed asthma-like symptoms and were restarted on ICS, whilst the other 66 had no issues after stopping steroids. After one year the remaining 205 (of 354) patients who had an unclear reason for ICS use and the 78 patients who did not return for repeat spirometry (total of 283) were asked to return for spirometry. 49 of these had stopped ICS with no problems, 89 were still taking ICS for no clear reason, 79 failed to re-attend and 66 had indications to continue with ICS therapy. In all at least 11% of those originally using steroids did not require them and at least 15% of those still taking ICS at the end of the study had unclear reasons for doing so. The authors estimate that up to 26% of all patients in the study probably did not require ICS treatment.

1.5.2.1 Summary

Having previously been under-diagnosed there are compelling data suggesting that asthma is now over-diagnosed. This not only leads to unnecessary confusion and anxiety on behalf of patients labelled with an incorrect diagnosis but also to overuse of expensive inhaled medication with cost implications for the NHS and potentially unnecessary exposure to ICS side effects.

The accuracy of asthma diagnosis may be improved by the use of objective tests that are capable of measuring and quantifying the degree of the ongoing pathological processes and these will now be reviewed.

1.5.3 Comparison of investigations for the diagnosis of asthma:

1.5.3.1 Sensitivity and Specificity

Before discussion of the various investigations used to support a diagnosis of asthma it is important to clarify the terms used to compare the diagnostic usefulness of these investigations. These terms include the *sensitivity* (the percentage of subjects who "have" asthma and test "positive"); *specificity* (the percentage of subjects who "do not have" asthma and test "negative"); *positive predictive value* (percentage of subjects who test "positive" and "have" asthma) and *negative predictive values* (percentage of subjects who test "negative" and "do not have" asthma) of these tests.

In order to determine the usefulness of investigations for diagnosing asthma and "optimal" cut-points with maximum sensitivity and specificity for this purpose many studies have used Receiver Operator Characteristics (ROC) analysis. This statistical technique uses values calculated for the sensitivity and specificity of a test at regular unit intervals to produce a ROC curve, the area under which is essentially a measure of the diagnostic utility of that test. An area under the curve (AUC) of 1 would be consistent with a perfect test that was capable of distinguishing between a subject with the incident condition and one without the condition correctly every time. An area under the curve of 0.5 would be consistent with a test that is no better at correctly identifying if a subject has the incident condition or not than randomly guessing.

It is worth noting that assessment of the sensitivity and specificity of any of these investigations in diagnosing asthma is problematic owing to the lack of an independent confirmatory (or 'gold standard') test against which a comparison can be made. In subjects who have mild symptoms suggestive of asthma investigations are often compared against

"physician diagnosis" of asthma, which is based on clinical history, examination and pulmonary function testing. However, as it is the difficulty in making a clinical diagnosis of asthma that has led to the need for further investigation, it is clear that this is not a reliable gold standard test against which all other investigations should be judged.

1.5.3.2 Tests for variable airflow obstruction

a) Spirometry

i) Description of test

It is generally accepted that spirometry should be the initial investigation in any subject above 5 years of age with a suspected diagnosis of asthma.

The test measures the volume of air that can be exhaled by the subject, allowing the measurement of certain parameters including the forced expiratory volume in the 1st second (FEV_1), the maximum volume of air expelled from the lungs following maximum inhalation (vital capacity or VC) and another similar measure, the forced vital capacity (FVC), which is the same as the VC but the subject is asked to exhale as forcefully and rapidly as possible.

ii) Significant ("positive") result

Airflow obstruction is determined by the presence of a reduced FEV_1:VC ratio or a reduced FEV_1:FVC ratio. The European Respiratory Society (ERS)/ATS spirometry guidelines recommend that a subject's calculated FEV_1:VC ratio should be compared to a predicted value based on their age, height, gender and race. Values that are below the 5th centile of the frequency distribution for the relevant reference population are considered below the "normal range" (217). In contrast to this the GOLD guidelines for spirometry consider airflow obstruction to be present if the FEV_1/FVC ratio is <70% (218).

A combination of symptoms suspicious of asthma together with a reduced FEV_1:VC or FEV_1:FVC ratio is consistent with a high probability of a diagnosis of asthma. A proviso to this is that as asthma is an intermittent condition with variable airflow obstruction, spirometry may be normal in asthmatic individuals in between symptomatic episodes.

iii) Sensitivity/specificity for asthma diagnosis

Spirometers are superior to peak flow meters for the assessment of airflow obstruction, which should not be used for diagnosing asthma as they are less accurate and more effort dependent (388-390).

Spirometry has a good specificity for asthma (90%) but a low sensitivity (29%) making it possible to 'rule in' asthma but virtually impossible to 'rule out' the diagnosis when spirometry is normal (391).

b) Peak flow variability

i) Description of test

The peak expiratory flow rate (PEFR) is the maximal exhalation rate of a subject after a full inspiration (392). It is most commonly measured using a peak flow meter, which is a small portable flow-gauge device. Peak flow measurements are of use in assessing suspected variable airflow obstruction, although peak flow is predominantly determined by the calibre

of large airways, as opposed to FEV_1 which is determined by the calibre of both large and medium sized airways (393).

Owing to the relative ease of use, portability and inexpensiveness of peak flow meters they can be used by patients to obtain self-assessed peak flow measurements in the community. Recording peak flow measurements at least twice daily over a specified period of time allows the calculation of peak flow variability, which can be used to estimate the degree of AHR experienced by a patient on a daily basis under normal work/life conditions.

ii) Significant ("positive") result

There are different strategies for interpreting peak flow values but one of the most common of these is to calculate a peak flow variability index. These indices are normally calculated by determining the difference between the highest and lowest PEFR readings in a day, then dividing this difference by the mean of all the PEFR readings taken in that day (392, 394).

Significant values for peak flow variability are disputed due to large overlaps in values between asthmatic and non-asthmatic subjects (393, 395) but population studies of non-asthmatic adults suggest that the upper limit of normal PEFR variability (assessed using 4 or more peak flow readings per day) is <20% (396, 397).

iii) Sensitivity/specificity for asthma diagnosis

Peak flow variability has been shown to have a low diagnostic value for asthma in a primary care setting (398-400). A clinical study of 3074 patients using a calculated PEFR variability value of ≥20% on ≥2 days in a 3 week period to diagnose asthma found this cut-point to have a sensitivity of just 36% with a specificity of 90% and a positive predictive value of 16.4% (395). Again, this means PEFR variability is useful for diagnosing asthma when positive but it is not useful to 'rule out' asthma.

c) Bronchodilator response testing ("Reversibility")

i) Description of test

Patients found to have airway obstruction on spirometry should undergo bronchodilator response testing to determine the degree of reversibility of their bronchoconstriction to an inhaled β2-agonist. Following baseline readings, 400 µg of salbutamol is administered from a pressurised inhaler device via a spacer and after waiting 15 min spirometry is repeated.

ii) Significant ("positive") result

There is no clear consensus on what a significant response to a bronchodilator constitutes but this is most often taken as "an increase of 12% and 200 mL in FEV_1 or FVC over the baseline value as recommended by the ATS (217).

Results from the Burden of Obstructive Lung Disease (BOLD) study (401) seem to broadly support the clinical significance of these criteria with the estimated 95^{th} centile values (with 95% CI) for change in FEV_1 post bronchodilator in a population of 3922 healthy never smokers found to be 284 mL (263-305 mL) and 12% (11.2-12.8%) above baseline with a corresponding average increase in FVC of 322 mL (271-373 mL) and 10.5% (8.9-12%).

iii) Sensitivity/specificity for asthma diagnosis

Unfortunately the traditional paradigm of separating asthma (classically thought of as a disease with 'reversible' airway obstruction) and diseases thought to display fixed airway obstruction (especially COPD) has been demonstrated to be unreliable with many asthmatic subjects displaying a degree of fixed airway obstruction and a large proportion of COPD patients demonstrating significant reversibility (402).

Indeed as a diagnostic test for asthma, bronchodilator response testing suffers from similar problems to spirometry in that it has a low sensitivity and hence a low negative predictive value, leading to a high false negative rate (400, 403, 404).

A comparative study by Hunter *et al.* (403) of different diagnostic tests in a population of adults with mild asthma (in comparison to a gold standard of clinical diagnosis based on history and presence of airflow obstruction) found reversibility testing (using a much lower cut-off of >3% increase in FEV_1) to have a sensitivity of 49% with a specificity of 70% for asthma diagnosis. Goldstein *et al.* (400) compared the diagnostic utility of peak flow variability, MCT and reversibility in 57 patients with suspected asthma. Only 3 of these subjects exhibited post-BD FEV_1 responses ≥12%, meaning reversibility had almost 100% specificity but only 6% sensitivity for asthma diagnosis.

1.5.3.3 Tests for airways inflammation

a) Sputum differential cell count

i) Description of test

Airway inflammation is a characteristic pathological feature of asthma and can be assessed directly through the microscopic examination of induced sputum.

Sputum induction is a well described technique (405) consisting of the inhalation of hypertonic saline of increasing concentrations by the subject in order to encourage the expectoration of sputum. The mechanisms for this effect are not entirely clear but are thought to be either due to the osmotic effects of hypertonic saline in the airways (406, 407) or an enhancement of mucociliary clearance (408, 409). Unfortunately, hypertonic saline is also known to cause bronchoconstriction in asthmatic subjects (410), and this sometimes occurs despite pre-treatment with a β2-agonist (411). This is possibly due to mast cell activation (412) or neurogenic reflexes (413).

Therefore despite the relatively non-invasive nature of this technique in comparison to bronchoscopic methods to obtain samples that give a direct measure of airway inflammation, it is not suitable for use outside a closely monitored setting (411). Reports of the success rate of sputum induction in producing an adequate sputum sample for analysis range between ~70-100% in both adults (264, 414) and children (415, 416). Also, special expertise is required to process sputum samples in order to perform a cell count and processing must take place within hours of obtaining the sample in order to obtain a reliable result, further limiting the applicability of this technique outside a specialist centre (417).

Microscopy of induced sputum from asthmatics has allowed the identification and study of different inflammatory subtypes, based on the inflammatory cells identified in the sputum. The four main inflammatory subtypes identified are the eosinophilic, neutrophilic, granulocytic and paucigranulocytic types (260), of which, with regards to the *diagnosis* of asthma only the eosinophilic type will be discussed further in this section.

ii) Significant ("positive") result

Different definitions have been used for sputum eosinophilia from 1% to 3% eosinophils/total sputum cell count. Studies investigating the mean % eosinophil count in the induced sputum of healthy non-smokers found this to be around 0.4% (335, 418) and it has been recommended a value of ≥3% be used to identify patients with eosinophilic inflammation with optimum reproducibility (263).

iii) Sensitivity/specificity for asthma diagnosis

Studies that have used sputum eosinophil count for asthma diagnosis include Hunter *et al.* (403) (as described above) who calculated a sensitivity and specificity using a cut-off value of >1% of 72% and 80% respectively when compared to physician diagnosis of asthma.

Di Lorenzo *et al.* (419) compared the validity of sputum eosinophil count with MCT, PEF variability, FEV_1/FVC ratio, serum eosinophil and ECP levels in diagnosing asthma in a population of 60 mild asthmatics, 30 patients with GORD and asthma-like symptoms and 25 healthy volunteers. These workers concluded that a sputum eosinophil count of >1% had a sensitivity of 90% and a specificity of 92% for the diagnosis of asthma, which was superior to all the other tests assessed except the MCT which was roughly equivalent with a sensitivity of 90% and specificity of 89%.

In summary, the sputum differential cell count is useful for identifying eosinophilic (and neutrophilic) inflammation, although not necessarily asthma per se. However, this test is difficult to administer outside a specialist centre due to the safety aspects of sputum induction and the expertise required to process sputum samples and produce a valid cell count.

1.5.3.4 Tests for airway hyper-responsiveness

a) Principles of AHR testing

AHR is one of the pathological hallmarks of the classic asthma syndrome (Section 1.4.3). Despite being a vague term, it can be defined as an "exaggerated narrowing of airways through airway smooth muscle mediated bronchoconstriction following exposure to a direct or indirect stimulus" (161).

The response of the airway to bronchoconstrictor stimuli is measured clinically by bronchial challenge testing, which exposes subjects to a stepwise increase in the dose of the selected stimulus whilst measuring their lung function. This allows a dose-response curve to be produced from which the different components of AHR can be elucidated.

i) Types of bronchoconstrictor stimuli

The stimuli that cause a bronchoconstriction response can be divided into 2 groups; direct and indirect.

Direct bronchoconstrictor stimuli are those which act directly on receptors present in airway smooth muscle causing contraction. These include methacholine which acts on muscarinic (M3) receptors and histamine which acts on H1 receptors as well as leukotrienes C4 and D4 (420) and prostaglandins D2 and F2α (421).

Indirect stimuli cause bronchoconstriction via the release of constrictor mediators (such as histamine and prostaglandins) from inflammatory cells (such as mast cells). These mediators then act on receptors in airway smooth muscle. Such stimuli include allergens, exercise (422), osmotic agents such as mannitol or hypertonic saline (423) or adenosine monophosphate (AMP) (424).

ii) Dose response curve

There are 2 relatively independent components of AHR; airway sensitivity and airway reactivity. Airway *sensitivity* refers to the minimum level/dose of a stimulus that causes bronchoconstriction, i.e. the greater the airway sensitivity to a substance, the greater the left-shift of the dose response curve from 'normal'. Airway *reactivity* refers to the incremental relationship between the level/dose of stimulus and the degree of bronchial constriction i.e. the higher the airway reactivity to a substance, the steeper the gradient of the dose response curve (see Fig. 2.1).

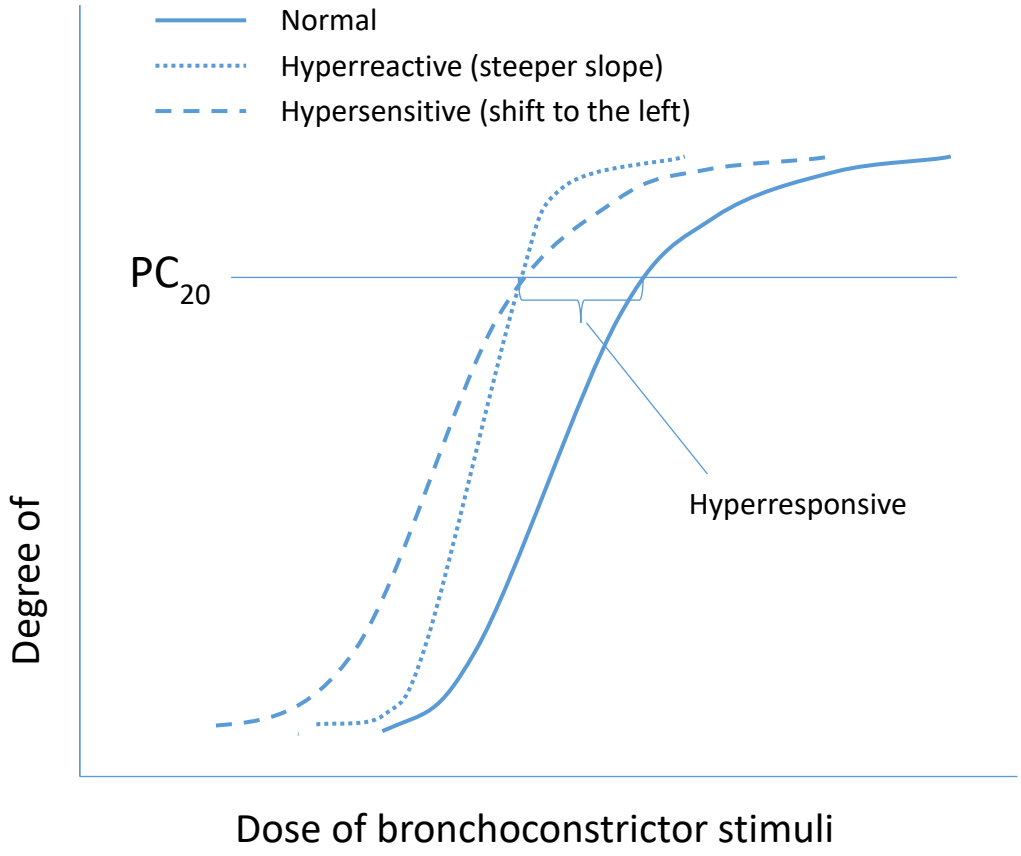


Figure 1.2: A dose-response curve from bronchial challenge testing demonstrating the components of AHR of airways 'hypersensitivity' and 'hyperreactivity' (adapted from Lotvall et al. (425))

As shown in Fig. 2.1, the same degree of hyper-responsiveness may result from either an increase in airways hypersensitivity or hyperreactivity, although these two different mechanisms may reflect different pathological components that lead to increased bronchoconstriction (426, 427). In general however, AHR testing does not tend to consider these components differently and is used to determine whether a subject has increased AHR in relation to a "normal" cut-off point to certain types of bronchoconstrictor stimuli, as discussed below.

b) Fixed and variable direct AHR

It is thought there may be two components of the "hyper-response" to direct stimuli: fixed and variable.

The "fixed" component is traditionally considered to reflect chronic persistent structural changes in the airway i.e. airway remodelling, and it has been suggested that this may be the predominant mechanism for AHR in conditions of fixed airway obstruction (428). A number of investigators have demonstrated that the lower the baseline FEV_1, the greater the magnitude of AHR (429-431), and it was thought that increased airway wall thickness, at a given degree of airway smooth muscle contraction, resulted in greater airway narrowing

(425). However, the situation may be more complicated than this, and a more recent study (427) using HRCT to assess airway thickness in asthmatics found that airway wall thickness was inversely correlated with airway reactivity (whereas airway sensitivity correlated with eosinophilic airway inflammation).

The "variable" component of direct AHR is thought to reflect the degree of airway inflammation and can therefore change rapidly in response to pro-inflammatory stimuli such as allergen inhalation or direct challenge or anti-inflammatory stimuli such as ICS.

This proposed combination of these two components of the airway response to direct stimuli may explain certain clinical observations. These include (1) the short term improvement, though incomplete inhibition of AHR, of certain asthmatic patients with ICS to direct bronchoconstrictors, presumably due to reversal of variable AHR with some persistent degree of fixed AHR (432); (2) the continuing persistence of AHR in some groups of asthmatic patients despite long periods of high dose ICS (252, 432) due to fixed AHR and (3) the positive AHR response of individuals with airway remodelling but without a clinical diagnosis of asthma due to fixed AHR (168). However, this relationship is yet to be fully validated and may prove more complex than outlined here.

c) Direct bronchial provocation tests

i) Description of test

The methacholine bronchial provocation test (MCT) is the most widely used bronchial challenge test, and there are standardised protocols for its administration through tidal breathing using a nebuliser (433) or deep inhalations using a dosimeter (434).

These techniques differ slightly in their methodology but both basically involve the inhalation of saline as a baseline control, followed by doubling concentrations of methacholine (from 0.03 mg/mL to 16 mg/mL) with measurement of the FEV_1 after each inhalation until either the highest dose has been inhaled or the FEV_1 has fallen by 20% (PC_{20}) (434). The tidal breathing method may be preferable as some evidence suggests the dosimeter/deep inhalation method induces bronchodilatation leading to a lower diagnostic sensitivity for asthma (435-438).

Asthmatic subjects are often both more sensitive and more reactive to methacholine than those without asthma, and hence have a lower PC_{20}. However, individuals with other airway diseases in which permanent airflow obstruction is a feature, such as COPD, may also exhibit an increased response to direct stimuli.

ii) Significant ("positive") result

Defining absolute values of PC_{20} to conclusively rule out or rule in asthma is not possible due to the overlap in values observed when comparing results from healthy volunteers and subjects diagnosed with asthma.

Two differing statistical approaches may be used when interpreting the results of a MCT with regards to determining the likelihood of a subject having asthma or not.

The first of these is through the process of *decision analysis*, in which a pre-test probability of a subject having asthma is calculated, as well as a post-test probability that takes into account the pre-test probability and results of the MCT (439). The difference between the pre and post-test probabilities reflects the usefulness of the MCT results in helping to determine if a subject has asthma or not. The pre-test probability in this situation is influenced by the clinical history of the subject. This means that if the subject was chosen at random from the general population, the pre-test probability of that subject having asthma would be very low (i.e. around 5-10% which is the prevalence of asthma in the general population (12)), whereas if the subject had symptoms suggestive of asthma the pre-test probability would be much higher (although difficult to provide an exact value) (440). Approximate values of post-test probabilities can be estimated from pre-test probability values and different values of PC_{20}. For example, a series of curves demonstrating post-test probability values for given pre-test probability values at different values of PC_{20} can be used, as shown in Fig. 2.2, adapted from the ATS guidelines for Methacholine and Exercise Challenge testing (434).

The alternative approach for using MCT results to assess a subjects likelihood of having asthma can be described as a "categorical" method, which assumes that (1) asthma is present or absent (2) that the MCT result is either positive or negative for AHR and (3) that there is a gold standard test for asthma (434). This allows definitions for the sensitivity and the specificity of the test to be used, and this approach is much more commonly used than decision analysis in assessing the utility of MCT in diagnosing asthma. Different PC_{20} cut-points have been used by different authors to signify "positive" and "negative" MCTs and these are discussed further in the next section.

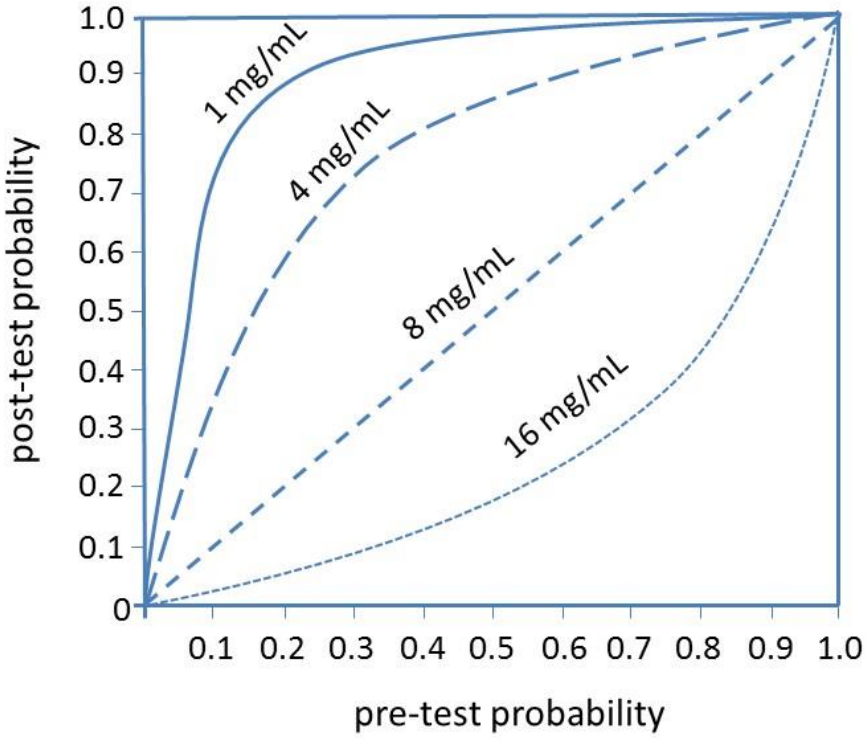


Figure 1.3: Curves illustrating pre-test and post-test probability of asthma after a methacholine challenge test with four PC_{20} values. (adapted from ATS guidelines on methacholine challenge testing (434))

iii) Sensitivity and specificity of direct AHR testing/MCT

Table 1.4 summarises the results of a literature review concerning the determination of the diagnostic utility for asthma of direct bronchial challenge testing. Some of these studies are discussed in further detail here.

In one of the first studies to assess the utility of direct bronchial challenge testing for asthma diagnosis in a non-selected population Cockcroft *et al.* defined a PC_{20} cut-point for the histamine challenge of <8 mg/mL (433) (Table 1.4). In a study of 500 randomly selected college students to identify those with current symptomatic asthma this cut-point had a high sensitivity (100%), specificity (93%) and NPV (100%), although the PPV was poor (29%), which may have been due to the low prevalence of asthma in the population (441). By lowering the cut-point to <1 mg/mL, the specificity and positive predictive value of the test were increased to ~100% (441).

This often quoted study is frequently used to support the assertion that direct bronchial challenge testing is the most accurate diagnostic test for asthma. However, the main weakness of this investigation is that a diagnosis of asthma ("current symptomatic asthma") was defined by questionnaire rather than by physician diagnosis or any objective testing. As questions on symptoms alone are poor diagnostic indicators of asthma and are unlikely to

discriminate well between asthma and conditions leading to similar symptoms (e.g. PNDS or respiratory infection both of which may also cause a degree of AHR (168)) the certainty of the "diagnoses" of asthma in this study could be questioned.

Hunter *et al.* (403) found similarly high levels of both sensitivity (91%) and specificity (90%) using MCT with a PC_{20} cut-point of <8 mg/mL for asthma diagnosis. However, this study assessed the diagnostic utility of the same objective tests it used to define asthma (PEF variability, reversibility and positive MCT). Virtually all of the subjects classified as having asthma exhibited a positive MCT, suggesting that this was the most important criterion in classification. Therefore, even though MCT clearly outperformed PEF variability, reversibility and other investigations not used to define asthma (including blood and sputum eosinophil counts), these results should be assessed with some caution as the sensitivity and specificity values are largely based on the ability of a positive MCT to identify subjects with a positive MCT.

More recent studies examining the use of the MCT in certain patient groups have not found a similarly high level of diagnostic sensitivity including studies by Hedman *et al.* (442), Anderson *et al.* (443), Sverrild *et al.* (444), Sumino *et al.* (445) and Backer *et al.* (446) as shown in Table 1.4.

Sumino *et al.* (445) assessed the influence of factors such as the use of ICS, race and atopic status on the sensitivity of methacholine to identify asthma and found that in asthmatics taking regular ICS the sensitivity of the test was 77%. These workers also determined that the sensitivity using a cut-off (PC_{20}) of 8 mg/mL was significantly lower in Caucasian (69%) in comparison to African American subjects (95%) and in non-atopic (52%) in comparison to atopic subjects (82%).

Backer *et al.* (446) assessed the sensitivity and specificity of the MCT, along with the mannitol challenge test, PEFR variability and reversibility, to diagnose asthma in 190 individuals with 'suspected asthma' in comparison to asthma diagnosis made by a panel of three independent respiratory physicians (based on symptoms, presence of atopy and baseline spirometry). None of the tests provided good combinations of sensitivity and specificity for asthma diagnosis, although MCT had the highest sensitivity of 69% (with specificity of 57%). Interestingly, the clinical diagnoses of asthma made by respiratory physicians generated a low level of agreement, with agreement between all three observers occurring in only 42% of cases and poor agreement between different pairs of observers as assessed by the kappa statistic (a measure of inter-observer agreement).

The MCT does not appear to be a particularly sensitive test for asthma in paediatric populations with the 'best' combinations of sensitivity and specificity for the test in a study by Liem *et al.* (447) for cohorts of atopic boys (67% sensitivity 75% specificity using a PC_{20} of ≤2 mg/mL) and girls (71% sensitivity 69% specificity using a PC_{20} of ≤4 mg/mL) being relatively low. These values were even lower for non-atopic individuals.

In conclusion the MCT, although not as sensitive in diagnosing asthma as originally described, is still probably the most sensitive test for asthma diagnosis. Owing to this higher sensitivity and hence NPV it is best employed as a test to "rule out" rather than "rule in"

asthma, and its sensitivity will be highest when the pre-test probability of asthma is between 30-70% (441, 448).

iv) Indirect challenge tests

Indirect challenge testing using stimuli including exercise and inhaled mannitol challenge is thought to be less sensitive but more specific for diagnosing asthma (428, 444, 446), although some studies have demonstrated comparable values for both measures (443, 444, 449).

Study author	Population	Type of bronchial challenge testing	Criteria for asthma diagnosis	Method of analysis	Optimal cut-point for diagnosis	Sensitivity, specificity, NPV and PPV
Cockcroft *et al.* (441)	500 randomly selected young (20-29) students	Histamine challenge test	"current symptomatic asthma" as defined by ATS "Adult Questionnaire on Respiratory Disease"	Calculated sensitivities/specificities for certain cut-points	Using ≤8 mg/mL Using ≤1 mg/mL	Sensitivity 100%, Specificity 93% NPV 100% PPV 29% Sensitivity 41% Specificity 100% NPV 98% PPV 86%
Nieminen *et al.* (448)	791 consecutive adult patients referred to pulmonary clinic with symptoms of dyspnoea, wheezing, prolonged cough or history of asthma	MCT (dosimeter)	Physician diagnosis with objective test. Objective tests were: 1) Documented variation in FEV_1/PEFR of ≥15% post BD OR 2) Repeatedly ≥20% spontaneous daily variation in PEFR over 2 week period 3) IN ADDITION TO (1) or (2) ≥15% decrease in FEV_1 after specific	Calculated sensitivity etc. for MCT cut-point of 2600 µg. Test was considered to be positive (for bronchial hyperreactivity) if PD_{20} FEV_1 ≤2600 µg	≤2600 µg	Sensitivity 89%, Specificity 76% NPV 91% PPV 71%

			allergen provocation or exercise test			
Hedman *et al.* (442)	230 consecutive adult patients referred to pulmonary clinic with symptoms of dyspnoea, wheezing or cough of unknown cause. Patients with previous asthma diagnosis or ICS use in last 4 weeks excluded	MCT (dosimeter)	As per Nieminen *et al.* (85)	ROC analysis Methacholine positivity/bronchial hyperresponsiveness defined as PD_{20} FEV_1 ≤6900 µg	≤6900 µg	Sensitivity 77%, Specificity 82% NPV 91% PPV 60%
Popovic-Grle *et al.*(450)	195 patients referred by GP with dyspnoea	MCT (details unclear)	Diagnosis based on questionnaire	Calculated sensitivity/specificity/PPV and NPV	≤8 mg/mL	Sensitivity 97%, Specificity 85% NPV 92% PPV 94%
Hunter *et al.* (403)	69 patients diagnosed with asthma, 20 subjects referred to outpatient clinic and found to have 'pseudoasthma'	MCT (tidal breathing)	Physician diagnosis with symptoms consistent with asthma and FEV_1 > 65% predicted with ≥1 of: (1) PC_{20} FEV_1 < 8 mg/mL (2) >15% increase in post BD FEV_1	Calculated sensitivity/specificity/PPV and NPV	≤8 mg/mL	Sensitivity 91%, Specificity 90% NPV 75% PPV 97%

	and 21 healthy controls		(3) > 20% maximum within-day variability of PEF when measured twice daily for > 14 days			
Koskela *et al.* (451)	37 consecutive patients with a new diagnosis of asthma from outpatient clinic	Mannitol challenge Histamine challenge test (dosimeter)	Physician diagnosis based on clinical assessment plus ≥1 of: 1) Documented variation in FEV_1/PEFR of ≥15% post BD 2) Repeatedly ≥20% daily variation in PEFR over a 2 week period 3) ≥15% decrease in FEV_1 after specific allergen provocation or exercise test	Calculated sensitivity of both challenge tests	Histamine PD_{15} ≤0.4 mg ≤1 mg Mannitol ≤635 mg	Sensitivity 49% Sensitivity 81% Sensitivity 51%
Anderson *et al.* (443)	509 subjects (6-50) with signs and symptoms of asthma according to NIH questionnaire but without previous diagnosis of asthma	Mannitol challenge (commercially available test kit – Aridol, Pharmaxis Ltd, Australia) MCT (dosimeter)	Physician diagnosis based on clinical assessment, FEV_1 reversibility and exercise challenge results	Calculated sensitivity and specificity of both challenge tests	MCT PC_{20} ≤16 mg/mL Mannitol PD_{15} ≤635 mg	Methacholine Sensitivity 51%, Specificity 75% NPV 46% PPV 78% Mannitol Sensitivity 55%, Specificity 73% NPV 48% PPV 79%

Sverrild *et al.* (444)	238 randomly selected young adults	Mannitol (commercially available test kit – Aridol, Pharmaxis Ltd, Australia) MCT	Diagnosis based on asthma symptoms within the last 12 months in combination with either a FE_{NO} level >30 ppb, a history of allergic rhinoconjunctivitis, dermatitis, a +ve skin prick test, a familial predisposition to atopic disease, nonallergic rhinoconjunctivitis, or an FEV_1/FVC ratio < 75%	ROC analysis	MCT PD_{20} ≤8 µmol Mannitol PD_{15} ≤635 mg	Methacholine ROC AUC 0.849 Sensitivity 69%, Specificity 80% NPV 90% PPV 49% Mannitol ROC AUC 0.891 Sensitivity 59%, Specificity 98% NPV 91% PPV 90%
Sumino *et al.* (445)	126 “asthmatic” patients receiving controller medications	MCT (dosimeter method)	Physician diagnosed stable asthma; current treatment for asthma in the preceding 12 months with regular use of controller medications (ICS, leukotriene receptor modifiers, or both); no asthma exacerbation in the prior 4 weeks; and pre-bronchodilator FEV_1 ≥70%	Calculated sensitivity and specificity of MCT	MCT PC_{20} ≤8 mg/mL	Sensitivity 77% Specificity 96% PPV 96% NPV 75%
Kim *et al.*	50 “asthmatic”	MCT	“Asthmatic” subjects	ROC analysis	MCT	Methacholine

(449)	patients	(Dosimeter method) Mannitol (commercially available test kit – Aridol, BL&H Co Ltd, Seoul, S.Korea)	had received previous physician diagnosis of asthma, had recurrent symptoms of asthma (wheezing and dyspnoea) and were using medication for asthma ≥6 months before enrolment		PC_{20} ≤16 mg/mL Mannitol PD_{15} ≤635 mg	ROC AUC 0.89 Sensitivity 44%, Specificity 98.1% NPV 65.4% PPV 95.7% Mannitol ROC AUC 0.77 Sensitivity 48%, Specificity 92.6% NPV 65.8% PPV 85.7%
Backer *et al.* (446)	190 patients with "suspected asthma"	MCT (Dosimeter method) Mannitol (commercially available test kit – Aridol™)	Physician diagnosis based on symptoms, presence of atopy and baseline spirometry	Calculated sensitivity/specificity/PPV and NPV	Methacholine PD_{20} ≤7.8 µmol Mannitol PD_{15} ≤635 mg	Methacholine Sensitivity 69%, Specificity 57% NPV 48% PPV 74% Mannitol Sensitivity 38%, Specificity 82% NPV 42% PPV 79%

Table 1.4: The utility of bronchial challenge testing for the diagnosis of asthma

v) Airway hyperresponsiveness and airway inflammation

The relationship between AHR and airway inflammation is a complicated one. Crimi *et al.* (452) found no correlation between AHR to methacholine and airway inflammation (as quantified by numbers of inflammatory cells in sputum, BAL or bronchial biopsy) in a cohort of 71 mild to moderate atopic asthmatic patients. Rosi *et al.* (275), using the method of factor analysis to determine the relatedness of AHR to histamine, reversible airway obstruction and eosinophilic airway inflammation in a cohort of 99 patients with chronic stable asthma also concluded there was no correlation between AHR and airway inflammation and that these should be considered as separate dimensions of disease. Other investigators have found only a weak correlation between the two parameters (300, 453, 454).

Evidence suggests that the correlation is stronger between airway sensitivity to indirect agents and the proportion of eosinophils in induced sputum than for sensitivity to direct agents (455, 456). Both Scollo *et al.* (457) and Porsbjerg *et al.* (458) also identified significant relationships between airway sensitivity to indirect agents (exercise and mannitol) and FE_{NO} values, although a certain proportion of asthmatic subjects responsive to indirect agents have normal FE_{NO} values (456, 459) and these individuals may have non-eosinophilic asthma (458).

1.5.3.5 Summary: Asthma over-diagnosis and comparison of investigations for the diagnosis of asthma

Recent data suggest asthma, a diagnosis of which is often based on clinical assessment in a primary care setting, is now over-diagnosed. A number of objective measures of well described pathological features of asthma with cut-points based on the optimal separation of 'asthmatic' and 'non-asthmatic' populations have been proposed to try and objectively define the condition. Unfortunately although these investigations may be useful in 'confirming' a label of asthma in subjects with commensurate symptoms if they are positive (high specificity), a negative test result is often not helpful (low sensitivity). AHR testing in the form of the MCT was thought to be the closest to a 'gold standard' test for asthma, but more recent studies in unselected populations have revealed this test may have a more modest sensitivity value than initially believed. One caveat to this is the sputum eosinophil count, which appears to be sensitive at identifying ongoing eosinophilic inflammation, rather than asthma per se, and this test has demonstrated utility at guiding management decisions in eosinophilic (Th2 high) asthma. Another investigation used to assess airway inflammation, exhaled nitric oxide testing, has also been proposed as a diagnostic test for asthma, and the utility of this test will now be examined.

1.5.4 Exhaled Nitric Oxide (FE_{NO}) in asthma diagnosis

1.5.4.1 Introduction

Nitric Oxide (NO) is a gaseous signalling molecule with multiple critical roles in human physiology. As well as regulating airway function, NO is a mediator of vasodilation, a

neurotransmitter and an important molecule in the immune response, where it is generated by phagocytes to kill invading bacteria.

NO is synthesised by three different isoforms of the NO synthase (NOS) enzyme: the neuronal (nNOS/NOS1), inducible (iNOS/NOS2) and endothelial (eNOS/NOS3) isoforms, each of which have different physiological functions.

A brief review of NO production by these NOS isoforms and the physiology and pathophysiology of NO in the context of airways disease will now be discussed.

1.5.4.2 NO in airways disease

a) Production of NO

All three NOS isoforms are found in the airways and produce differing amounts of NO. The neuronal and endothelial isoforms are usually collectively referred to as the 'constitutive' NOS isoenzymes, in comparison to the inducible (NOS2) isoenzyme. The significance of this is that whereas the constitutive isoenzymes are dependent on the influx of calcium ions and produce small amounts of NO, the inducible NOS2 type is "induced" by infectious or inflammatory stimuli to produce much larger amounts of NO, independent of calcium influx.

Neuronal NOS enzymes are found mostly in cholinergic nerves in the airways, where they serve to inhibit bronchoconstriction by the production of NO, which inhibits cholinergic bronchoconstriction of the airways by acting as a functional antagonist to acetylcholine in airway smooth muscle (460). Low concentrations of NOS 1 are also found in airway epithelial cells.

Endothelial NOS enzymes are predominantly found in the endothelial cells of the bronchopulmonary circulation and have a role in regulating vascular blood flow (461). However, eNOS is also expressed in airway epithelial cells, where it may contribute to the regulation of ciliary beating (462).

Inducible NOS enzymes are mostly found in airway epithelial cells, although they are also expressed in alveolar macrophages and nasal endothelial and epithelial cells. Several studies have reported increased NOS2 expression in the airway epithelial cells of asthma patients, which is reduced by ICS (463-465). Lane *et al.* (465) also found higher levels of iNOS mRNA in the airway epithelial cells of asthmatic children as well as a significant correlation between iNOS expression and FE_{NO} levels, suggesting that increased expression of the iNOS isotype is responsible for the higher FE_{NO} levels observed in asthmatic patients. This finding was supported by the results of a placebo-controlled double blind RCT by Hansel *et al.* (466) in which the investigators determined that the oral administration of an iNOS selective NOS inhibitor to groups of healthy subjects and mild asthmatics reduced exhaled nitric oxide levels by >90% from baseline.

iNOS enzymes are known to be induced by a variety of pro-inflammatory cytokines including IL-4 (464, 467, 468) and IL-13 (469, 470), via activation of signal transducer and activator of transcription (STAT)-6. Both IL-4 and IL-13 have prominent roles in the Th2 mediated inflammation known to occur in allergic airway inflammation. Hence, FE_{NO} can be

considered a proxy marker for Th2 mediated inflammation, an important finding that will be further explored later.

Other potential sources of excess NO in asthma have been suggested, including the release of NO from S-nitrosothiols (471, 472) and the protonation of nitrites in airway lining fluid forming nitric acid which releases NO with acidification (473, 474).

b) Exhaled nitric oxide and eosinophilic inflammation

Traditionally FE_{NO} has been viewed as a marker of eosinophilic airway inflammation. Some of the earlier studies investigating the relationship between these two variables found no correlation (475, 476) or only a weak correlation (477) between them. However, the majority show a good correlation between FE_{NO} levels and sputum eosinophils (300, 478-481), blood eosinophils (482, 483), serum ECP (482, 483), eosinophils in BAL fluid (484) and eosinophil count in endobronchial biopsy specimens (89, 278, 485).

Nevertheless, more recent evidence from clinical trials of monoclonal antibodies in asthma have suggested that FE_{NO} might be more accurately be described as a marker of Th2 mediated airway inflammation, of which eosinophilic inflammation is a prominent feature.

Halder *et al.* (319) showed that treatment with mepolizumab, an anti-IL-5 monoclonal antibody, significantly decreased both sputum and blood eosinophil counts but had no effect on FE_{NO} levels.

Conversely, Corren *et al.* (325) found that treatment with the anti-IL-13 monoclonal antibody lebrikizumab significantly increased peripheral blood eosinophils and significantly reduced FE_{NO} levels, especially in subjects with high levels of ongoing Th2 inflammation at baseline (as defined by high serum periostin levels).

The reason for this disconnect between FE_{NO} levels and eosinophilic inflammation may be because IL-4 and IL-13 regulate iNOS induction and hence NO production via STAT-6 dependent mechanisms. In contrast, IL-5 activates eosinophils through mechanisms that do not involve STAT-6 (and hence do not induce iNOS) and this activation occurs mostly in the systemic circulation (486).

As FE_{NO} levels seem to reflect the degree of ongoing Th2 inflammation, it is not surprising that they are a sensitive marker of corticosteroid-responsiveness (487). The synthesis of IL-4 and IL-13 is inhibited by corticosteroids, and this effect is likely due to steroid inhibition of transcription factor GATA-3 (488).

c) Detection of NO

Exhaled NO can be detected by several different techniques which can generally be categorised as spectroscopic (including chemiluminescence and laser spectroscopy) (489) or electrochemical detection (490).

Spectroscopic detection methods involve the measurement of products of a reaction involving NO. These include a chemiluminescence technique which relies on the reaction between NO and ozone which produces NO2 in an excited state. A photon is emitted as the NO2 molecule returns to its ground state, which is detected by a photon multiplier tube and

converted into an electrical current. The output voltage of the detector is therefore proportional to the NO concentration (491). Despite the extremely high sensitivity of this technique (down to a concentration of 1 ppb) ensuring it is the "gold standard" for NO detection, the method requires sophisticated expensive equipment which is too large to be portable and therefore only of use in a research setting (492).

By contrast, electrochemical detection methods directly detect NO, and although the sensitivity of these devices is not yet as high as chemiluminescence devices, they show a good level of agreement for all ranges of values (493-495). Their use is rapidly increasing in clinical studies as they are portable, relatively inexpensive and easy to use and maintain (492).

d) Measurement of NO

Detailed discussion of the models of NO excretion from the lungs and measurement of NO levels from different lung compartments is beyond the scope of this thesis. Briefly FE_{NO} concentration is inversely proportional to the exhalation flow of air from the lungs (496), although this is a complex relationship with NO elimination rates varying throughout different phases of exhalation, as certain structures in the lung such as the alveoli are not rigid and change volume during exhalation (497).

Certain models have been formulated to account for the relative contribution of NO from the airway and alveolar compartments in exhalation (498-500), and interest in the area of NO exchange dynamics remains. This is due to the potential usefulness of being able to discriminate between ongoing eosinophilic inflammation in the large airways or small airways/alveoli (501).

Largely however, most ongoing clinical studies measuring FE_{NO} levels tend to do so according to the ATS/ERS guidelines (502), which recommend a standardised exhaled flow rate of 50mL/s and specify the other technical considerations critical in obtaining standardised and reproducible FE_{NO} measurements.

1.5.4.3 Reference values for exhaled nitric oxide

Several investigations have attempted to determine reference values of FE_{NO} for populations of "healthy" adults. Some of the earlier studies in this field measured the NO values of "healthy" populations, without considering potential confounders and it has since become clear that there are a number of independent factors (discussed further below) that significantly affect NO values which need to be taken into account when trying to estimate population reference values.

Some of the most reliable estimates of "normal" ranges of NO in various selected populations therefore come from studies that have measured and adjusted for confounders including age, sex, height, atopic status, smoking status and inhaled steroid usage. The results of the largest of these studies in adult populations with clearly specified subgroups are summarised in Table 1.5 below, whilst the findings from studies of subjects with asthma are summarised in Table 1.6:

Authors	No of subjects	Group studied	Mean ± SD (ppb)	FE_{NO} device and flow rate	Factors not considered
Olivieri *et al.* (503)	204	Healthy non-smoking male adults (n=102) Healthy non-smoking female adults (n=102)	(5^{th} – 95^{th} centiles) 4.5 – 20.6 3.6 – 18.2	Online chemiluminescence analyser (CLD88, Ecomedics, Switzerland) 250 mL/s	Atopy
Olin *et al.* (504)	1131	<u>By atopic status</u> Healthy non-smoking non-atopic individuals (n=845) Non-smoking atopic individuals (n=286) <u>By gender</u> Non-smoking males (n=558) Non-smoking females (n=573)	Mean and (5^{th}-95^{th} centiles) 16 (5.9 – 58.8) 18.8 (5.9-47.1) 18.5 (16.7-57.1) 14.9 (5.4-41.5)	NiOX online chemiluminescence analyser (Aerocrine, Solna, Sweden) 50 mL/s	
Travers *et al.* (505)	528	'Healthy' controls (n=193)	17.9 (7.8 – 41.1)	NiOX online chemiluminescence analyser (Aerocrine, Solna, Sweden) 50 mL/s	Atopy, smoking
Sundy *et al.* (506)	994	'Healthy' non-smokers (n=895) 'Healthy' smokers (n=99)	20.5 ± 213 13.9 ± 18	Sievers 280i Nitric Oxide Analyzer (NOA; GE Analytical Instruments, Boulder, CO, USA) 50 mL/s	Atopy
Levesque *et al.* (507)	895	'Healthy' non-smoking males (n=271) 'Healthy' non-smoking females (n=587)	27 ± 26 18 ± 18	Sievers 280i Nitric Oxide Analyzer (NOA; GE Analytical Instruments, Boulder, CO, USA) 50 mL/s	Atopy

Table 1.5: FE_{NO} reference values from the largest studies carried out in 'healthy' subjects

Authors	No of subjects	Group studied	Mean ± SD (ppb)	FE_{NO} device and flow rate	Factors not considered
Olin *et al.* (508)	1090	Asthmatics (never smoked) (n=1038) Non-asthmatics (never smoked) (n=52)	Median (IQR) 19.9 (14.6-31.4) 17 (12.7-23.5)	NiOX online chemiluminescence analyser (Aerocrine, Solna, Sweden) 50 mL/s	
Shaw *et al.* (509)	118	2 groups of non-smoking asthmatics Group 1 (n=58) Group 2 (n=60)	Mean (68% CI) 29.2 (14 - 61) 31.2 (13.3-73.1)	NiOX online chemiluminescence analyser (Aerocrine, Solna, Sweden) 50 mL/s	
Travers *et al.* (505)	137	Asthmatics	25 ± 15.2	NiOX online chemiluminescence analyser (Aerocrine, Solna, Sweden) 50 mL/s	Atopy, smoking
Michils *et al.* (510)	341	Non-smoking asthmatics Total (n=341) ICS naïve (142) ICS dose >500 µg BDP equivalent	 32.9 49.8 20.5	LR 2000 online chemiluminescence analyser (Logan Research Ltd, Rochester, UK) 50 mL/s	Atopy

Table 1.6: FE_{NO} reference values from the largest studies carried out in cohorts of asthma patients

Due to these multiple confounding factors in interpreting NO levels and the significant overlap between values for "healthy" subjects and asthmatic subjects, standard reference ranges for NO cannot be applied to patients in a clinical setting. Instead, the use of "cut-points" has been proposed to try and delineate subjects with an abnormally high NO, in whom ongoing Th2 airways inflammation is likely, and those with a low NO, in whom active Th2 inflammation is unlikely.

Multiple investigations have attempted to use various FE_{NO} cut-points for the diagnosis of asthma, as discussed further in the following section.

1.5.4.4 Previous studies using FE_{NO} for diagnosis of asthma

Table 1.7 summarises the results of a literature review relating to the diagnostic utility for asthma of measuring FE_{NO} levels. Some of these studies will be discussed in further detail below.

Dupont *et al.* (511) assessed the measurement of FE_{NO} as a diagnostic tool in asthma in 240 consecutive non-smoking patients referred to an outpatient clinic with symptoms of obstructive airways disease. The cut-off point of FE_{NO} >13 ppb was associated with the highest combination of specificity (80%) and sensitivity (85%) i.e. the best test accuracy. There was a significant overlap in this study in the FE_{NO} levels recorded from those patients diagnosed as asthmatic and those not thought to have asthma. This was reflected by the marked variation in sensitivity and specificity (steep gradient on ROC curve) over the narrow range of FE_{NO} values from values of 92.5% and 50% respectively using a cut-off of >10 ppb to values of 73.8% and 85% when using a cut-off of >15 ppb. Despite the good reproducibility of the FE_{NO} technique, values measured in healthy individuals may vary by up to 10% (or ~4 ppb) (512, 513) and the within-subject variation of FE_{NO} values in asthmatics may be up to 20% (512-514). This variation makes it difficult to recommend the use of a single cut-off point in trying to rule asthma in or out as a diagnosis.

Smith *et al.* (515) compared the diagnostic utility of a range of investigations including clinical asthma assessment, FE_{NO} measurement, spirometry, reversibility, hypertonic saline challenge, induced sputum, peak flow measurements in a group of 47 subjects referred by their GPs with symptoms suggestive of asthma for a minimum of 6 weeks. The effect of a trial of oral prednisolone on these measurements was also assessed. Subjects were seen on three separate occasions at 2 week intervals and underwent a fixed sequence of these investigations, and at the final visit were diagnosed with asthma if they had a relevant history (as defined by ATS criteria) *and* a positive test for AHR *and/or* reversibility to a bronchodilator. Seventeen of the 47 patients (36%) were diagnosed with asthma at the end of the study (i.e. positive AHR/reversibility) with the other 30 classified as non-asthmatic. These 30 patients were given diagnoses including chronic rhinosinusitis (13 patients; 28%), extended post-viral respiratory syndrome (8 patients; 17%), GORD (6 patients; 13%), EB (2 patients; 4%) and COPD (1 patient; 2%). The mean FEV_1 and FEV_1/FVC ratio were significantly lower in the asthmatic group than the non-asthmatic group and FE_{NO} and sputum eosinophils were significantly higher in the asthmatic group. Sensitivities for FE_{NO} > 20 ppb and sputum eosinophil count > 3% were 88% and 86% respectively with corresponding specificities of 79% and 88%. Using ROC curve analysis these two tests were significantly more accurate in diagnosing asthma than any of the tests based on lung function, including any change in these parameters following a course of an oral steroid. As this study used reversibility and airways hyper-responsiveness (to hypertonic saline) as diagnostic "gold-standard" tests, these could not be compared to FE_{NO} in terms of diagnostic utility.

Berkman *et al.* (516) compared FE_{NO} against methacholine and adenosine 5'-monophosphate bronchial provocation tests for asthma diagnosis in a group of 85 patients with non-specific respiratory symptoms of over 3 months duration. The optimal FE_{NO} cut-off

point for diagnosis (based on clinical features, spirometry/reversibility or response to treatment) was >7 ppb which gave a sensitivity of 82.5% and a specificity of 88.9%. This compared favourably to the optimal cut-off values for MCT of ≤3 mg/mL, with a sensitivity of 87.5% and specificity of 86.7% for diagnosing asthma and a cut-off value for the adenosine 5'-monophosphate bronchial provocation test of ≤150 mg/mL with a sensitivity of 89.5% and a specificity of 95.6%. The cut-off point of >7 ppb for diagnosing asthma was found to be optimal also when using either of the bronchial challenge tests as the gold standard diagnostic test. The value of >7 ppb is low in comparison to other studies, but this provides an example of the difficulties in comparability of absolute FE_{NO} values determined in different studies owing to the different techniques and FE_{NO} analysers used, as well as possible differences in ambient NO levels (502). In this latter study, an expiratory flow rate of 250 mL/s was used, which is much higher than the ATS recommended expiratory flow rate of 50 mL/s, and due to the recognised inverse relationship between FE_{NO} values and exhaled flow rate, lower FE_{NO} values would be expected (502).

Arora *et al.* (517) measured FE_{NO} levels in a population of 172 basic military trainees with symptoms suggestive of asthma. These trainees each had FE_{NO} levels measured before undergoing a clinical history and examination, spirometry and a histamine bronchoprovocation test. A diagnosis of asthma was made on the basis of these other investigations and FE_{NO} levels in all patients were reviewed. The 80% of trainees who were diagnosed as having asthma had significantly higher FE_{NO} levels than the non-asthmatic trainees, with mean values of 30 ppb for diagnosed asthmatics compared to 19 ppb for non-asthmatics ($p<0.001$). However, a FE_{NO} cut-off with high values for both sensitivity and specificity could not be obtained. At the highest value for sensitivity (86%) at a cut-off of 10.5 ppb the specificity was only 21%, whereas using a higher cut-off point of 46 ppb to give 100% specificity reduced the sensitivity to 17%.

Schneider *et al.* (518) attempted to determine the diagnostic accuracy of FE_{NO} as part of the routine diagnostic assessment of 393 patients attending respiratory private practice with symptoms suggestive of obstructive airway disease. For the whole population the optimal single cut-off point for asthma diagnosis was 25 ppb with a sensitivity/specificity of 49% and 75% respectively. Using a "high" and "low" cut-off point to try and "rule in" and "rule out" asthma diagnosis, a "high" cut-off of >71 ppb had a PPV of 80% and the "low" cut-off of <9 ppb had a NPV of 82%. Subgroup analysis was performed to determine the effect of various factors on the diagnostic accuracy of FE_{NO} and FE_{NO} levels were found to be lower in current smokers and also in instances when diagnoses were made solely using whole body plethysmography with no clinical assessment of patients. By omitting patients with sputum neutrophilia from analysis (although only a third of patients managed to produce sputum), the diagnostic accuracy of FE_{NO} was much improved with a PPV of 82% at a high cut-off of 31 ppb and NPV of 81% at 12 ppb. These results demonstrate that the predictive value of FE_{NO} is low in a general population with a low pre-test probability of asthma. The diagnostic value may be improved if the value is interpreted with prior knowledge of a subject's inflammatory subtype. However obtaining a sputum sample to determine inflammatory subtype would likely require sputum induction, which as mentioned above, is not a readily

available technique and needs careful patient monitoring. This limits the implementation of such an approach in many community or clinic settings.

Cordeiro *et al.* (519) assessed the utility of FE_{NO} to diagnose asthma in a population of 114 atopic individuals presenting to allergy clinic. Subjects with histories suggestive of asthma underwent histamine challenge. 42 subjects were diagnosed with asthma and 72 were diagnosed as non-asthmatic and the asthmatic patients were found to have a higher average FE_{NO} level (44 ppb vs 17 ppb; $p < 0.001$). ROC analysis determined the optimal FE_{NO} cut-point to distinguish between asthma and "non-asthma" was 27 ppb with a sensitivity of 78%, specificity of 92%, PPV of 86% and NPV 87%.

1.5.4.5 Summary

In summary, it would appear that FE_{NO} has greater sensitivity and specificity for the diagnosis of asthma than investigations such as spirometry, reversibility and peak flow monitoring, although it seems to be less sensitive than AHR testing.

However, assessing the literature as a whole it is clear that FE_{NO} cannot be used to diagnose asthma in subjects with symptoms suggestive of asthma based on a single cut-point. When comparing different studies using FE_{NO} detection devices with a flow rate of 50 mL/s the cut-points with optimal sensitivity and specificity for asthma diagnosis vary between 20 ppb (515) to 64 ppb (520). When this is limited further to studies using the same FE_{NO} detection device (NiOX MINO), the optimal cut-point still varies between 25 and 46 ppb, even though the two studies advocating these values were performed by the same authors (518, 521).

A meta-analysis of the literature has been performed (522), which included 19 of the studies reviewed here and 6 studies assessing the diagnostic utility of FE_{NO} in children. The pooled results and summary ROC curve (AUC 0.84) produced suggested FE_{NO} is insufficiently sensitive (sensitivity 78%) and specific (specificity 74%) as a single investigation, but may be used in combination with other tests, to diagnose asthma. However, it seems that the pooled figures for sensitivity and specificity have been derived by combining studies with a significant range in values of FE_{NO} cut-points so the practical utility of this advice seems limited.

The difficulties in using FE_{NO} for asthma diagnosis include: (1) the heterogeneous nature of the asthma syndrome with Th2-high (raised FE_{NO}) and Th2-low (low FE_{NO}) phenotypes (2) the variability of measured FE_{NO} levels between FE_{NO} detection devices, (3) the significant number of confounding factors that affect measured FE_{NO} such as smoking and ICS use and (4) the overlap in values between asthmatics and non-asthmatics with conditions such as EB and atopy.

Study author	**Population**	**FE_{NO} device and flow rate**	**Criteria for asthma diagnosis**	**Method of analysis**	**Optimal FE_{NO} cut-point for diagnosis**	**Sensitivity, specificity, positive and negative predictive values**
Chatkin *et al.* (523)	38 consecutive patients referred to outpatient clinic with cough for ≥ 3 weeks, a normal chest radiograph and FEV_1 > 80% of predicted	Online chemiluminescence analyser 45 mL/s	Physician diagnosis based on significant reversibility (≥12% of FEV_1) or positive MCT (PC_{20} ≤8 mg/mL)	Calculated sensitivity, specificity, PPV and NPV at 10th, 25th, 50th, 75th, and 90th percentiles of the NO distribution	>30 ppb	Sensitivity 75% Specificity 87% PPV 60% NPV 93%
Dupont *et al.* (511)	240 consecutive non-smoking patients referred to outpatient clinic with symptoms of obstructive airways disease	Online chemiluminescence analyser 200 mL/s	Physician diagnosis based on significant reversibility (≥12% of FEV_1) and/or positive histamine challenge (PC_{20} ≤8 mg/mL)	ROC analysis	>13 ppb	Sensitivity 85% Specificity 80% PPV 89.5% NPV 89.5%
Smith *et al.* (515)	47 subjects referred by GPs to outpatient clinic with symptoms suggestive of asthma	Device not listed 50 mL/s	Significant reversibility (≥12% of FEV_1) and/or provocative dose of hypertonic saline resulting in a 15% fall in FEV_1 (PD_{15}) of <20 mL	ROC analysis (AUC 0.864)	>20 ppb	Sensitivity 88% Specificity 79% PPV 70% NPV 92%
Berkman *et al.* (516)	85 subjects with non-specific respiratory	Chemiluminescence analyser (LR 2000,	Physician diagnosis based on significant reversibility	ROC analysis (AUC 0.896)	>7 ppb	Sensitivity 82.5% Specificity 88.9%

	symptoms >3 months duration	Logan Research, Rochester, UK) 250 mL/s	(≥12% of FEV_1) or documented variability of FEV_1 ≥12% at any time over follow up period OR Diagnosis based on +ve MCT (defined as PC_{20} ≤3 mg/mL)			PPV 89.1% NPV 85.4% Sensitivity 66.7% Specificity 72.9% PPV 68.3% NPV 71.4%
Arora *et al.* (517)	172 military trainees with symptoms suggestive of asthma	Niox-Flex 50 mL/s	Physician diagnosis based on history, examination, spirometry and positive histamine challenge	Calculated sensitivity/ specificity for different cut-points	Unable to determine optimal cut-point	Cut-point 10.5 ppb Sensitivity 86% Specificity 21% Cut-point 46 ppb Sensitivity 17% Specificity 100%
Heffler *et al.* (524)	48 consecutive patients referred to allergy outpatients clinic with symptoms of rhinitis and lower airway symptoms	NiOX online chemiluminescence analyser (Aerocrine, Solna, Sweden) 50 mL/s	Significant reversibility (≥12% of FEV_1) and/or positive MCT (PD_{20} ≤800 µg)	ROC analysis (AUC 0.78)	>36 ppb	Sensitivity 77.8% Specificity 60% PPV 54% NPV 81.8%
Fortuna *et al.* (525)	50 patients respiratory outpatients clinic with symptoms suggestive	Chemiluminescence analyser 50 mL/s	Positive MCT (PD_{20} ≤16 mg/mL)	ROC analysis (AUC 0.8)	>23 ppb	Values for 23 ppb cut-point not stated.

	of asthma (dry cough, wheeze, dyspnoea)					For ≥20 ppb Sensitivity 77% Specificity 64% PPV 62% NPV 78%
Sato *et al.* (526)	71 consecutive patients attending respiratory clinic with prolonged cough or wheeze ≥3 weeks	Chemiluminescence analyser 50 mL/s	Diagnosed as 'bronchial asthma' if (1) symptoms of cough and wheeze ≥3 weeks (2) sputum eosinophilia (3) positive MCT/reversibility Diagnosed as 'cough variant asthma' if (1) cough without wheeze ≥3 weeks (2) sputum eosinophilia (3) positive MCT/reversibility	ROC analysis	>38.8 ppb (to distinguish bronchial asthma or cough variant asthma from non-asthmatics)	Sensitivity 79.2% Specificity 91.3%
Bommarito *et al.* (527)	109 symptomatic individuals from ECHRS cohort who consented to take part in study and have FE_{NO} levels measured	Offline chemiluminescence analyser 350 mL/s	Subjects with 'current asthma' were defined as those reporting asthma in life and ≥1 asthma-like symptom in the last 12 months: wheezing or whistling, tightness in	ROC analysis (AUC 0.79)	>18.7 ppb	Sensitivity 69.2% Specificity 71% PPV 24% NPV 95%

			chest, asthma attacks or treatment for medically diagnosed asthma			
Kowal *et al.* (528)	540 young adults with chronic cough (≥8 weeks) referred to outpatient clinic	No details of device 50 mL/s	All patients had normal baseline spirometry. Asthma diagnosis based on positive histamine challenge (PC_{20} ≤8 mg/mL)/significant PEF variability/significant reversibility	ROC analysis (AUC 0.92)	>40 ppb	Sensitivity 88.3% Specificity 82.6% PPV 72.6% NPV 93.1%
Schneider *et al.* (521)	160 patients presenting to GPs with symptoms suggestive of asthma (dyspnoea, cough or phlegm) ≥2 months duration	NiOX MINO (Aerocrine, Solna, Sweden) 50 mL/s	Physician decision based on medical history, examination, spirometry, whole body plethysmography and MCT (PC_{20} ≤16 mg/mL) results	ROC analysis (AUC 0.65)	>46 ppb	Sensitivity 32% Specificity 93% PPV 80% NPV 61%
Pedrosa *et al.* (529)	114 consecutive adult subjects with symptoms suggestive of asthma	NiOX MINO (Aerocrine, Solna, Sweden) 50 mL/s	Positive MCT (PC_{20} ≤8 mg/mL)	ROC analysis (AUC 0.76)	>40 ppb	Sensitivity 74.3% Specificity 72.5% PPV 54.2% NPV 86.6%
Cordeiro *et al.* (519)	114 atopic individuals presenting to allergy clinic (symptoms not specified)	Niox-Flex 50 mL/s	Referred by physician for histamine challenge if clinical assessment consistent with asthma. Positive histamine	ROC analysis	>27 ppb	Sensitivity 78% Specificity 92% PPV 86% NPV 87%

			challenge (PC_{20}) cut-off not stated			
Fukuhara *et al.* (530)	61 subjects presenting to outpatient clinic with ≥1 of recurrent cough, wheeze or dyspnoea	NA623N, Chest MI, Tokyo, Japan Online chemiluminescence 50 mL/s	Asthma diagnosis based on (1) ≥1 of above symptoms (2) ≥2 of induced sputum eosinophilia, AHR and reversible airway obstruction (3) other diseases ruled out using CT and 'other tests' Compared diagnosis by these criteria against diagnosis using "FE_{NO} based criteria" based on (1) symptoms as above (2) FE_{NO} level ≥40 ppb (derived from prior studies) (3) other diseases ruled out	Comparison of "conventional criteria" and "FE_{NO} based criteria"	Pre-specified cut-point of 40 ppb based on previous studies	Sensitivity 78.6% Specificity 89.5% Concordance rate of 0.62 between two sets of criteria 9/42 (21%) of patients 'misdiagnosed' as not having asthma according to FE_{NO} based criteria
Matsunaga *et al.* (531)	142 subjects with respiratory symptoms referred to outpatient clinic and 224 subjects with no current	NiOX MINO (Aerocrine, Solna, Sweden) 50 mL/s	Based on presence of "significant airway reversibility and or airway hyperresponsiveness" (not further specified) during	ROC analysis For non-smokers without	>22 ppb	Sensitivity 92% Specificity 90%

	respiratory symptoms		the follow up period.	rhinitis (n=126) AUC 0.92 For non-smokers with rhinitis (n=136) AUC 0.88 For smokers without rhinitis (n=49) AUC 0.94 For smokers with rhinitis (n=55) AUC 0.87	 >28 ppb >18 ppb >22 ppb	 Sensitivity 90% Specificity 77% Sensitivity 100% Specificity 87% Sensitivity 80% Specificity 86%
Schleich *et al.* (67)	174 patients referred to a pulmonary function laboratory with suspected asthma but normal spirometry and reversibility	NiOX chemiluminescence analyser (Aerocrine, Solna, Sweden) 50 mL/s	Positive MCT (PC_{20} ≤16 mg/mL)	ROC analysis (AUC 0.62)	>34 ppb	Sensitivity 35.4% Specificity 95.4% PPV 88% NPV 62%
Malinovschi *et al.* (532)	282 subjects from a group of 686 subjects	NiOX MINO (Aerocrine, Solna,	Physician diagnosis based on symptoms plus ≥1 of	ROC analysis	For non-smokers	Sensitivity 77.8% Specificity 63.5%

	who reported ≥2 ongoing respiratory symptoms on an asthma questionnaire sent to a random population sample of 10,400 subjects	Sweden) 50 mL/s	the following: 1) Positive MCT (PC_{20} ≤8 mg/mL). 2) ≥250 mL increase in post BD FEV_1 3) Daily use of oral steroid, ICS, or inhaled β2-agonist 4) Asthma symptoms during pollen season, eventually supported by allergic rhinitis, although no objective signs of asthma outside season	For all subjects AUC 0.72 Excluding current ICS users AUC 0.73 Subjects divided into non, ex-smoking and current smoking groups	(n=108) >15 ppb For ex-smokers (n=62) >22 ppb For current smokers (n=112) >17 ppb	PPV 60% NPV 80% Sensitivity 63.2% Specificity 86.1% PPV 67% NPV 84% Sensitivity 56.3% Specificity 82.5% PPV 57% NPV 82%
Voutilainen *et al.* (533)	Sedentary patients referred to outpatient clinic with symptoms suggestive of asthma	NiOX chemiluminescence analyser (Aerocrine, Solna, Sweden) Flow rate not stated	Positive histamine challenge (cut-off not stated)	ROC analysis (AUC 0.83)	Pre-specified cut-point of >30 ppb as "high FE_{NO}"	Not stated
Katsoulis *et al.* (534)	112 subjects with asthma-like symptoms and negative reversibility	NiOX MINO (Aerocrine, Solna, Sweden) 50 mL/s	Positive MCT (PD_{20} <800 µg)	ROC analysis (AUC 0.69)	>32 ppb	Sensitivity 47% Specificity 85%
Schneider *et al.* (518)	393 patients presenting to GPs with	NiOX MINO (Aerocrine, Solna, Sweden)	Physician decision based on medical history, physical examination,	ROC analysis (AUC 0.66)	>25 ppb	Sensitivity 49% Specificity 75% PPV 56%

	symptoms suggestive of asthma (dyspnoea, cough or phlegm) ≥2 months duration	50 mL/s	spirometry, whole body plethysmography and bronchial provocation (PC_{20} ≤16 mg/mL) results			NPV 69%
Wang *et al.* (520)	923 consecutive patients referred to outpatient clinic with symptoms suggestive of asthma (recurrent wheezing, dyspnoea, chest tightness and/or cough, duration over 6 months),	Nano Coulomb nitric oxide analyser 50 mL/s	+ve MCT (cut-point not listed) OR +ve reversibility (considered +ve if post BD FEV_1 15% and 200 mL higher than pre BD FEV_1)	ROC analysis (AUC 0.76) AUC 0.78	>64 ppb >41 ppb	For MCT +ve Sensitivity 52% Specificity 94.4% PPV 80.2% NPV 72.8% For reversibility +ve Sensitivity 72.4% Specificity 74.9% PPV 61.8% NPV 82.9%

Table 1.7: The utility of FE_{NO} testing for the diagnosis of asthma

1.5.5 Guidelines for the Diagnosis of Asthma

1.5.5.1 British Thoracic Society (BTS) guidelines (2016)

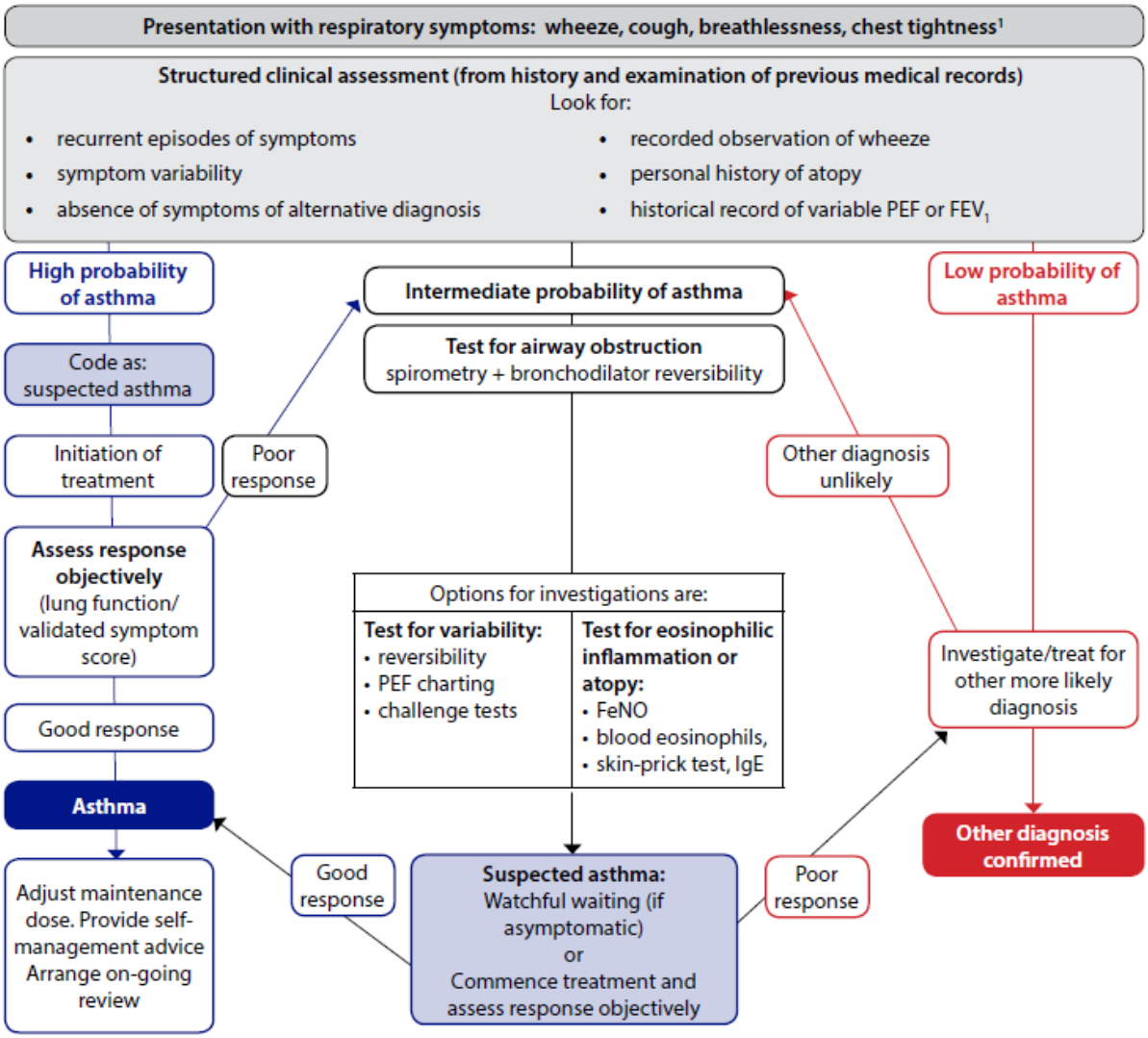
The BTS/Scottish Intercollegiate Guidelines Network (SIGN) guidelines recommend that a person presenting with suspected asthma should be clinically assessed and the probability of asthma determined (535).

The clinical assessment should include careful enquiry regarding the following symptoms (Fig. 1.4):

Episodic symptoms *(see sections 3.2.1 and 3.2.2)*[13,21-24,64,74,75] More than one of the symptoms of wheeze, breathlessness, chest tightness and cough occurring in episodes with periods of no (or minimal) symptoms between episodes. Note that this excludes cough as an isolated symptom in children.[76] For example: • a documented history of acute attacks of wheeze, triggered by viral infection or allergen exposure with symptomatic and objective improvement with time and/or treatment • recurrent intermittent episodes of symptoms triggered by allergen exposure as well as viral infections and exacerbated by exercise and cold air, and emotion or laughter in children • in adults, symptoms triggered by taking non-steroidal anti-inflammatory medication or beta blockers. An historical record of significantly lower FEV_1 or PEF during symptomatic episodes compared to asymptomatic periods provides objective confirmation of the obstructive nature of the episodic symptoms.
Wheeze confirmed by a healthcare professional on auscultation *(see section 3.2.1)*[23,25] It is important to distinguish wheezing from other respiratory noises, such as stridor or rattly breathing. Repeatedly normal examination of chest when symptomatic reduces the probability of asthma.
Evidence of diurnal variability[21-23,34,74] Symptoms which are worse at night or in the early morning.
Atopic history *(see section 3.2.4)*[19,23,64,75,77,78] Personal history of an atopic disorder (ie eczema or allergic rhinitis) or a family history of asthma and/or atopic disorders, potentially corroborated by a previous record of raised allergen-specific IgE levels, positive skin prick tests to aeroallergens or blood eosinophilia.
Absence of symptoms, signs or clinical history to suggest alternative diagnoses (including but not limited to COPD, dysfunctional breathing, obesity) *(see section 3.3.3)*.

Figure 1.4: Clinical features to be enquired about during clinical assessment for asthma. From BTS guidelines (535).

This assessment should be used to classify whether the patient has a high, intermediate or low probability of asthma. Patients with a high probability should be offered a trial of treatment, and those with a low probability investigated for other conditions, with those in the intermediate group being assigned to a treatment trial or further investigation depending on their FEV_1/FVC ratio, reversibility and possibly further investigations as shown in Fig. 1.5.



[1] In children under 5 years and others unable to undertake spirometry in whom there is a high or intermediate probability of asthma, the options are monitored initiation of treatment or watchful waiting according to the assessed probabilityof asthma.

Figure 1.5: Algorithm for further investigation or treatment of asthma following initial clinical assessment and spirometry. From BTS guidelines (535).

For patients with intermediate probability of asthma the guidelines recommend further investigation of patients following spirometry, which may include tests to assess airflow variability or tests for eosinophilic inflammation/atopy prior to strategies of either watchful waiting or a treatment trial.

The formal inclusion of investigations such as challenge tests and FE_{NO} for consideration in the diagnostic algorithm are a new feature of the 2016 guidelines and were previously not included in the last 2014 update. Probably owing to the lack of clear evidence about the exact role of the tests in 'diagnosing' asthma they are included as 'options' that may be considered to provide extra evidence to support a diagnosis of asthma.

1.5.5.2 National Heart, Lung, and Blood Institute (NHLBI) guidelines (2007)

This guidance states that (536):

In order to establish a diagnosis of asthma the clinician should determine that:

— Episodic symptoms of airflow obstruction or airway hyperresponsiveness are present.
— Airflow obstruction is at least partially reversible.
— Alternative diagnoses are excluded

The diagnosis should be made from a medical history, physical examination and spirometry with reversibility. If no clear diagnosis can be made, further investigations should be used to exclude asthma or consider other alternative diagnoses.

Some of the "key indicators" from the medical history suggestive of a diagnosis of asthma include:

- *Wheezing – high pitched whistling sounds when breathing out – especially in children. (Lack of wheezing and a normal chest examination do not exclude asthma)*
- *History of any of the following :*
    - *Cough, worse particularly at night*
    - *Recurrent wheeze*
    - *Recurrent difficulty in breathing*
    - *Recurrent chest tightness*
- *Symptoms occur or worsen in the presence of:*
    - *Exercise*
    - *Viral infection*
    - *Animals with fur or hair*
    - *House-dust mites (in mattresses, pillows, upholstered furniture, carpets)*
    - *Mold*
    - *Smoke (tobacco, wood)*
    - *Pollen*
    - *Changes in weather*
    - *Strong emotional expression (laughing or crying hard)*
    - *Airborne chemicals or dusts*

Figure 1.6: Key indicators from medical history suggestive of a diagnosis of asthma. From NHLBI guidelines (536).

Features that should be assessed for on physical examination include:

- Hyperexpansion of the thorax; use of accessory muscles or chest deformity
- Sounds of wheezing on chest auscultation/prolonged phase of forced expiration
- Increased nasal secretion, mucosal swelling, and/or nasal polyps
- Signs of allergic skin condition such as atopic dermatitis/eczema

The guidelines then recommend that all patients over the age of 5 in whom asthma is being considered as a diagnosis should undergo spirometry with reversibility testing. As well as measuring FEV_1 and FVC these guidelines also recommend measurement of the volume of air exhaled after the first 6 seconds of expiration (FEV_6). This parameter should be measured instead of FVC in patients who might find sustaining maximal expiratory effort until complete expiration too arduous, such as patients with severe airflow obstruction.

Based on the results of spirometry it can be determined if the patient has airways obstruction and whether or not this is reversible. Significant reversibility is defined as per ATS/ERS guidelines (537) as an increase in FEV_1 of >200 mL and ≥12% from the baseline measure after inhalation of a short acting beta agonist (SABA).

These guidelines are not explicit regarding the use of further investigations for the diagnosis of asthma and merely state that "additional studies are not routinely necessary but may be useful when considering alternative diagnoses" which should be considered "as appropriate".

1.5.5.3 Canadian Thoracic Society guidelines (2012)

These guidelines state that "*asthma is diagnosed by the combination of a comparable clinical history and objective measures of lung function*" (538).

Features of the clinical history should include "*paroxysmal or persistent symptoms such as dyspnoea, chest tightness, wheezing, sputum production and cough, associated with variable airflow limitation and airway hyper-responsiveness to endogenous or exogenous stimuli*".

The recommended measures of lung function are those in Fig. 1.7:

Pulmonary function measurement	Children (6 years of age and over)	Adults
Preferred: Spirometry showing reversible airway obstruction Reduced FEV_1/FVC **AND** Increase in FEV_1 after a bronchodilator or after course of controller therapy	Less than lower limit of normal based on age, sex, height and ethnicity (<0.8–0.9)* **AND** ≥12%	Less than lower limit of normal based on age, sex, height and ethnicity (<0.75–0.8)* **AND** ≥12% (and a minimum ≥200 mL)
Alternative: Peak expiratory flow variability Increase after a bronchodilator or after course of controller therapy OR Diurnal variation†	≥20% OR Not recommended	60 L/min (minimum ≥20%) OR >8% based on twice daily readings; >20% based on multiple daily readings
Alternative: Positive challenge test a) Methacholine challenge OR b) Exercise challenge	PC_{20} <4 mg/mL (4–16 mg/mL is borderline; >16 mg/mL is negative) OR ≥10%–15% decrease in FEV_1 post-exercise	

**Approximate lower limits of normal ratios for children and adults. †Difference between minimum am pre-bronchodilator value in 1 week and maximum pm value as % of recent maximum. FEV_1 Forced expiratory volume in 1 s; FVC Forced vital capacity; PC_{20} Provocative concentration of methacholine producing a 20% fall in FEV_1. Reproduced from reference 4, with permission*

Figure 1.7: Recommended measures of lung function when considering diagnosis of asthma. From Canadian Thoracic Society Guidelines (538)

These guidelines explicitly recommend bronchial challenge testing (with both methacholine and exercise) as an alternative objective diagnostic criterion for asthma. There is no further guidance on how to proceed if a patient has a clinical history consistent with asthma but normal pulmonary function tests and no mention of using measures of airway inflammation for asthma diagnosis.

1.5.5.4 Comparison of guidelines

a) Similarities

All of these guidelines include recommendations for the clinical assessment of patients with suspected asthma and highlight certain features that make asthma more likely. These include symptoms of wheeze, dyspnoea, chest tightness and cough (especially nocturnal cough) that worsen in relation to recognised stimuli such as exercise and allergen exposure.

All guidelines agree on performing spirometry, but after this they vary in the approach to further confirmation of a diagnosis of asthma.

b) Differences

There are a number of important differences between these sets of guidelines which are most apparent following the clinical assessment of patients and spirometry.

The BTS/SIGN guidelines recommend classifying patients into groups with different probabilities of asthma prior to performing further investigations or instigating a treatment trial. The use of spirometry/reversibility and further investigations is reserved for those with an intermediate probability of asthma. In contrast, the NHLBI guidelines recommend all patients should undergo reversibility testing in addition to basic spirometry and then are not

explicit regarding the use of further investigations. CTS guidelines state that patients should preferably have spirometry and reversibility testing following clinical assessment, but that the results of other investigations including variability in serial peak flow measurements or positive methacholine/exercise challenge testing may be used instead to make a diagnosis of asthma.

The BTS guidelines are the only one of the three sets of guidelines that endorse a trial of treatment in suspected asthma patients before performing further investigations such as reversibility testing. This may be a concession to pragmatism with evidence suggesting that many patients are started on ICS treatment for presumed asthma without even having spirometry performed (382).

As the BTS guidelines are more extensive and more frequently updated than the other two guidelines they contain much more information about the further investigations that may be used in asthma. They also provide some comparison of their relative sensitivities/specificities. Investigations including challenge tests with methacholine, exercise and mannitol and FE_{NO} measurement are generally recommended in patients where the diagnosis is unclear but there is no guidance on the systematic use of these tests. The CTS guidelines also recommend bronchial challenge testing but only as an alternative to spirometry with reversibility for asthma diagnosis rather than in addition to this.

1.5.5.5 Proposed NICE guidelines for asthma diagnosis

This proposed set of guidelines, originally due for full release in 2015, are an attempt by the National Institute for Health and Care Excellence (NICE) to improve the efficiency and accuracy of diagnosing asthma (539). The use of a series of objective tests in the format of a diagnostic algorithm is recommended based on review of the literature and an economic analysis assessing the likely cost implications of using these tests to diagnose asthma.

In its current format, the algorithm recommends the use of spirometry followed by combinations of peak flow variability testing, FE_{NO} measurement and bronchial challenge testing to diagnose asthma. FE_{NO} measurement is suggested to "rule out" asthma (FE_{NO} <25 ppb) or "rule in" asthma (FE_{NO} >40 ppb) with values between these two cut-points being labelled as intermediate, and the patient requiring further investigation. However, this approach is still likely to suffer from the problems of multiple confounding factors affecting FE_{NO} levels, including high FE_{NO} levels in subjects with other airway diseases and low FE_{NO} levels in subjects with neutrophilic asthma.

Release of these guidelines is currently on hold while the "impact and feasibility" of measuring "quality-assured spirometry" and FE_{NO} in primary care to diagnose asthma in primary care is assessed, with an estimated release date of 2017.

1.5.5.6 Summary

All of the published guidelines appear to lack detail that may help guide the diagnostic process for asthma. Although the clinical assessment and use of spirometry in diagnosing asthma is well defined in this guidance, the role of further investigations that are well characterised such as reversibility testing and bronchial challenge testing is unclear. Tests of airways inflammation do not yet have an explicit role in the diagnosis of asthma in any

published guidance to date. The proposed NICE guidelines are an attempt to utilise further objective measures including FE_{NO} to diagnose asthma, although the feasibility of using objective tests for asthma diagnosis in the algorithm described requires validation and potential problems with this approach have been described above. Owing to its ease of measurement and the fact that results are available almost instantaneously FE_{NO} is clearly an attractive test to help guide asthma management but there is, as yet, no clear evidence it has a role in the diagnosis of asthma. However, an alternative use has been suggested for the technique in the assessment of subjects with suspected airways disease.

1.5.6 The use of FE_{NO} to predict steroid response

As FE_{NO} is a biomarker of ongoing Th2 inflammation and Th2 inflammation is usually responsive to corticosteroids a small number of studies have investigated the potential of FE_{NO} to predict subjects' treatment response to corticosteroids irrespective of their underlying diagnosis.

1.5.6.1 Previous studies assessing FE_{NO} to predict steroid response

Smith *et al.* (540) aimed to evaluate the role of FE_{NO} measurements in predicting treatment response to ICS in a cohort of 52 patients with undiagnosed respiratory symptoms. Subjects were assessed in a respiratory clinic on 5 separate occasions over a 10 week period. A series of sequential diagnostic tests (including spirometry, reversibility, methacholine and adenosine monophosphate challenges, symptom diary/peak flow measurements and multiple FE_{NO} measurements) were carried out whilst subjects were being treated with a 4 week course of inhaled placebo therapy, followed by a 4 week course of inhaled fluticasone.

A response to steroid treatment was defined by:

- an improvement in FEV_1 of >12% **or**
- an improvement in mean morning peak flow (over 7 day period) by >15% **or**
- a reduction in composite symptom score by 1 point **or**
- an improvement in PC_{20} AMP by ≥2 doubling dose shift

The steroid response also took into account any response to the placebo treatment as any "placebo response" for any of these endpoints was subtracted from the steroid treatment response. Patients were also diagnosed with asthma if they had a corresponding symptom history (which all patients did) **and** either significant reversibility to short acting β-agonist, significant FEV_1 or peak flow response to inhaled steroids (using same criteria as above) or a positive MCT.

FE_{NO} was compared to the other baseline measurements (FEV_1, FEV_1 bronchodilator response, peak flow variation and methacholine PC_{20}) in its ability to predict a response to steroids, based on the 4 defined measures of steroid response. This parameter was demonstrated to be significantly more accurate than all of the other baseline measurements for at least one of the steroid response measures and inferior in none, as measured by greater ROC AUC for comparative ROC curves. The optimum FE_{NO} cut-point for predictive purposes for all 4 steroid response measures was found to be >47 ppb, although there were patients with FE_{NO} levels lower than this who responded to treatment. For FE_{NO} >47 ppb

using ≥2 doubling dose increase in AMP PC_{20} as a response measure the sensitivity was 82%, specificity 91%, PPV 82% and NPV 91%.

The results of this study were clearly dependent on the measure used to determine a "steroid response". This was illustrated by the range of values for the specificity of FE_{NO} levels >47 ppb to predict steroid response from 71-91% for different response measures, suggesting that up to 30% of subjects in this group did not demonstrate a response to steroids. The authors suggested this may be because many of this group had near to normal lung function and hence had limited room for improvement in this metric, making the cut-points used to define steroid response inappropriate for this population. It is possible that these patients as well as patients with lower FE_{NO} levels may also have demonstrated a response to steroid treatment if different measures of response such as change in FE_{NO}, long term symptom measures or quality of life data had been collected.

Little *et al.* (541) assessed FE_{NO} levels as a marker of oral steroid response in 37 patients with chronic stable asthma. All had a diagnosis of asthma according to ATS criteria for ≥5 years and all but one of the subjects were using regular ICS. A FE_{NO} level of >10 ppb at baseline was found to have a high specificity (90%) and positive predictive value (83%) for an improvement in FEV_1 of >15% but a low sensitivity (59%). Interestingly, all of these values had better predictive accuracy than sputum eosinophilia of ≥4% (although 7 patients did not produce sputum), but it could be argued that both tests may have performed better if an outcome measure more reflective of the activity of Th2 inflammation had been chosen i.e. a measure related to number of exacerbations rather than lung function.

Prieto *et al.* (542) also assessed the utility of FE_{NO} measurement to predict response to ICS in 43 non-smoking subjects with chronic cough and FEV_1 >80%. The cohort in this study had chronic cough of at least 8 weeks duration with no evidence of any lung disease on clinical or radiological assessment and had not previously received any treatment for pulmonary conditions including inhaled or oral corticosteroids. At the three baseline visits investigations including a high-resolution CT scan, spirometry with reversibility, FE_{NO} and bronchial challenge testing with methacholine and AMP were carried out. Subjects were then given 4 weeks treatment with inhaled fluticasone. The primary outcome of ICS response was defined by a >50% reduction in the mean daily cough symptom scores during the treatment period when compared with the baseline period. Interestingly, only 4/43 (9%) of these patients had a positive MCT so the frequency of "asthma" in this cohort was likely to be low. Nineteen patients (44%) responded well to ICS therapy but FE_{NO} was poor at predicting ICS response, with low sensitivity (53%), specificity (63%) and positive and negative predictive values (53% and 63% respectively) at the 'optimal' cut-off point of 20 ppb. It is likely that due to the low incidence of asthma (or, at least, AHR) in this group that a significant proportion of the cohort were less likely to respond to ICS and this may partially explain the poor performance of FE_{NO} in predicting ICS response in this study. Also, it is questionable whether the chosen response variable (mean cough score rated on an unvalidated 5 point scale) had the necessary sensitivity to accurately discern a significant response.

1.5.6.2 ATS guidelines for the interpretation of FE_{NO} levels

The ATS guidelines for the interpretation of FE_{NO} levels (543) published in 2011 concluded that FE_{NO} levels of <25 ppb (20 ppb in children) should be considered low and a strong indicator that responsiveness to steroids is unlikely. In addition, FE_{NO} levels >50 ppb (35 ppb in children) should be considered high and a strong indicator that steroid response is likely. The high cut-point of >50 ppb suggesting a likely response to steroids is advocated based on the results of only one study (540). In contrast, the lower cut-point of <25 ppb was suggested based on data from a variety of studies (458, 509, 511, 515, 517) and has been used because a high frequency of normal people are thought to have FE_{NO} levels up to around 22 ppb. The presence of ongoing Th2 inflammation at such levels is unlikely.

According to the guidelines FE_{NO} levels between 25 and 50 ppb should be interpreted cautiously and their significance is unclear. Measured FE_{NO} levels in this range should be interpreted in the context of the individual patient's clinical symptoms, concurrent medications, smoking status etc. and relative changes in FE_{NO} levels in this range, e.g. a reduction after the use of ICS, may be more important than absolute values.

1.5.6.3 Summary

Using FE_{NO} levels to predict steroid response would appear to be a logical approach given that FE_{NO} is a biomarker of Th2 inflammation and there is good evidence that this type of inflammation is responsive to steroid treatment. This approach avoids the complexities inherent in attempting to use the technique to classify heterogeneous airways diseases according to diagnostic labels.

The study by Smith *et al.* provides good evidence that this strategy may be useful, but the optimal cut-point derived by these authors to predict ICS response (>47 ppb) may risk missing subjects with measured FE_{NO} levels in the 'indeterminate' range of 25-50 ppb identified in the ATS FE_{NO} guidelines who would benefit from steroid treatment. It would be useful for clinical practice if a 'minimum' FE_{NO} cut-point below which steroid response is unlikely could be determined in order to guide the decision of whether or not to initiate steroid treatment in subjects with symptoms suggestive of airways disease.

1.6 Chronic Productive Cough and the use of Macrolides in Airways Disease

1.6.1 Introduction

A chronic 'productive' or 'wet' cough is a common presenting complaint for patients attending the adult respiratory clinic. Most reviews and guidelines suggest that the causes of a productive cough are the same as those of a non-productive cough and as such the same diagnostic pathway should be followed (559).

A cohort of adult patients presenting to respiratory clinic have been observed with chronic productive cough which improves with antibiotic treatment but usually relapses (560). Many patients in this cohort have suspected poorly controlled asthma but investigations including spirometry, bronchial challenges, chest X-ray, screen for immunodeficiency and HRCT scan are normal. Sputum culture is often positive for potentially pathogenic bacteria such as *Haemophilus influenzae*, but may be repeatedly negative. Initial observations suggest significant symptomatic improvement with a prolonged course of low dose azithromycin.

A literature review regarding the causes of chronic productive cough was undertaken to ascertain if this cohort had been described previously or if this presentation could be adequately explained by one of the recognised causes of chronic productive cough. Following this the mechanisms of action and previous uses of macrolides in respiratory disease were also reviewed.

1.6.2 Causes of Chronic Productive Cough

For the purposes of this review the definition of a "chronic productive cough" was considered to be a cough regularly leading to the expectoration of sputum with the same duration as the standard definition of chronic cough i.e. more than 8 weeks (57). Conditions causing productive cough have been listed in an approximate order of prevalence from most to least frequent.

1.6.2.1 Bronchiectasis

Bronchiectasis is defined in Section 1.2.3.1.

a) Epidemiology

The epidemiology of bronchiectasis is reviewed in Section 1.2.3.2.

b) Clinical Presentation

The condition usually presents as a chronic productive cough (561), with daily sputum production (42). Other factors that suggest the diagnosis include haemoptysis, systemic features of weight loss and fatigue and multiple positive sputum cultures (42, 562).

c) Pathology

Bronchiectasis may be secondary to a multitude of other conditions (as listed in Table 1.8), with the most common predisposing factor thought to be post-respiratory infection (563,

564). However, a significant proportion of cases have no obvious discernable cause, although the number of these idiopathic cases reported differs markedly between studies (563-565).

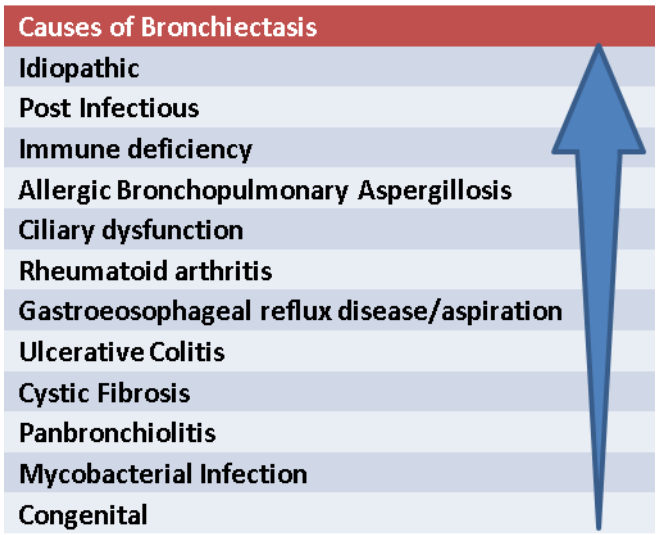


Table 1.8: Causes of bronchiectasis in approximate order of frequency from most to least common. Based on data from Pasteur et al. (563) and Shoemark et al. (564)

It has been suggested that bronchiectasis is largely a result of dysregulation of the immune system, as it is often seen in patients with either immunodeficiencies or 'hyperimmune' (autoimmune) conditions such as Rheumatoid Arthritis or Inflammatory Bowel Disease (565, 566). Although the initial step in the pathogenesis of the condition is not yet clear, it is broadly accepted that it progresses in a largely similar way, based on the ''vicious circle' hypothesis proposed by Cole (567). This describes a cycle of airway inflammation, leading to structural airway damage and resultant mucous stasis, with the pooled mucus becoming colonised with bacteria, which initiate further inflammation (Fig 1.8).

The most common sputum isolates, using standard microbiological approaches, from patients with bronchiectasis are the gram negative bacteria *H. influenzae* and *Pseudomonas aeruginosa* (568, 569). Colonisation of the sputum by first *H. influenzae*, and later *P. aeruginosa*, coincide with worsening of the clinical features of bronchiectasis including lung function and frequency of exacerbation (568, 569).

d) Treatment

Guidance on the treatment of bronchiectasis can be found in the BTS guidelines on bronchiectasis (570).
Broad principles in the management of the condition include treatment of the underlying cause, monitoring of disease activity using lung function and regular sputum cultures,

airway clearance techniques and antibiotic treatment. These principles are further outlined in Table 1.9.

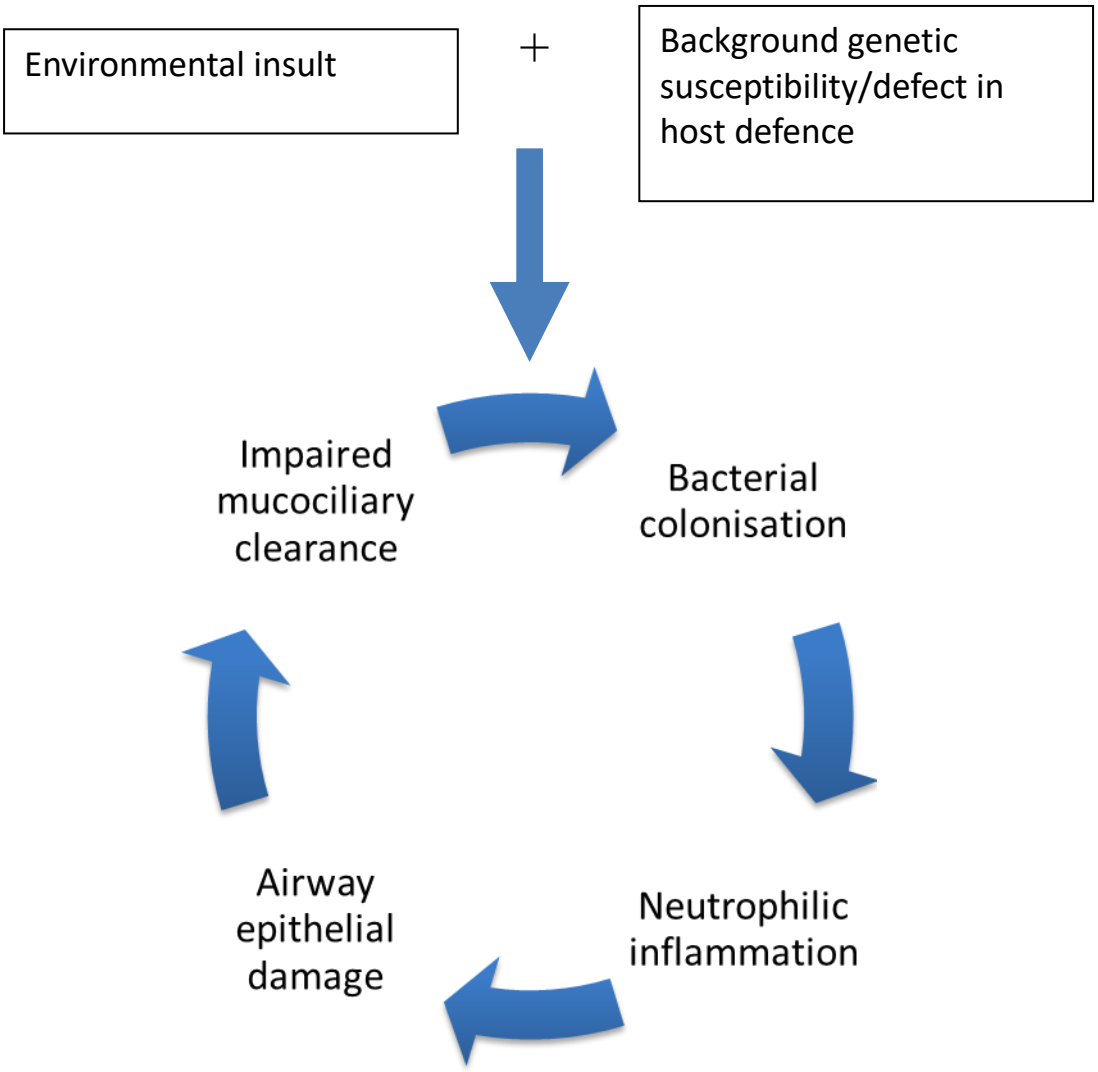


Figure 1.8: The 'vicious circle' hypothesis of bronchiectasis (after Cole (567))

Principle of management	***Specific management points***
Treatment of underlying cause	e.g. allergic bronchopulmonary aspergillosis (ABPA) treatment, immunoglobulin replacement, treatment of rheumatoid arthritis or inflammatory bowel disease
Monitoring of disease activity	• Lung function measured annually (571, 572) • Regular sputum cultures to determine colonising organisms and antibiotic resistance (568)
Airway clearance techniques	• Active cycle of breathing techniques (573) • Postural drainage (574) • Positive expiratory pressure devices e.g. Flutter (575), Acapella (576, 577) devices • High frequency chest wall oscillation devices (578) • Nebulised saline (579, 580)
Antibiotic treatment	Treatment of exacerbations • Definition of 'exacerbation' not universally agreed • No randomised controlled trials of antibiotic treatment for bronchiectasis exacerbations • Consensus opinion currently antibiotic treatment for 14 days (570) • Antibiotic choice based on likely causative organisms and sensitivities • Sputum culture should be sent prior to treatment *P. aeruginosa* eradication • If cultured for first time an attempt should be made to eradicate *P. aeruginosa* (581) Regular prophylactic antibiotic therapy • Patients having ≥3 exacerbations per year requiring antibiotic therapy or those with <3 exacerbations but with significant morbidity should be considered for long term antibiotics (570) such as macrolides (582)

Table 1.9: Principles of management of bronchiectasis

1.6.2.2 Chronic bronchitis

Chronic bronchitis is defined as "the presence of a chronic productive cough for more than 3 months in 2 successive years" (583). It is almost invariably described as a feature of COPD secondary to smoking (583).

a) Epidemiology

The prevalence of chronic bronchitis in the general population is unclear, with many estimates ranging from 3-7% of adults experiencing symptoms (61, 584-588), although higher rates of up to 22% have been reported (589, 590). This uncertainty is probably due to different definitions of the condition, variable reporting of symptoms and the inclusion of subjects in these estimates with other conditions such as bronchiectasis.

It is clear that individuals who are current or ex-smokers are more likely to have chronic bronchitis (61, 584, 587, 588, 590) and patients with COPD have a higher prevalence of chronic bronchitis, with up to 74% affected (591, 592). However, there seems to be a significant proportion of the general population experiencing these symptoms that do not have a formal respiratory diagnosis (587-590) and this group may be at greater risk of morbidity and mortality than healthy subjects. Guerra *et al.* (586) demonstrated that subjects under the age of 50 with symptoms of chronic bronchitis were significantly more likely to develop airflow limitation with increased risk of mortality than subjects without chronic bronchitis.

b) Clinical Presentation

Patients with chronic bronchitis present with a productive cough, although this symptom is often more unpredictable than the classic epidemiological definition of chronic bronchitis with much variation in the pattern of sputum production (593). Due to the large crossover of chronic bronchitis with COPD, many patients present with other features of COPD including dyspnoea and wheeze (594).

Weatherall *et al.* (595) used cluster analysis to classify 175 patients with airways disease into 5 separate phenotypes. They identified a 'chronic bronchitis in non-smokers' phenotype (n=38) with similarities to patients described in Section 3.1. This group tended to have relatively preserved lung function compared to the other phenotypic groups described in the study. However, these patients did not have HRCT scans to rule out bronchiectasis, so it is unclear if this may have been the cause for their symptoms.

c) Pathology

Productive cough in chronic bronchitis is secondary to excessive mucus secretions in the airways. Mucus is present in excessive amounts owing to over-production and hypersecretion from mucus-producing goblet cells and decreased airway clearance mechanisms.

Mucus *overproduction* is caused by exposure to inflammatory stimuli such as cigarette smoke (596, 597) and also viral (598) or bacterial (599) infection which lead to increased transcription of mucin genes due to activation of the epidermal growth factor receptor by inflammatory cells (597, 599). Unlike in asthma, in which mucous metaplasia is known to be a result of Th2 inflammation, the corresponding inflammatory response in COPD causing mucus over-production is not entirely clear, although it is thought to be Th17 mediated (366, 600).

Continual exposure to inflammatory stimuli leads to increased numbers of goblet cells and mucin storage in the airways (598, 599, 601). As the severity of disease, i.e. extent of airway obstruction, worsens in COPD, the degree of mucous metaplasia and occlusion of the small airways by mucus tends to increase (236, 602). Mucus *hypersecretion* is caused by increased goblet cell degranulation due to neutrophil elastase (603).

In conjunction with the increased amounts of mucus secreted into the airways, clearance of this mucus is impaired in patients with established COPD, owing to reduced ciliary function, occlusion of distal airways and respiratory muscle weakness leading to ineffective cough (236, 604).

d) Treatment

Treatment of chronic bronchitis is largely based on treatment of the underlying COPD, as per NICE COPD guidelines (605).

Certain treatment considerations that may particularly apply to patients with chronic bronchitis include the use of mucolytic therapy and judicious use of antibiotic therapy based on sputum colour and culture results. Another promising emerging treatment that has demonstrated efficacy in this patient group is the phosphodiesterase inhibitor roflumilast.

Mucolytic agents are widely prescribed to patients with chronic bronchitis in an attempt to improve their symptoms related to sputum production. The evidence for their use is mixed although a 2012 Cochrane review concluded that they may produce a small reduction in the exacerbation rate of patients with chronic bronchitis and COPD albeit with no difference in quality of life (606).There are some suggestions that chest physiotherapy (607) and inhalation of nebulised saline (608) may be beneficial in patients with patients with COPD but no RCT data assessing the impact of these interventions.

It is generally accepted that for subjects with chronic bronchitis a change in the amount or nature of sputum produced, beyond day-to-day variation, may signify an exacerbation (605, 609) and the production of green (purulent) sputum has been found to be highly sensitive (94.4%) and specific (77%) for the yield of a high bacterial sputum load (610). Guidelines therefore recommend antibiotic treatment following change in sputum quantity or quality (605, 609). Sending sputum for culture undoubtedly has a role in the management of chronic bronchitis, especially when there is a lack of response to an initial antibiotic treatment (609). However, potentially pathogenic bacteria that often permanently colonise

the respiratory tract of symptomatically stable patients with COPD are frequently not isolated on standard sputum cultures (611). These colonising bacteria, most frequently *H. influenzae*, are associated with increased levels of airway inflammation, higher symptom burden and increased risk of exacerbation (612). The lack of sensitivity of standard sputum cultures to detect these bacteria has led to increasing interest in DNA-based bacterial detection techniques (613, 614).

The long term use of low dose azithromycin has demonstrated efficacy in the treatment of patients with COPD with improved quality of life measures and decreased frequency of exacerbations (615). Long term macrolides should be used with some caution however owing to the recognised potential side effects including QT interval prolongation, disturbance of liver function, hearing loss and development of bacterial macrolide resistance (616).

Finally, roflumilast which is a phosphodiesterase 4 inhibitor has been employed. This drug has anti-inflammatory effects in the airways by preventing the breakdown of intracellular cyclic AMP, a substance that when degraded leads to the release of inflammatory mediators (617). Two clinical trials assessing the effects of roflumilast (in addition to either salmeterol or tiotropium) vs placebo in patients with moderate to severe COPD and symptoms of chronic bronchitis both found that roflumilast significantly improved pre-bronchodilator FEV_1 and exacerbation rate (618).

1.6.2.3 Asthma with productive cough

a) Epidemiology

Limited data are available regarding the prevalence of chronic productive cough (or "chronic mucus hypersecretion") in asthmatic patients, but there are reports of a significant subgroup of asthmatics in which these symptoms may be prominent. Two large scale European epidemiological studies reported the prevalence of chronic productive cough (≥ 3 months sputum production for 2 successive years) symptoms in populations of asthmatic non-smokers of 39% (619) and 42% (620). These proportions were significantly higher for smokers with asthma, a finding replicated in a recent cross-sectional study by Thomson *et al.* (621).

b) Clinical Presentation

The symptom of chronic productive cough seems to be associated with an accelerated decline in FEV_1 in asthmatic patients regardless of smoking status (619, 622). Thomson *et al.* found that asthmatic smokers with chronic productive cough had worse asthma control than those without a cough and asthmatic non-smokers with a productive cough had more exacerbations than those without cough (621).

c) Pathology

The cause of chronic productive cough in asthmatic patients is not entirely clear. Possible pathologies underlying this symptom include mucus hypersecretion or chronic bacterial infection/colonisation.

Mucus hypersecretion has long been recognised as a feature of asthma with mucus plugging of the airways acknowledged as a contributing factor in cases of fatal asthma (623). Pathophysiological features of mucus hypersecretion in asthma include goblet cell hyperplasia (624) and submucosal gland hypertrophy (625), both of which lead to increased sputum production. These changes are thought to be driven by Th2 lymphocyte release of cytokines IL-9 (626) and IL-13 (627) as well as mast cell infiltration of submucosal glands, with subsequent mast cell degranulation leading to increased amounts of luminal mucus (628).

Certain groups of asthmatic patients have been identified with stable clinical features of disease that have sputum cultures positive for potentially pathogenic organisms. Studies by Wood *et al.* (629) and Green *et al.* (630) both identified sub-groups of 'stable' asthmatic patients with significant loads of potentially pathogenic bacteria (including *H. influenzae*) in sputum culture with high sputum neutrophil counts. All of the patients within these groups were taking high dose ICS, which have been linked with increased risk of respiratory infection. For example, inhaled fluticasone propionate has recently been shown to increase the risk of lower respiratory tract infections in patients with COPD (631, 632) and also asthma (633). It is possible that ICS lead to chronic bronchitis in some patients by reducing host defence mechanisms, contributing to chronic infection. A more recent investigation by Zhang *et al.* (634) found that 29/56 (52%) of a cohort of patients with severe but stable asthma (and bronchiectasis excluded by HRCT) produced positive sputum cultures, with *H. influenzae* most commonly cultured. Of the 29 patients with positive sputum cultures 23 produced repeat sputum cultures and 16 of these were again positive, with 14 having the same bacteria isolated on both occasions; suggesting these bacteria were colonising the airways. The group with concurrent positive sputum cultures had a significantly longer duration of asthma and a greater number of exacerbations in the preceding year.

d) Treatment

Guidance on the treatment of asthma can be found in the BTS/SIGN asthma guidelines (535).

The association between severe neutrophilic asthma and airway colonisation by potentially pathogenic bacteria (635) may suggest a mechanism for the reduction in asthma exacerbations and lower respiratory tract infections (LRTI) in a sub-group of patients with non-eosinophilic asthma treated with a prolonged course of azithromycin in the AZIZAST study (636). However, this finding is yet to be verified. The use of antibiotics in asthmatics with sputum production as a main symptom should probably be guided by the results of sputum culture if possible, although the limitations of identifying micro-organisms from sputum cultures as described above (Section 1.6.2.2) should be considered.

1.6.2.4 Immunodeficiency

A small group of patients presenting with recurrent LRTI are shown to have immunodeficiencies, including IgG/IgA deficiency or Combined Variable Immunodeficiency (CVID). These patients may present with recurrent but discrete episodes of infection punctuated by periods of recovery, but over time are at risk of developing bronchiectasis (637).

The natural history of the clinical, pathological and radiological features displayed by these patients is unclear. Previous studies have reported significant rates of bronchitis symptoms in patients with primary immunodeficiencies (638-640), but it is uncertain if these patients have symptoms secondary to established bronchiectasis or if they progress through a state of 'pre-bronchiectasis' with bacterial airway colonisation and persistent cough but no significant bronchiectasis on HRCT scan.

1.6.2.5 IgA deficiency

Diagnosis of IgA deficiency has been defined by international consensus as "an IgA level of 0.07 g/L after the age of 4 years in the absence of IgG and IgM deficiency" (641).

a) Epidemiology

Selective IgA deficiency is the most common primary immunodeficiency with a prevalence in Caucasians of between 1/300 and 1/1200 (642).

b) Clinical Presentation

Although the majority of cases (estimated at around 85-90%) are asymptomatic, there are a significant number who develop clinical disease as listed in Table 3.3. This mostly consists of recurrent respiratory tract and gastrointestinal tract infections and autoimmune conditions such as coeliac disease (643). These complications are not unexpected given that IgA is the immunoglobulin found at the highest concentrations in secretions at mucosal surfaces, especially in the gut and respiratory tract (644).

Respiratory tract infections are usually caused by bacteria including *H. influenzae* and *Streptococcus pneumoniae*. Some patients go on to develop bronchiectasis presumably secondary to recurrent infection causing airway damage and scarring (643, 645).

Clinical Manifestation of IgA deficiency	Details
Asymptomatic	85-90% of patients may have no symptoms
Recurrent sino-pulmonary infections	Mostly bacterial e.g. *H. influenzae* and *S. pneumoniae*. May lead to bronchiectasis
Gastrointestinal infections/disorders	Infections include Giardiasis, other disorders linked with IgA deficiency include coeliac disease, lactose intolerance, malabsorption and ulcerative colitis
Allergic disorders	Increased frequency of asthma, atopy, food

	and drug allergies reported
Autoimmunity	Including idiopathic thrombocytopaenic purpura (ITP), haemolytic anaemia, juvenile rheumatoid arthritis and systemic lupus erythematosus (SLE)
Malignancy	There may be an association between IgA deficiency and malignancies including lymphoid and GI malignancies

Table 1.10: The clinical manifestations of IgA deficiency

c) Treatment

In general, IgA antibody replacement therapy is not indicated in patients with IgA deficiency, and such therapy may in fact be harmful (646). However a sub-group of patients with IgA deficiency and recurrent sino-pulmonary infections may benefit from extended courses of prophylactic antibiotics or sometimes intravenous gamma globulin (IVGG) therapy if they have other associated antibody deficiencies (643, 646).

1.6.2.6 Combined Variable Immunodeficiency

CVID is a disease defined by the defective production of immunoglobulins (647). Diagnosis of CVID can be made using internationally agreed diagnostic criteria, of which 1 of the 3 parts required for diagnosis states there should be "hypogammaglobulinaemia with IgG levels two standard deviations below the mean" (647).

a) Epidemiology

The epidemiology of the condition is unclear but the prevalence is thought to be around 1/30000 in Northern European populations (647, 648).

b) Clinical Presentation

Clinically the disease manifests with recurrent respiratory tract infections/pneumonias, progressing later in life to bronchiectasis (638, 649, 650). Patients with CVID may also experience repeated infections of other sites of the body including the skin, soft tissues, nervous system and gastrointestinal tract (638, 649). There is some evidence that asthmatics may be at greater risk of CVID than non-asthmatics, and this has been suggested as a potential reason for the increased risk of respiratory infection noted in asthmatic patients (651).

Respiratory infections are usually caused by encapsulated bacteria, especially *H. influenzae*, *S. pneumoniae* and *Staphylococcus spp*. (638, 652), due to the inability of the immune system to produce IgG antibodies against these pathogens. Usually, the cumulative effect of these repeated infections leads to complications such as empyema, lung abscesses or, most commonly bronchiectasis (638, 650).

However, despite the prominent burden of symptoms this condition can cause the sufferer, there may often be delays in the diagnosis and treatment of the condition due to either a lack of awareness of its existence, or the misperception that the condition only presents in childhood, when in fact the average age of presentation is thought to be around 30 years (638, 639).

c) Treatment

The management of pulmonary complications of CVID usually consists of regular immunoglobulin replacement and suppressive antimicrobial treatment, although there are no RCT data to support these measures (653). Several other interventions to maintain or improve lung function in patients with CVID have also shown some efficacy including the maintenance of higher IgG trough levels, chest physiotherapy techniques including postural drainage, azithromycin and nebulised antibiotics for eradication of *P. aeruginosa* (653).

1.6.2.7 Protracted Bacterial Bronchitis (PBB)

The cohort of patients described in Section 1.6 have many similar features in common with the paediatric diagnosis of protracted bacterial bronchitis (PBB).

a) Epidemiology

PBB is a common diagnosis in children and is thought to account for up to 40% of cases of paediatric chronic cough (654).

b) Clinical Presentation

PBB has been clinically defined as the presence of an isolated chronic 'wet' cough, in the absence of an alternative cause, which resolves with a prolonged course of antibiotic treatment (655). Children with the condition do not usually respond to bronchodilator therapy, but as with the patients described in Section 1.6 are often misdiagnosed as having asthma.

The condition is suspected to be a potential precursor to the development of bronchiectasis in adulthood (654) and some authors have suggested it should be renamed 'pre-bronchiectasis' (656). In retrospective studies the majority of adult patients with idiopathic bronchiectasis give a history of persistent wet cough from childhood (563, 657). There are very few, if any, descriptions of PBB in adults, although one previous study identified 15 adult subjects with chronic productive cough secondary to 'unsuspected bacterial suppurative disease of the airways' and grossly normal HRCT scans (658).

c) Pathology

The pathogenesis of PBB is as yet unclear, but the main finding on investigation of the condition is persistent infection of the airways with bacteria including *H. influenzae, S. pneumoniae and M. catarrhalis* and neutrophilic airway inflammation (659-661). It is thought that bacteria may colonise the airways from the upper respiratory tract following a period of impaired mucociliary clearance, as may occur following a viral respiratory tract

infection. Once present in sufficient numbers in the conducting airways, bacteria (especially non-typeable *H. influenzae*) may form biofilms as a means of defence against airway clearance mechanisms and antibiotics (662).

d) Treatment

Resolution of cough in PBB is typically seen only after a prolonged course of antibiotics (663). RCT evidence suggests treatment with at least 2 weeks of an appropriate agent, although in some cases a longer duration of treatment (4-6 weeks) may be required (664).

1.6.2.8 Summary: Causes of chronic productive cough

There are multiple causes for chronic productive cough with distinct pathologies and features of disease. Possible causes of productive cough in the cohort described in Section 1.6 may include early stage bronchiectasis without radiological changes, "chronic bronchitis" (i.e. excessive mucus production) with or without other features of underlying airways disease in non-smokers, immunodeficiency or an adult version of protracted bacterial bronchitis.

Initial observations suggest that the chronic productive cough in the described cohort responds well to a prolonged course of low dose azithromycin therapy. Hence the literature regarding mechanisms of action and previous use of macrolides in respiratory disease will now be discussed.

1.6.3 Macrolides: mechanisms of action and use in respiratory disease

1.6.3.1 Introduction

Macrolides are a clinically important group of antibiotics characterised chemically by the presence of a macrocyclic lactone ring (665). They exert bacteriostatic effects on a broad range of organisms by interfering with bacterial protein synthesis through binding to ribosomal RNA (666).

In addition to the well documented anti-microbial effects of macrolides, due to their good oral bioavailability, tissue penetration and broad spectrum activity, there is a growing recognition that macrolides also have immunomodulatory and anti-inflammatory properties (667-669). Although the mechanisms of these effects are not yet entirely clear, macrolides have proven efficacy in the treatment of a number of respiratory conditions and have been demonstrated to affect a number of pathophysiological processes that are likely to contribute to ongoing disease.

In this chapter the likely mechanisms of action of macrolides will be outlined followed by the use of macrolides in airways disease to date.

1.6.3.2 Mechanisms of action of macrolides in respiratory disease

The proven effectiveness of macrolides in reducing morbidity in a variety of respiratory diseases has prompted a great deal of research investigating the mechanisms by which they

convey their anti-inflammatory and immunomodulatory effects. Some of the main mechanisms for these effects recognised to date will now be discussed.

a) Anti-inflammatory effects

On neutrophils: Macrolide therapy appears to reduce airway neutrophil accumulation probably through a reduction in the expression of pro-inflammatory cytokines such as IL-8 (670-672). Macrolides have also been shown in a mouse model to reduce airway neutrophilia (673) with significant reductions in IL-1β, an inducer of neutrophil infiltration of the airways (674), and GM-CSF, which is a neutrophil survival factor (675).

On cytokine production: As well as their effect on IL-8, which is a potent chemotactic factor for neutrophils, macrolides have also been demonstrated to down regulate a number of other pro-inflammatory mediators, many of which serve as chemoattractants, survival factors and adhesion molecules for neutrophils. These include TNFα, IL-5 and soluble vascular cell adhesion molecule (sVCAM)-1 (676, 677).

On macrophages: Studies have demonstrated that macrolides are able to reduce by the pro-inflammatory effects of macrophages by switching classically activated M1 macrophage phenotypes to alternatively activated M2 phenotypes (678). Whereas the MI "killer" phenotype, which is activated by bacterial lipopolysaccharide and interferon-γ produces pro-inflammatory cytokines such as IL-6 and IL-12, the M2 "repair" phenotype refers to macrophages involved in tissue remodelling and immunosuppressive responses, which release anti-inflammatory cytokines such as IL-10 (679).

Azithromycin at 'sub-bactericidal' doses has also been shown to enhance clearance of dead (apoptotic) material from the airways through phagocytosis following inflammation (macrophage efferocytosis) in patients with COPD (680). Impaired efferocytosis and an impairment of the ability of alveolar macrophages to phagocytose bacteria has been noted in a variety of airways conditions including COPD (681) and non-eosinophilic asthma (682) and low dose macrolides may help in these conditions by restoring this function.

b) Effects on airway epithelial cells / mucus production

The bronchial epithelium plays a key role in host immunity, secreting cytokines and antimicrobial factors in response to infection and most importantly providing a mechanical barrier to pathogen infiltration (683). The integrity of this barrier is therefore paramount to prevent infection of the respiratory tract and a key component in maintaining this integrity are the 'seals' between intercellular spaces called tight junctions (684).

Several studies have demonstrated how bacteria including *P. aeruginosa* (685, 686) and *Vibrio cholerae* (687) are capable of producing toxins and compounds that compromise tight junction function leading to reduced epithelial integrity and bacterial infiltration. Azithromycin appears to be protective against tight junction rearrangement *in vitro*, which

helps to maintain integrity of the respiratory epithelium leading to greater resistance against pseudomonal infection (688).

Some of the chronic inflammatory conditions reviewed in Section 1.6.2 have the shared pathological characteristic of airway mucus hypersecretion leading to cough productive of sputum. This is due to inflammatory stimuli provoking Th2/Th17 mediated inflammation leading to increased transcription of mucin genes such as MUC5AC and mucus hypersecretion (366, 599, 624, 626). There is some evidence that bacteria including *Chlamydophila pneumoniae* are capable of inducing MUC5AC production in airway epithelial cells through the ERK-NF-κB pathway (689, 690).

Macrolides have been demonstrated to inhibit hypersecretion of mucus from rat nasal epithelial cells (691) and this may be due to downregulation of MUC5AC transcription through reduction of NF-κB activation (689, 690). Azithromycin directly inhibits hypersecretion of mucus from airway epithelial cells by inhibiting TNFα induced MUC5AC secretion from airway and human nasal epithelial cells (692).

c) Effects on Pathogens

Most of the work assessing the effects of macrolides on bacteria has focussed on the organism *P. aeruginosa*, one of the most virulent respiratory pathogens which has developed a number of methods to evade antibiotic treatment. Although *P. aeruginosa* often possesses a natural resistance to the antibiotic effects of macrolides, several 'non-antibiotic' mechanisms have been demonstrated through which macrolides may disrupt the colonisation and establishment of pseudomonal communities in the airways.

The process of *P. aeruginosa* infection, colonisation and biofilm formation will now be outlined in brief, followed by a summary of the mechanisms through which macrolides may disrupt this process at various different stages.

d) Pseudomonas infection and colonisation

Following entry to the lung, pseudomonal infections establish as the organism, which is able to mobilise owing to its tail-like flagellae and hair-like fimbriae, adheres to the respiratory epithelium via adhesion molecules such as lectins (693). Once adhered, it releases toxins, causing tissue damage, loses its flagellae and fimbriae and begins to produce a polysaccharide which will eventually form the matrix of a protective structure known as a biofilm (693). During biofilm formation and establishment, organisms are able to communicate with each other to co-ordinate the expression of certain genes such as tissue-damaging factors via a process known as quorum sensing (694).

Macrolides can affect this process in the following ways:
i) Mobility: Sub-inhibitory concentrations of macrolides including azithromycin seem to decrease motility of *P. aeruginosa* due to disruption of flagellae and fimbriae formation

(695, 696). This reduced mobility leads to an increased rate of phagocytosis by alveolar macrophages.

ii) Bacterial Adherence: Macrolides have been demonstrated to have significant effects on the adherence of *P. aeruginosa* to airway epithelial cells, which is a crucial stage in the establishment of bacterial infection. Both an *in vitro* investigation of pseudomonal adherence to collagen before and after erythromycin (697) and a study of the adherence of a *P. aeruginosa* strain to the buccal mucosal cells of patients with CF before and after azithromycin treatment (698) showed decreased adherence of the organism following macrolide treatment.

iii) Toxin release: The bacterial virulence of *P. aeruginosa* is determined partly by the bacterial toxins these micro-organisms release; these include the cytotoxic enzymes alkaline protease, elastase, exotoxin A and phospholipase C. Several macrolides including erythromycin and azithromycin suppress the release of these enzymes resulting in decreased bacterial virulence and tissue damage (699, 700).

iv) Biofilm construction: The formation of biofilms by *P. aeruginosa*, as well as other organisms such as *H. influenzae* can be disrupted by macrolides (695, 701). *In vitro* studies suggest that this may be due to the inhibition of production of polysaccharides (702, 703).

v) Quorum sensing: The effect of macrolides on quorum sensing is not yet clear, but it is thought that they may suppress transcription of quorum sensing genes resulting in reduced production of quorum sensing virulence factors (704, 705).

1.6.3.3 Use of macrolides in respiratory disease

a) Diffuse Panbronchiolitis

One of the first and most notably successful uses of macrolides in respiratory disease was in the treatment of Diffuse Panbronchiolitis (DPB). DPB is a chronic idiopathic condition which almost exclusively affects East Asians characterised by neutrophilic inflammation of the respiratory tract. The disease may progress if untreated to destruction of lung parenchyma and early mortality (706, 707).

From the late 1980s onwards courses of long term macrolide therapy were used to treat the condition with a resultant improvement in 5 year prognosis from around 63% in the 1970s to around 90% in the 1990s (708). The macrolide originally chosen for treatment of DPB was erythromycin, but similar benefits have been found with other macrolides including azithromycin and clarithromycin (709).

Although there are a large number of studies reporting significant improvement of DPB with macrolide therapy, a recent Cochrane review (710) in the subject did not find comprehensive evidence to substantiate their use for this purpose owing to a lack of large RCTs. Only one of the studies assessed in the review was deemed to be of sufficient quality

to include, albeit with significant methodological limitations (711). However, despite these reservations and the small number of participants in the trial the results of this study were compelling. This was because all of the 12 patients randomised to receive low dose erythromycin treatment showed improvement on their post-treatment CT scans compared to none of the seven patients who received no treatment, 5 of whom actually showed progression of DPB on their second CT scan.

b) Bronchiectasis

Bronchiectasis is a condition that has historically been treated with long courses of antibiotics in order to improve the persistent symptoms of cough with production of sputum positive for bacteria. Clinical trials of long term antibiotics in patients with bronchiectasis were first conducted in the 1950s, with seemingly positive results. These included reduced sputum volume and reduced number of days off work in a group of bronchiectatic patients taking a year-long course of oxytetracycline compared to the placebo group (712). However, no formal statistical analysis on these data was ever performed.

Since this initial study, many investigators have conducted clinical trials in patients with bronchiectasis to assess the effect of various antibiotics including oral amoxicillin (713) and nebulised tobramycin (714, 715), gentamicin (716) and ciprofloxacin (717). These studies produced mixed results, although in general seemed to demonstrate that long term non-macrolide antibiotics decreased exacerbation frequency and reduced bacterial load in non-CF bronchiectasis, with no effect on pulmonary function.

Koh *et al*. (718) performed one of the first trials of macrolides in subjects with bronchiectasis in a double-blind placebo-controlled RCT of roxithromycin in 25 children with bronchiectasis. The primary outcome for the trial was AHR as measured by MCT, but it should be noted that one of the entry requirements for the study subjects was increased AHR at baseline. After 12 weeks of roxithromycin treatment (4 mg/kg twice daily), AHR was found to be significantly reduced in comparison with the placebo group and there was also an improvement in sputum features (sputum purulence and leucocyte scores). Despite the positive results of this study, the primary outcome of AHR was a strange endpoint to measure in subjects with bronchiectasis, as this is not one of the key pathological features of the disease. Also, due to the intentional selection of children with significant AHR prior to treatment, the generalisability of these results may be in question.

Tsang *et al.* (719) conducted a small double-blind RCT of 8 weeks of low dose erythromycin (500 mg twice daily) vs placebo in 21 patients. The erythromycin group demonstrated a significantly improved FEV_1, FVC and 24 h sputum volume compared to the placebo group. No significant difference was found following erythromycin in any of the multiple measurements taken from sputum including sputum pathogens, leucocyte count, IL-1α, IL-8, TNFα or leukotriene B4.

Cymbala *et al.* (720) carried out a pilot study of 11 patients with bronchiectasis comparing exacerbation frequency of patients on their usual medications vs those taking additional

azithromycin. Despite the low number of patients in the study, azithromycin was found to significantly reduce the incidence of exacerbation and 24 h sputum volume, although there was no discernible change in lung function or peak flow recordings.

Yalcin *et al.* (721) studied the effects of 3 months of low dose clarithromycin treatment (15 mg/kg once daily) on inflammatory markers in bronchiectasis in a double-blind placebo-controlled trial of 34 children. The inflammatory markers assessed were IL-8, TNFα, IL-10 levels and cell profiles in BAL fluid as well as pulmonary function and amount of sputum production. Significantly reduced levels of IL-8, total cell count and neutrophil ratios in BAL fluid and daily sputum production were found in the clarithromycin group in comparison to the placebo group, although there was no difference in pulmonary function.

Diego *et al.* (722) carried out an open label study of azithromycin treatment in patients with bronchiectasis (n=30) to determine its effect on markers of airway oxidative stress in exhaled breath condensate (EBC). The selected markers included FE_{NO}, 8-isoprostane, pH, nitrites and nitrates. Patients were allocated to treatment with azithromycin (250 mg three times weekly) for 3 months or to a control group who received no intervention. There was no significant difference in the markers of airway oxidative stress between the 2 groups at the end of the study, although some of the secondary outcomes such as sputum volume, number of exacerbations and St George's respiratory questionnaire (SGRQ) symptom score were significantly improved in patients in the azithromycin group.

The best evidence so far for the use of macrolides in bronchiectasis comes from three large scale clinical trials carried out in the last few years.

The first of these by Wong *et al.* (723) was a multicentre double-blind RCT of 141 patients with bronchiectasis (confirmed by HRCT scan) who were assigned to receive 500 mg of azithromycin or placebo three times a week for 6 months. The co-primary endpoints of the study were exacerbation rate, FEV_1 and SGRQ score. After the 6 month treatment period, a significantly lower rate of exacerbations occurred in the azithromycin group in comparison to the placebo group, although there was no significant difference between the two groups in FEV_1 and symptom scores. Sputum microbiology at baseline and after treatment was also documented, and although bacterial resistance to macrolide was not tested routinely, two patients in the azithromycin group developed macrolide resistant *S. pneumoniae* following treatment.

Altenburg *et al*. (724) also conducted a multicentre double blind RCT of azithromycin vs placebo. This trial comprised fewer participants (83) but assessed the effect of azithromycin (250 mg once daily) over a longer 12 month period, and the primary endpoint to the study was also exacerbation rate. Again, the study demonstrated a reduction in the number of exacerbations in the group treated with azithromycin compared to the placebo group. However, another significant finding was the increased bacterial macrolide resistance rate in the azithromycin group with 88% of cultured organisms from the treatment group demonstrating macrolide resistance compared to 26% of those from the control group.

Lastly, Serisier *et al.* (725) have evaluated the use of low dose erythromycin therapy over a 12 month period in a double-blind placebo-controlled RCT in 117 patients with bronchiectasis. The primary outcome measure was exacerbation rate and secondary outcome measures included lung function and macrolide resistance rate of oropharyngeal bacteria. Erythromycin was found to significantly decrease exacerbation rate as well as 24 h sputum production with a borderline significant improvement in lung function compared to placebo. This study also provided evidence of increasing bacterial macrolide resistance as a result of long term macrolide therapy. The proportion of macrolide-resistant oropharyngeal streptococci isolated from patients in the azithromycin group (median change of 27.7%) was significantly increased in comparison to those from the placebo group (median change 0.04%).

c) COPD

There is an abundance of trial data regarding the use of long term macrolide therapy in COPD and only a brief summary of the evidence to date is reported here. A number of studies conducted prior to 2001 on this subject are not reviewed owing to the significant heterogeneity in patient groups. For example, spirometric criteria were often not used to diagnose COPD, antibiotics were taken for short time periods and some of the studies were simply of poor quality.

Suzuki *et al.* (726) conducted an unblinded RCT investigating the effect of erythromycin treatment (200-400 mg once daily) over a 12 month period in 109 patients with COPD. Outcome measures were the frequency of COPD exacerbations and the frequency of episodes of common cold. Patients in the erythromycin group experienced significantly fewer COPD exacerbations and episodes of the common cold than the placebo group and the rate of hospitalisation was reduced. However, the lack of blinding in this study is a potential source of bias in these results.

Banerjee *et al.* (727) carried out a double-blind placebo-controlled RCT examining the effect of 3 months clarithromycin (500 mg once daily) on the health status, sputum bacterial load and exacerbation rate of 67 moderate to severe COPD patients. None of these measures were improved in comparison to those found in the placebo group. This trial had the shortest duration of treatment of those included here, and this may have influenced the results.

Seemungal *et al.* (728) in another double-blind RCT, this time assessing 12 months of erythromycin (250 mg twice daily) in 109 patients with moderate COPD, found that there was a significant reduction in the exacerbation rate in the erythromycin group in comparison to the placebo group. Erythromycin had no effect however on FEV_1, serum or sputum inflammatory markers or bacterial composition of sputum. A smaller RCT by He *et al.* (729) (n=36) also examining the role of erythromycin treatment (125 mg three times daily) over a 6 month period similarly found a lower exacerbation rate in the erythromycin group. Another finding from this trial was a decreased sputum neutrophil and neutrophil elastase count in the erythromycin group in comparison to the placebo group.

A further double-blind RCT by Mygind *et al.* (730) assessed the use of azithromycin in 575 COPD patients using an intermittent or 'pulsed' dosing regimen over a 3 year time period. Azithromycin, at a dose of 500 mg per day, was given for 3 days every month over the 36 month treatment period and its effect on exacerbation frequency and duration, hospital admission, quality of life, pulmonary function and mortality determined. The azithromycin group experienced significantly fewer total days of exacerbation and required significantly fewer antibiotics and systemic steroids than the placebo group, although there was no difference in terms of pulmonary function, quality of life or mortality.

Finally, the most compelling evidence so far for the use of macrolides in COPD comes from a large scale clinical trial carried out in 2011 by Albert and co-investigators (615). This multi-centre double-blind RCT assessed the use of azithromycin (250 mg daily) over a 12 month period. The trial included 1142 patients at risk of acute exacerbations of COPD, 570 of which received azithromycin whilst the other 572 received placebo in addition to standard care. The primary outcome for the study was time to first exacerbation which was significantly increased in the azithromycin group compared to the placebo group (226 days vs 174 days). Azithromycin also reduced the frequency of exacerbations and significantly improved quality of life scores, although there was no effect on hospitalisation or mortality. However, unwanted effects of azithromycin were also noted including an increased bacterial macrolide resistance rate and increased hearing loss in the azithromycin group.

d) Asthma

The first reported use of macrolides as an anti-inflammatory agent for use in the management of asthma was in the 1960s and was conducted using troleandomycin. This drug was investigated for some decades as an additional therapeutic agent for asthma owing to its apparent "steroid sparing" effect. Although early trials showed promising results (731), recognition of hepatic adverse effects (732) and a systematic review of available trial data showing no benefits in terms of steroid dose reduction or lung function (733) seem to have limited any potential further use of this agent.

Several studies have investigated the potential of clarithromycin therapy in asthma, with mixed results. Gotfried *et al.* (734) conducted a double-blind RCT of clarithromycin versus placebo in 21 oral corticosteroid dependent asthmatics. Although the mean prednisolone requirement of the clarithromycin group decreased by 30%, they showed no improvement in lung function, asthma quality of life or symptom scores compared to those in the placebo group.

Kostadima *et al.* (735) reported an improvement in AHR with the addition of short term clarithromycin treatment (250 mg twice daily or 250 mg three times daily) vs placebo to adult asthma patients on moderate doses of inhaled budesonide, although again there was no significant improvement of lung function.

Simpson *et al.* (670) studied 45 patients with severe refractory asthma in a double blind placebo-controlled RCT evaluating clarithromycin (500 mg twice a day for 8 weeks). The primary outcome measure for this study was sputum IL-8 concentration, as IL-8 is a potent chemotactic factor which attracts and activates neutrophils, the levels of which are elevated in non-eosinophilic asthma (NEA). After 8 weeks of clarithromycin therapy sputum IL-8 levels, IL-8 gene expression and neutrophil activation (as measured by sputum neutrophil elastase levels) were significantly reduced in the clarithromycin group. Levels of these mediators were lower in the subgroup of patients with NEA, suggesting an anti-inflammatory mechanism by which clarithromycin may have effect in this sub-group. The clarithromycin group also demonstrated a significant improvement in quality of life scores, and this effect was most profound in the NEA sub-group, although there was no significant improvement in presence of symptoms or asthma control score.

A number of trials of macrolides in subjects with asthma have been conducted, since there is evidence implicating the presence in the airways of the atypical bacteria, such as *Mycoplasma pneumoniae* and *C. pneumoniae*, in the pathogenesis of asthma. The objective was to determine whether subjects with microbiological evidence of these bacteria in the airways formed a sub-group of asthmatics whose disease would respond to macrolides owing to their antibiotic properties.

Kraft *et al.* (736) conducted a double-blind RCT of clarithromycin (500 mg twice daily for 6 weeks) in 55 subjects with chronic stable asthma. Subjects were assessed pre and post treatment for the presence of *M.* and *C. pneumoniae* in their airways via PCR of BAL samples, in conjunction with standard microbiological culture. In all, 55% of patients were PCR positive for *M. or C. pneumoniae* (although interestingly all were culture negative), and only these positive subjects responded to clarithromycin with improvement in their lung function (as measured by FEV_1).

A similar larger double-blind RCT (n=92) by Sutherland *et al.* (737) which also assessed PCR positivity for *M.* or *C. pneumoniae* failed to replicate these results and found no improvement in lung function or asthma control in the clarithromycin group. Interestingly PCR negative patients showed an improvement in AHR which was not seen in PCR positive patients alone.

Data from trials using roxithromycin in asthma are also inconclusive. Shoji *et al.* (738) failed to show any difference in lung function or AHR in a small RCT (n=14) of subjects with aspirin-sensitive asthma receiving roxithromycin (150 mg twice daily), although the roxithromycin group showed some improvement in asthma symptom score.

Black *et al.* (739) in a large multicentre RCT (n=232) of asthma patients with serological evidence of *C. pneumoniae* (Raised serum IgG or IgA titres against *C. pneumoniae*) receiving roxithromycin (150 mg twice daily for 6 weeks) found a significant improvement in evening PEFR readings post treatment. However, no improvement in morning PEFR values or asthma

symptom scores was found and at 6 months follow-up, the improvement in evening PEFR had returned to baseline.

One of the largest studies of macrolides in subjects with asthma carried out to date was the 'Telithromycin in Acute Exacerbations of Asthma' (TELICAST) study (740). The objective of this study was different to those discussed above, as it examined the effect of short term courses of telithromycin in patients with asthma exacerbations. However, owing to its large size (n=278) and findings it merits some further discussion here. The primary endpoints determined were asthma symptom scores and morning PEFR values. This study also attempted to ascertain subjects' infection status with *M.* and *C. pneumoniae* by culture and PCR of sputum or nasopharyngeal samples in conjunction with serological evidence of *M.* or *C. pneumoniae* infection (i.e. raised serum IgG, IgM or IgA titres against *M.* and *C. pneumoniae*). In comparison to the placebo group, subjects in the telithromycin group reported a significant improvement in asthma symptom scores, although there was no difference in morning PEFR. Baseline FEV_1 in the telithromycin group appeared significantly improved in comparison to the placebo group but this was a secondary outcome. No relationship was observed between *M.* or *C. pneumoniae* infection status and treatment response.

Azithromycin has also been considered as an additional treatment in asthma. Strunk *et al.* (741) investigated its use as a steroid-sparing agent in children with moderate to severe asthma. The design of the study included a 6 week run-in period in which the budesonide dose needed to achieve stable asthma control was determined before randomisation to azithromycin or montelukast. Unfortunately this trial had to be stopped early due to lower than expected recruitment and the difficulty pre-randomisation of stabilising the child subjects' asthma control. This was either due to non-adherence with treatment before randomisation or an improvement in asthma control under medical supervision suggesting non-adherence prior to trial entry and hence less severe disease than previously thought. A futility analysis of the subjects recruited suggested that azithromycin was unlikely to have a steroid-sparing effect.

Hahn and colleagues (742) carried out a placebo-controlled RCT in stable asthma patients (n=45) evaluating the use of azithromycin (600 mg for 3 days, then a further 600 mg weekly for 5 weeks). *C. pneumoniae* serology was also assessed. Patients in the azithromycin group with high titres of IgA against *C. pneumoniae* reported some symptomatic improvement using an unvalidated symptom score, but there was no significant difference between azithromycin and placebo groups using the AQLQ (743).

Hahn and the 'Azithromycin Asthma Trial in Community Settings' (AZMATICS) study group performed another RCT using azithromycin in adults with persistent asthma symptoms (744). There were three treatment arms in the study of patients randomised to azithromycin or placebo with a 3rd group of patients taking azithromycin on an open label basis. The group randomised to azithromycin showed no statistically significant improvement in asthma outcomes compared to placebo, although the study was underpowered to detect a

significant difference in its primary outcome (AQLQ scores). The open label group however, who had greater disease severity than subjects randomised to azithromycin which was usually treatment refractory, demonstrated improvements in asthma symptoms, asthma quality of life scores and control. This improvement appeared to persist after completion of azithromycin therapy.

Finally, and perhaps most importantly, the most recent trial data for azithromycin use in asthma comes from the AZIZAST study by Brusselle *et al*. (636). This multicentre double-blind RCT compared the efficacy of azithromycin vs placebo in prevention of asthma exacerbations in severe asthmatics (as an additional treatment to inhaled corticosteroids and LABA) over a 6 month period. Although there was no significant difference in the rate of exacerbation between the azithromycin and placebo groups, a predefined subgroup analysis stratifying patients by inflammatory phenotype determined a significant reduction in exacerbation rate in patients with a non-eosinophilic asthma subtype.

1.6.3.4 Macrolides in Chronic Cough

Two studies have assessed the use of macrolides in chronic cough. An RCT by Yousaf *et al.* (745) evaluated the effect of 12 weeks of low dose erythromycin therapy in 30 subjects with chronic cough. Chronic cough was defined as a cough lasting ≥8 weeks in a subject with normal spirometry, a PC_{20} >8 mg/mL, a normal sputum eosinophil count and HRCT scan whose symptoms had failed to improve despite trials of treatment for GORD and PNDS. There was no significant difference in the primary outcome measure of 24 h cough frequency (as assessed by a cough monitor) or any other measures except for sputum neutrophil count, which decreased significantly in the erythromycin group compared to the placebo group (mean difference 16.8%; 95% CI 1.6 to 32.1; p=0.03).

Hodgson *et al*. (746) studied the effect of 12 weeks of low dose azithromycin in an RCT of 44 subjects with chronic cough. Subjects had ongoing cough with normal spirometry and HRCT. All subjects underwent a MCT and sputum induction prior to entering the trial and underwent a treatment trial of 2 weeks of oral steroid treatment if they had a positive MCT or sputum eosinophils >3%. Subjects who did not have sputum eosinophils >3% or a positive MCT and those who failed to respond to oral steroids also received treatment trials for GORD or PNDS if symptomatic. If their cough symptoms were refractory to these interventions they were entered into the study. There was a clinically important improvement in Leicester Cough Questionnaire (LCQ) score in the azithromycin group (mean change 2.4; 95% CI 0.5 to 4.2) but the difference between azithromycin and placebo groups was not significant. When the characteristics of the responders to azithromycin were assessed, a large significant improvement in LCQ score was noted in subjects with a concurrent diagnosis of asthma (mean change 6.19; 95% CI 4.06 to 8.32), implying azithromycin may be useful in the treatment of chronic cough associated with asthma.

1.6.3.5 Summary: Macrolides in Airways Disease

Macrolide antibiotics are a clinically useful class of antibiotics, which are already used widely for their antibacterial properties. Evidence is accumulating of their useful anti-inflammatory and immunomodulatory effects which may explain their proven benefit in a growing number of chronic inflammatory respiratory conditions including DPB, COPD and bronchiectasis. Macrolides also appear to benefit subgroups of asthmatic patients with non-eosinophilic asthma and chronic cough, for whom there are currently few treatment options available.

1.7 Microbiota in Asthma

1.7.1 Introduction

The human body is host to trillions of resident microbes, which outnumber our own cells and have a significant influence on many aspects of human physiology. Collectively, these micro-organisms make up what has been termed the human "microbiota". Recent advances in DNA sequencing technology allowing fast and accurate assessment of these complex microbial communities have led to a rapidly growing interest in this area, with investigators attempting to discover the significance of the "microbiota" in states of health and disease.

1.7.2 Microbiota in health

The human microbiota is comprised of vast numbers of micro-organisms that colonise the skin and mucosal surfaces of the body. In the human gut alone there are estimated to be as many as 100 trillion microbes of over 1000 different species (771). Other body sites with distinctive microbial commensal populations include the skin (772, 773), oral cavity (774) and nasopharynx (775), urogenital tracts (776) and the lower respiratory tract (777).

The term "microbiota" encompasses all of the micro-organisms including bacteria, viruses and fungi found in a specified location, be that the human body as a whole, or the mucosal surface of a particular organ. As yet few studies have attempted characterisation of the viral or fungal components of the microbiota owing to their relative scarcity in comparison to the bacterial element and also a relative lack of expertise in their detection and classification. Therefore further discussion of the microbiota will be based on current knowledge regarding the composition of bacterial communities at sites within the human body.

The composition of the microbiota is usually described using well defined microbial ecological terms and an increasing number of statistical measures. Familiarity with some of the basic ecological and statistical terms used in this field is therefore essential to understand descriptions of the human microbiota to date and a brief glossary of these terms is provided in Table 1.11.

Term	Definition
Microbiome	The organisms, collective genomes of all these organisms and environmental conditions in a specified microbiota
Metagenome	The collection of genomes and genes in a specified microbiota
Operational Taxonomic Unit (OTU)	A cluster of DNA/RNA sequences that share more than a specified level of similarity (97%) which would be expected to correspond to a particular species
Richness	The number of different types of organisms present in a sample
Abundance	The relative representation of an organism

	in a sample (Relative species abundance = The number of organisms in one group/the total number of organism in all groups)
Evenness	The distribution of organisms across types
Diversity	A combination of richness and evenness to form a summary statistic measuring the variety present in a community
Resilience	The rate at which a community recovers to its native structure following a perturbation
Resistance	The ability of a community to resist change to its structure following an environmental challenge

Table 1.11: A glossary of common microbial ecological terms used in discussion of the microbiota

1.7.3 Gastrointestinal (GI) microbiota

There are an estimated 10^{14} bacteria in the GI tract (778). Owing to these vast numbers of bacteria and their significant role in the development of the immune system, the majority of the current knowledge regarding the importance of the microbiota in health and disease comes from studies relating to the microbiota of the gut. As such, it is important to recognise some of the significant findings from this field and how they might influence and impact upon studies involving the airway microbiota. These include observations regarding the emergence of a bacterial community, the development of this community over time and the effect of perturbations on the community structure.

Establishment of the human microbiota begins at birth with bacterial colonisation of the newborn gut occurring hours after delivery, with the mechanism of delivery immediately affecting the initial microbiota composition. Children born by vaginal delivery seem to develop a GI microbiota redolent of the vaginal flora of their mother, whereas children delivered by Caesarean section develop a gut microbiota consisting of organisms mostly found on the skin (779). Diversity of the infant GI microbiota gradually increases over time and is influenced by factors including type of feeding (breast vs formula milk feeding), infant hospitalisation and antibiotic use (780).

As the interaction between the gut and the intestinal microbiota plays a critical role in the development of the immune system, it has been speculated that differences in the microbiota at this time of life may lead to disrupted immunotolerance of certain micro-organisms and the development of allergic diseases including asthma in later life (781).

This period of gradually increasing microbial diversity and "training" of the developing immune system lasts for around 3 years (782) after which the "core" members of the GI microbiota are established, and become less sensitive to perturbation, more closely resembling the adult gut microbiota (783).

Once established, the adult GI microbiota is thought to be fairly stable, demonstrating high levels of resilience to serious perturbations, including high dose antibiotic treatment and significant dietary changes (784, 785). However, following recurrent "high-impact" events such as repeated courses of high dose antibiotics the bacterial community may become permanently disordered (786), leading to the overgrowth of opportunistic strains of bacteria. This may lead to the overgrowth of opportunistic bacteria such as *Clostridium difficile* in the intestine following extended periods of antibiotic treatment, which aggressively colonises mucosal surfaces at the expense of typical commensal bacteria, and causes overt symptoms of colitis (787, 788).

This disruption in the balance of a bacterial community leading to overgrowth of certain species which may in turn cause further disruption of other smaller, more beneficial members of that community is known as dysbiosis. Dysbiosis of the GI microbiota has been linked to a variety of diseases including inflammatory bowel disease (789), type 2 diabetes (790), bowel cancer (791) and obesity (792).

1.7.4 Airway microbiota

Although the GI tract microbiota is the largest and most complex in the body there is a growing recognition of the potentially significant influence of the composition and interactions of the microbiota at other mucosal surfaces, including that extant within the airways.

Classically, except in advanced stages of airways disease such as bronchiectasis or cystic fibrosis, the lower respiratory tract was considered to be sterile. The first study to demonstrate this was not the case by Hilty *et al.* (777) used non-culture bacterial DNA detection techniques on samples taken at bronchoscopy to elucidate that the airways of the lungs are not sterile even in healthy subjects and that there appears to be a microbiota unique to the lower airways. Subsequently it has been determined that the bacterial biomass of the lower airways in healthy subjects is comparatively low with studies demonstrating a BAL bacterial load of $10^{3\text{-}4}$/ml (793, 794). It is unclear whether or not the airway microbiome in healthy subjects is resident and distinct, or whether it simply consists of a transient collection of organisms aspirated from the upper airways (795, 796).

The Hilty study also demonstrated a significant difference in the bacterial communities present in the lungs of healthy subjects as compared to those detected in COPD patients and asthmatic patients treated with high dose inhaled steroids. A much higher frequency of the potentially pathogenic *Haemophilus* species, including the organism *H. influenza*e which is one of the most common pathogens isolated in respiratory tract infections, was detected in samples from the bronchi of asthmatic and COPD patients in comparison to control subjects, who were more likely to be colonised with multiple species of *Prevotella*.

Following this finding by Hilty and colleagues the number of studies of the lung microbiota in different disease states has expanded rapidly. A summary of the results so far based on a full literature review of this area will now be presented, followed by a more in depth look at the studies to date that have assessed the significance of the microbiota composition in asthma.

1.7.5 Microbiota in Cystic Fibrosis (CF)

Owing to the well-recognised role of colonising micro-organisms in morbidity and mortality in cystic fibrosis, many different investigators have studied the lung microbiota of CF patients in the hope of further understanding the dynamics of the CF lung bacterial community and its role in the clinical features and progression of the disease. A table detailing the full literature review can be found in Appendix I. The bacterial load in the CF lung is many orders of magnitude higher than that of the healthy lung at around 10^9/ml (797). Clearly, therefore, there is a significant difference in the airway microbiota between health and disease states but the processes that lead to this great disparity are not yet fully understood. It is likely that the physicochemical changes within the airways in individuals with CF as the disease progresses serve to create ecological niches that favourably support the growth of certain organisms (798). Such changes include the increasing amount and tenacity of respiratory secretions, airway wall inflammation and damage with subsequent development of bronchiectasis and potentially effects of treatments for the condition including antibiotics and steroids (799). However, the extent to which the microbiota composition is determined by such processes and to which the selected micro-organisms are then able to further alter the characteristics of their environment and actively cause disease progression is unclear.

Some general concepts have emerged from studies of the CF microbiota to date which may help to guide further work relating to the airways microbiota.

Firstly, as recognised previously in studies of the GI microbiota, the CF microbiota seems to be fairly stable and resilient to short term perturbation such as antibiotic treatment or disease exacerbation (800-803). Over the longer term i.e. ≥5 year periods, the community diversity of the CF microbiota in patients with progressive disease may decrease significantly but tends to remain relatively stable in patients with a mild lung disease phenotype (801, 804). It has been suggested that antibiotic use is the primary cause of any decreasing diversity over time, rather than age or lung function (801).

Secondly, also in common with previous GI microbiota studies, multiple investigators have found that the variability between the lung bacterial communities of different subjects (inter-subject variability) is greater than the variability between longitudinal samples from the same subject (intra-subject variability) (800, 801). This indicates that although subjects with the same disease process may have similarities in the composition of their lung microbiota, such that the bacterial communities in samples from these subjects could broadly be distinguished from those from healthy controls or subjects with other disease processes, each individual possesses their own unique microbiota.

Several studies of the CF microbiota have demonstrated that decreased richness of the bacterial community of the CF lung is associated with decreased lung function (805-807). Zemanick *et al.* (808) found a significant negative correlation between bacterial diversity and relative abundance of *Pseudomonas spp.*, which as expected, was found to be the most dominant organism in virtually all patients in CF microbiota studies to date.

Finally, several investigators have assessed the composition of the airway microbiota prior to and during disease exacerbations and also tried to determine how the microbiota is affected by antibiotic treatment. Generally these studies have showed decreased abundance of Pseudomonas spp. with antibiotic treatment but no change in the overall bacterial community composition (797, 800, 808).

1.7.6 Microbiota in COPD

Following the original description by Hilty *et al.* of the distinct bacterial microbiome of the COPD lung in comparison to that of healthy controls a number of further studies have attempted to characterise the bacterial COPD microbiota in greater detail. A table detailing the full literature review can be found in Appendix J.

Erb-Downward *et al.* (809) compared the bacterial microbiota isolated in bronchial lavage (BAL) samples from healthy non-smoking controls (n=3), healthy smokers (n=7) and COPD patients (n=8) with that detected in lung tissue samples from patients with severe COPD (n=6) . They found that the diversity of the bacterial microbiota was similar in the non-smoking and smoking controls groups to that of patients with mild COPD whereas the microbiota in moderate to severe COPD patients was much less diverse and was more commonly dominated by *Pseudomonas spp*. Despite these differences in diversity, the total bacterial load in each subject was not significantly different. Another interesting finding from this study was the heterogeneity of the bacterial communities between different anatomical sites in the same lung from patients with severe COPD. The authors suggested this may be due to either local differences in lung airway microarchitecture leading to the favourable development of certain bacteria or the anatomical heterogeneity in the development of the disease meaning areas with different amounts of inflammation and/or tissue damage may favour the growth of particular species.

Huang *et al.* (614) attempted to characterise the bacterial composition of endotracheal aspirates from mechanically ventilated severe COPD patients using a bacterial 16S PhyloChip. Although this study included only 8 patients in total, two distinctly different bacterial populations were detected from the samples; a ‘more diverse’ and a ‘less diverse’ population. The ‘less diverse’ population identified in samples from 4 of the patients tended to contain more members of the *Pseudomonadaceae* group (containing *Pseudomonas spp.*) and these patients had been intubated for significantly longer than the others. The ‘more diverse’ population identified in samples from 3 of the patients intubated for a shorter duration demonstrated an increased abundance of the phylum *Firmicutes*.

In order to negate the effect of contamination from the upper airways on estimates of the lung microbiota, Sze *et al.* (810) analysed the microbiota present in lung tissue samples from patients with severe COPD (n=8), CF (n=8), smokers (n=8) and non-smokers (n=8). This study confirmed there were significant differences in the bacterial microbiota found in the COPD lung compared to the lungs of healthy controls. There was also a significant difference in the bacterial communities of the COPD and the CF lung, with a higher bacterial density and lower diversity of organisms in the CF subjects, although both of these groups had relatively high abundance of the *Firmicutes* phylum in comparison to controls. Overall the lung tissue

samples had lower bacterial densities than those obtained by bronchial lavage or bronchial brushing.

Pragman *et al.* (811) assessed the microbiota present in BAL samples from 22 patients with moderate to severe COPD and 10 healthy controls. This study again demonstrated a distinct bacterial community in the lungs of COPD patients compared to healthy controls, but was unable to discern any significant difference between COPD patients of different severity. There were consistent differences in microbiota composition between patients who used inhaled corticosteroids or bronchodilators and those who did not, although causality could not be determined due to the cross-sectional nature of the study.

1.7.7 Microbiota in Asthma

Bacteria have long been implicated in the pathogenesis of asthma (812) especially atypical organisms such as *Mycoplasma spp.* and *C. pneumoniae* (813).

As discussed above Hilty *et al.* (777) detected a much higher frequency of *Haemophilus* spp. in samples from the bronchi of asthmatic and COPD patients than controls, whereas controls were more likely to be colonised with multiple species of *Prevotella*, which have previously been shown to directly inhibit the growth of a number of other bacteria.

Huang *et al.* (814) also found differences in bacterial community composition in bronchial lavage samples from asthmatic patients compared to those from healthy control subjects. The diversity of the microbiota in asthmatic patients was found to be significantly higher than that of controls and a significant positive correlation between diversity and bronchial hyper-responsiveness was observed. In particular the relative abundance of certain bacterial taxa primarily belonging to the *Proteobacteria* phylum were highly correlated with AHR.

A further study by Marri *et al.* (815) investigated the differences between the respiratory tract microbiota composition in the induced sputum of mild asthmatics (8 out of 10 of whom were not using inhaled corticosteroids) and non-asthmatic adults. This demonstrated that even subjects with mild asthma on minimal inhaled therapy exhibit a significantly different respiratory tract microbial composition to healthy subjects. Again, the microbiota of asthmatic patients were found to have a greater bacterial diversity than those of healthy subjects with increased levels of the *Proteobacteria* phylum. Healthy subjects tended to have higher relative abundances of *Firmicutes* and *Actinobacteria*, although these differences were not statistically significant.

Recent data assessing the effect of azithromycin treatment on the lung microbiota of moderate and severe asthmatics revealed potentially pathogenic organisms, including *Pseudomonas*, *Haemophilus* and *Staphylococcus* species were amongst the most abundant bacteria detected in pre-treatment bronchoscopy samples. The abundance of each of these bacteria was reduced following azithromycin treatment (816).

Goleva *et al.* (817) examined the potential contribution of the lung microbiota composition to the development of resistance to corticosteroid treatment in asthmatic subjects. Subjects were categorised as corticosteroid sensitive or corticosteroid resistant on the basis of their response to a treatment trial of oral prednisolone. Subjects were classified as corticosteroid

sensitive if their predicted FEV_1% value increased by ≥15% or corticosteroid "resistant" if their predicted FEV_1% improved <10%. BAL samples were obtained from 39 asthmatic subjects, 29 of whom were corticosteroid resistant and 10 of whom were corticosteroid sensitive, as well as from 12 healthy controls. The microbiota composition of the corticosteroid resistant and corticosteroid sensitive subjects did not differ at the phylum level. However, at the genus level 14 corticosteroid resistant patients displayed 'expansions' (i.e. sequences > 5% of the total 16S rRNA gene sequences) of bacteria not present in the corticosteroid sensitive group including the potential pathogen *Haemophilus parainfluenzae*. This organism was then demonstrated to inhibit the corticosteroid response of asthmatic airway macrophages *in vitro*. Other *in vitro* work also suggests *H. influenzae* may induce a steroid resistant inflammatory response by reducing histone deacetylase (HDAC) activity (818).

Huang *et al.* (819) used a 16S rRNA Phylochip to characterise the microbiota in 40 patients with severe asthma, specifically to delineate any relationships between microbiota composition and disease features. The investigators found significant correlations between the presence in the microbiota of certain taxa and certain features of disease, including BMI, asthma control, sputum leukocyte values and bronchial biopsy eosinophil values. Poor asthma control (i.e. between visit differences in the Asthma Control Questionnaire) and increased sputum leucocyte values were associated with a high relative abundance of *Proteobacteria*, whereas high BMI was associated with high relative abundance of *Bacteroidetes/Firmicutes*. The presence of certain families of *Proteobacteria* was also positively associated with increased expression of Th17-related genes. In comparison to healthy controls or subjects with mild to moderate asthma, subjects with severe asthma had significantly higher levels of *Actinobacteria*.

Green *et al.* (635) obtained sputum from 28 stable treatment resistant severe asthmatics and assessed the microbiota in these samples using T-RFLP profiling. Seventeen of the 28 asthmatics were predominantly colonised by a potentially pathogenic bacterium (*M. catarrhalis*, *Haemophilus spp.* or *Streptococcus spp.*) and these subjects had significantly lower post-bronchodilator percent predicted FEV_1 and higher sputum neutrophil differential cell counts. This suggests that colonisation of the airways by potentially pathogenic bacteria may lead to more severe airway obstruction and neutrophilic inflammation, both of which are features of a previously described phenotype of treatment resistant neutrophilic asthma (344).

Further support for the association of potentially pathogenic bacteria in the airways with neutrophilic asthma comes from an investigation by Simpson *et al.* (820). In this study induced sputum samples from 30 subjects with stable asthma were taken and bacterial DNA extracted and profiled to allow comparison of microbiota composition between different asthma inflammatory subtypes. Microbiota analysis revealed reduced bacterial diversity and species richness in a group of 7 patients with neutrophilic asthma as compared to the 20 patients with non-neutrophilic asthma. A significantly higher abundance of *Proteobacteria* were found in the sputum samples from subjects with neutrophilic asthma, the majority of which were consistent with *Haemophilus spp.*, and these were particularly abundant in 4 of the 7 neutrophilic subjects. Other differences noted between subjects with neutrophilic and non-neutrophilic airway inflammation included a significantly lower abundance of

Actinobacteria and *Firmicutes* in those with neutrophilic asthma with a particular difference noted in the distribution of sequences corresponding to the *Tropheryma* genus (from the phylum *Actinobacteria*). Five of the 7 subjects in whom *Tropheryma* sequences were detected had eosinophilic inflammation.

Zhang *et al.* (821) examined the microbiota content of sputum from 26 "severe" and 18 "non-severe" asthmatics and 12 healthy controls using 16S rRNA gene sequencing. Severe asthmatics were defined as those who required "either continuous or near-continuous oral corticosteroids, high-dose inhaled corticosteroids, or both" and non-severe asthmatics defined as those with no symptoms and minimal use of rescue medication using ≤2000 µg BDP. These study results suggested an increased abundance of *Proteobacteria* in the non-severe asthma group in comparison to the severe asthma group and controls and increased *Firmicutes* abundance in the severe asthma group in comparison to the other two groups.

1.7.8 Sampling the lung microbiota

One of the key questions in the study of the lung microbiota is the appropriateness of different techniques for sampling lung organisms. A literature review of this topic is summarised in Appendix K. The initial studies in this area performed sampling by direct bronchoscopic methods (777, 814) in order to minimise potential contamination from the mouth and upper airways, although this technique is invasive and uncomfortable for subjects. Induced sputum using hypertonic saline is far less invasive and very well tolerated, making repeat sampling on large numbers of subjects possible. However, the upper respiratory tract (URT) has been shown to contain its own unique microbiota, and there are concerns that the microbial profile in sputum samples (whether spontaneous or induced) from the lower respiratory tract (LRT) may either be contaminated with URT organisms or that the LRT 'microbiota' as sampled by bronchoscopy merely represents bronchoscopic carryover of URT organisms.

Charlson *et al.* (794) assessed the microbial populations present at different locations throughout the respiratory tract of healthy individuals including the oral cavity, oro and naso pharynx and the upper and lower airways. Measures to minimise contamination of samples from the airways were taken through the use of a two bronchoscope/protected brush technique. The study concluded that in healthy individuals a bacterial community is present in the lungs, but this is much less abundant than that of the URT. The composition of the bacterial communities from these two sites was very similar, suggesting that the bacteria present in the lungs of healthy individuals may arise through aspiration of these bacteria in the upper airways.

A study by the same group comparing the bacterial content of oral wash vs BAL fluid for 6 subjects with different lung diseases found no significant difference between these samples in 3 out of the 6 subjects, whereas the BAL samples from the other 3/6 subjects showed a number of sequences that were significantly more abundant in BAL compared to oral wash (822). This suggests that contamination of a bronchoscope with upper airway bacteria or repeated microaspirations does not fully explain the detection of bacterial communities in the lung.

Further evidence for the existence of a lung specific bacterial community comes from studies that analysed the microbial content of whole-lung tissue samples such as those by Sze *et al.* (810) and Erb-Downward *et al.* (809) as described above. These both concluded that there are detectable bacterial communities in the human lungs (in healthy subjects and subjects with COPD), although the total number of bacteria detected in the lungs is small compared to that of the airways as a whole.

Despite the seeming inability to be able to exclude a degree of URT contamination from samples ostensibly from the LRT, the most abundant bacteria in the asthma microbiota do not seem to be prominent members of the typical microbiota found in saliva, the nostrils or the oropharynx (823, 824). This was supported by the results of Marri *et al.* (815) who used the induced sputum method to sample the lower airways.

1.7.9 Sequencing the microbiota

The current study will detail the microbiota of the lower airways using state-of-the-art massively parallel pyrosequencing (825) of bacterial 16S ribosomal RNA gene region amplicons (826). This non-culture dependent technique provides an unprecedented level of detail regarding the bacterial community of the lower airways. Pyrosequencing is an increasingly recognised technique for studies of microbial communities due to its ability to rapidly and accurately sequence large numbers of bacterial species.

Several previous investigations have confirmed the applicability of this technique in the sequencing of the bacterial microbiota in the gut (827) and the lung (809, 828), but it has only been employed once in studies of asthma patients to date (815). It is expected that utilising this method will allow a greater depth of sequencing than in similar previous investigations (777, 814).

The steps involved in sequencing the microbiota from sample collection to data processing are summarised in Figure 1.9 (829).

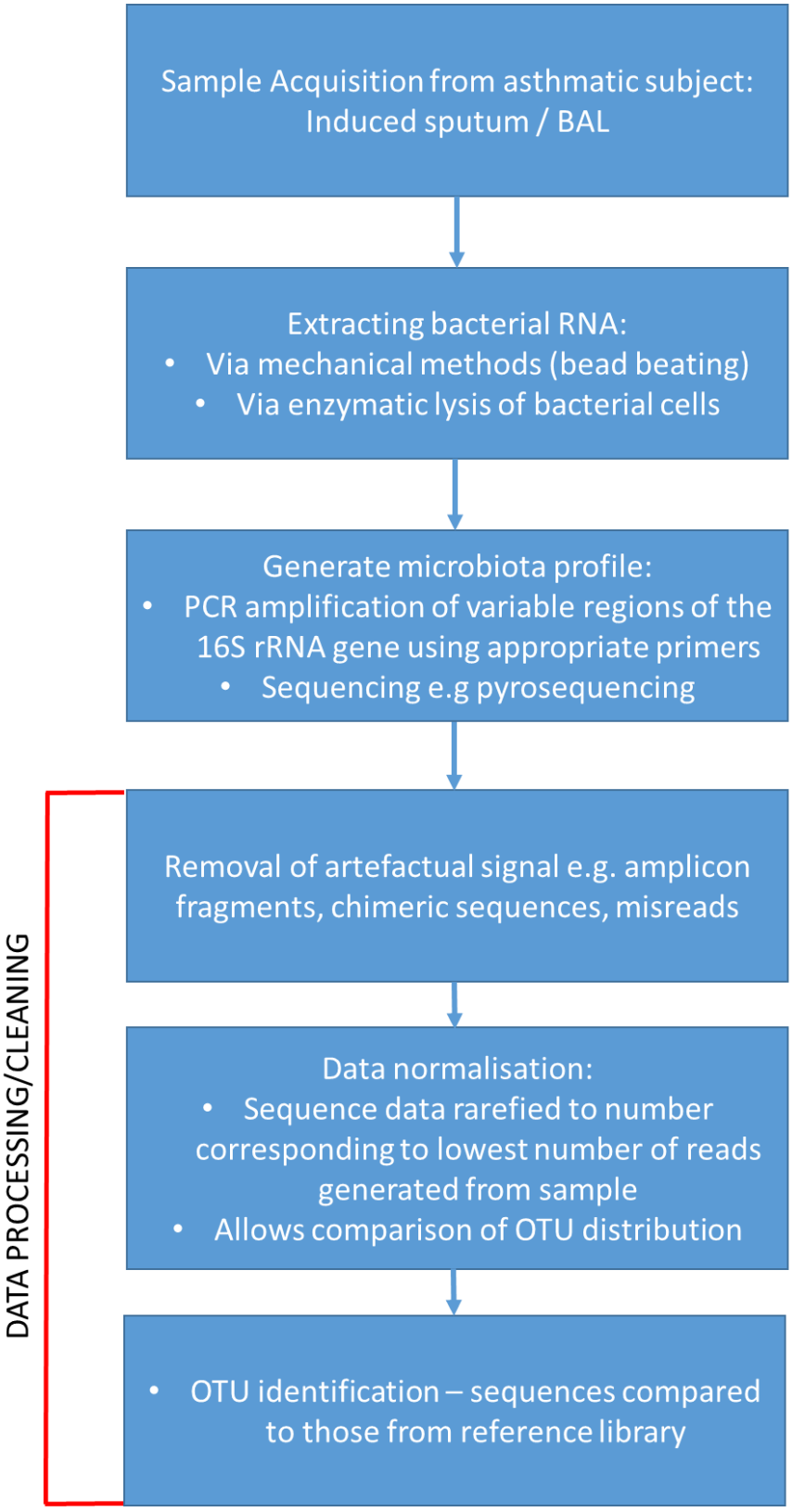


Figure 1.9: Flow diagram demonstrating the necessary steps in studies of the microbiota from sample acquisition to data analysis

1.7.10 Summary and rationale for study

Analysis of the human microbiota is a promising and rapidly expanding field. Studies to date of the lung microbiota have led to new insights into the pathogenesis and progression of lung disease. Generally, findings so far in most respiratory conditions indicate that reducing bacterial diversity and dysbiosis of the microbiota with dominance of certain pathogenic species correlate with worsening disease severity and outcomes. Studies of the bacterial microbiota in asthma have revealed differences in its composition to that of healthy controls, suggesting that an increased abundance of potentially pathogenic species such as *Proteobacteria* are associated with asthma, particularly the neutrophilic asthma subgroup, and with certain clinical measures.

As yet, it has not been possible to establish whether the presence of these organisms is due to disease itself, or whether this may represent a treatment effect of high dose inhaled steroids. It is hypothesised that the use of high dose ICS could alter the microbiota in asthma, acting as a selective pressure that favours the establishment of colonising species of potentially pathogenic species such as *H. influenzae* (Fig 1.10). Possible mechanisms for such an effect could include (1) a local immunosuppressive effect of ICS allowing the overgrowth of pathogenic species (2) a selective inhibitory effect on the growth of certain organisms to the benefit of others (3) the utilisation of ICS by certain organisms capable of steroid degradation as a source of energy.

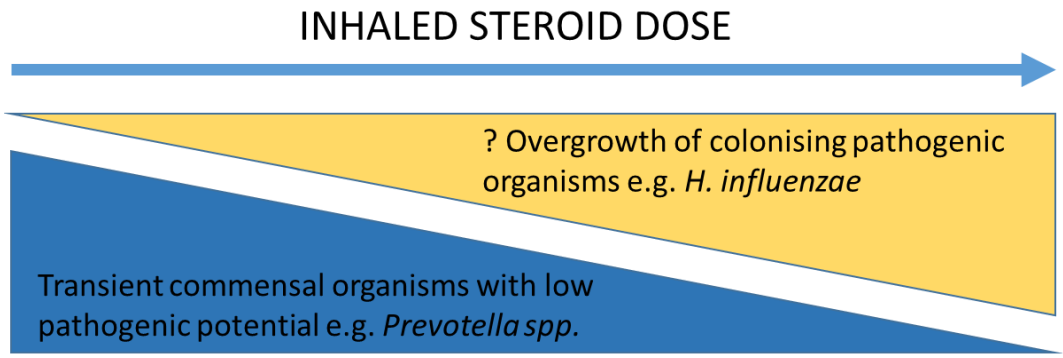


Figure 1.10: Illustrating hypothesised changes in the microbiota with increasing ICS dose

It is further speculated that the type of ICS used by an individual may have an effect on the composition of the airway microbiota in asthma. Evidence suggests that fluticasone use leads to an increased risk of pneumonia (632, 830) and non-tuberculous mycobacterial disease (831) in subjects with airways disease compared to budesonide. It is possible this increased risk may be due to a selective pressure caused by fluticasone that favours the overgrowth of potentially pathogenic species that is not observed with budesonide.

The proposed study aims to investigate two important questions regarding the microbiota composition in patients with a diagnosis of asthma. The first main aim of the study is to compare the microbiota composition in sputum samples from subjects with mild (BTS Step 2) and moderate/severe asthma (BTS Step 4). Although initial studies have suggested

subjects with different severities of asthma may have similar lower respiratory tract microbiota composition, there is no direct evidence to support this assertion. The second main aim is to compare microbiota composition between samples from subjects with asthma using the inhaled steroid fluticasone and those using budesonide.

Other aims of the study include assessment of the reproducibility of the induced sputum method for assessing the lung microbiota, assessment of the longitudinal stability of the bacterial population and comparison of the bacterial load of two common respiratory pathogens (*H. influenzae* and *S. pneumoniae*) in the BTS Step 2 and 4 groups. Clinical measurements of different components of airways disease; airflow obstruction, AHR and airway inflammation will be performed to assess correlation between bacterial community composition and these metrics and also to enable subgroup/phenotype analysis based on these characteristics.

1.8 Hypothesis of Thesis and Aims

1.8.1 Hypothesis

Strategies based on the measurement of selected phenotypic and biological characteristics of airways disease can help to improve the understanding of their pathogenesis and targeting of treatment.

1.8.2 Aims

1.8.2.1 Aim for Study 1

To establish whether there is a set of baseline characteristics which can reliably distinguish which patients will not benefit from ICS treatment.

1.8.2.2 Aims for Study 2

1) To describe the clinical, pathological and radiological features of a cohort of patients with unexplained chronic productive cough

2) To determine the response of this cohort to a 12 week course of low dose azithromycin therapy and assess if any of the baseline characteristics measured could predict response to azithromycin.

1.8.2.2 Aims for Study 3

1) To compare the microbiota composition in sputum samples from subjects with mild (BTS Step 2) and moderate/severe asthma (BTS Step 4)

2) To compare microbiota composition between samples from subjects with asthma using the inhaled steroid fluticasone and those using budesonide.

Chapter 2: The utility of exhaled nitric oxide in patients with suspected asthma

2.1 Background

2.1.1 Summary of background (Chapter 1.5)

Despite the established clinical importance of identifying cases of airways disease and the wealth of studies evaluating a range of objective measures for their detection the prospect of an accurate diagnostic test for "asthma" is still elusive. Some tests clearly perform better in identifying characteristics of the classical asthma syndrome than others. However, uncertainties owing to the poor definition of "asthma" and heterogeneity of the condition in addition to the often transient nature of the symptoms may always make the attempt to define and identify features of disease according to one diagnostic label futile.

However, with increasing availability of objective tests capable of identifying the pathophysiological processes underlying airways disease and a growing range of effective treatment agents targeting these processes the need for "diagnosing asthma" *per se* may ultimately be made redundant in favour of a "characteristic-targeted" treatment approach.

The proposed study will attempt to investigate the value of objectively measured features of airways disease (with a particular emphasis on FE_{NO}) to predict ICS treatment response in a cohort of steroid naïve patients with symptoms of asthma.

2.1.2 Rationale for study

Using FE_{NO} levels to predict steroid response would appear to be a logical approach given that FE_{NO} is a biomarker of Th2 inflammation and there is good evidence that this type of inflammation is responsive to steroid treatment. This approach avoids the complexities inherent in attempting to use the technique to classify heterogeneous airways diseases according to diagnostic labels.

The study by Smith *et al.* provides good evidence that this strategy may be useful, but the optimal cut-point derived by these authors to predict ICS response (>47 ppb) may risk missing subjects with measured FE_{NO} levels in the 'indeterminate' range of 25-50 ppb identified in the ATS FE_{NO} guidelines who would benefit from steroid treatment. It would be useful for clinical practice if a 'minimum' FE_{NO} cut-point below which steroid response is unlikely could be determined in order to guide the decision of whether or not to initiate steroid treatment in subjects with symptoms suggestive of airways disease.

2.2 Methods

2.2.1 Purpose and Design

2.2.1.1 Aim

To establish whether there is a set of baseline characteristics which can reliably distinguish which patients will not benefit from ICS treatment.

2.2.1.2 Hypothesis

A minimum FE_{NO} cut-point can be determined which reliably excludes a clinical response to inhaled corticosteroids in a cohort of steroid naïve patients with symptoms suggestive of asthma.

2.2.1.3 Study Design

A single centre non-interventional study was designed.

2.2.1.4 Ethical Approval

This study was approved by the National Research Ethics Committee East Midlands – Derby 1 (Ref 12/EM/0241) and Nottingham University Hospitals NHS Trust Research and Innovation department (Ref 11RM001).

2.2.2 Study Population:

2.2.2.1 Eligibility criteria

Patients were recruited according to the following eligibility criteria:

Inclusion criteria

- Male or Female aged between 18 and 80 years old
- Suspected asthma diagnosis and prescribed a new ICS

Exclusion criteria

- Subjects already using inhaled or oral corticosteroid
- Pregnant females
- Subjects with other significant respiratory diagnosis

2.2.2.2 Study setting and participant recruitment

This study was conducted at the Nottingham Respiratory Research Unit (Nottingham City Hospital UK). Subjects with respiratory symptoms suggestive of asthma who were deemed by general practitioner (GP) to require ICS treatment were prospectively identified. Interested subjects were provided with full written information about the study and given contact information for the study team. Upon contacting the study team they were invited to a screening visit to ensure they met the eligibility criteria specified above.

2.2.3 Outcome measures

2.2.3.1 Primary endpoint

- The sensitivity and specificity of low levels of FE_{NO} at predicting a lack of clinical benefit from ICS after 4 weeks of treatment

2.2.3.2 Secondary endpoints

- To determine the sensitivity and specificity of FE_{NO} for diagnosing asthma
- To determine whether there are other baseline characteristics which if used alone or in combination can exclude a response to ICS.

2.2.3.3 Sample size calculation

As this was a pilot study a formal power calculation was not possible. An initial target of 100 participants was set.

2.2.4 Summary of study protocol

2.2.4.1 Visit 1

Subjects attended a baseline study visit (visit 1) as soon as possible after being prescribed an ICS by their GP but before the treatment was started. FE_{NO}, skin prick testing (SPT), spirometry, MCT, asthma control questionnaire (ACQ), asthma control test (ACT), full blood count (FBC) and sputum induction were performed, as outlined in Fig. 2.1. Subjects were also questioned about their presenting symptoms, i.e. the symptoms that led them to consult with their GP.

2.2.4.2 Visit 1a

Subjects prepared to make a second visit on the day after visit 1 underwent reversibility testing with 400 µg salbutamol via a spacer.

Subjects were instructed to start their GP prescribed ICS treatment after visit 1 (or visit 1a if they also attended this) which was predominantly inhaled beclomethasone (100 µg per puff, 2 puffs twice daily) via a metered dose inhaler.

2.2.4.3 Visit 2

Visit 2 occurred 4 weeks post initiation of ICS treatment. At this visit subjects were asked about treatment adherence and FE_{NO}, spirometry, MCT, ACQ, ACT and FBC were performed (Fig. 2.7).

2.2.4.4 Visit 3

Visit 3 was after 12 weeks of ICS treatment. At this visit subjects were again asked about treatment adherence and FE_{NO}, spirometry, MCT, ACQ, ACT and FBC were performed.

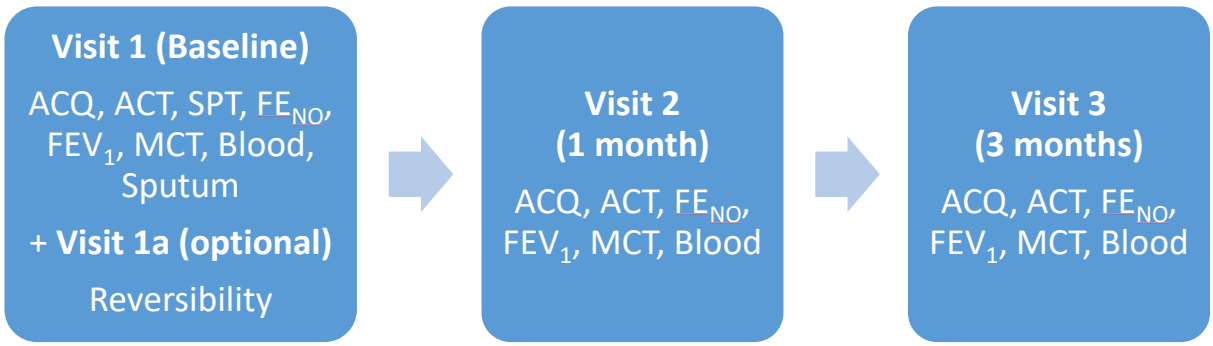


Figure 2.1: Demonstrating investigations performed at each study visit.

ACQ = asthma control questionnaire, ACT = asthma control test, SPT = skin prick test, FE_{NO} = Fractional exhaled nitric oxide level, FEV_1 = spirometry, MCT = methacholine challenge, Blood = full blood count (including blood eosinophil count), Sputum = differential sputum eosinophil count

2.2.5 Clinical Measurements

2.2.5.1 Spirometry and Reversibility

Spirometry was performed using a Vitalograph™ dry wedge bellows spirometer (Vitalograph™ model 2150, Buckinghamshire, England) and the FEV_1 and FVC were calculated. The best of 3 technically acceptable manoeuvres were recorded where the values of the largest and the next largest FEV_1 results were ≤150 mL and within 5% of each other as per ERS guidelines (537). Percentage predicted values were calculated using reference values from the ERS prediction equations (544). Bronchodilator reversibility was assessed 15 min after administration of 400 µg of salbutamol inhaled via a Volumatic® spacer as per ERS guidelines (537).

Reversibility was defined as:

$$\frac{(Post\ bronchodilator\ FEV_1 - Pre\ bronchodilator\ FEV_1)}{Pre\ bronchodilator\ FEV_1} \times 100$$

with a 12% increase considered significant as per ATS guidelines (217).

2.2.5.2 Skin Prick Tests (SPTs)

Atopy was assessed by SPTs to a panel of common aeroallergens which included *Dermatophagoides pteronyssinus* (house dust mite), tree and grass pollen, cat and dog fur and *Aspergillus fumigatus* with normal saline and histamine controls (Alk-Abello™, Berkshire, UK). A small drop of each solution was placed on the skin of the volar aspect of the lower forearm. Disposable sterile lancets (Alk-Abello™, Berkshire, UK) were used to puncture the epidermis under each drop in turn and the diameters of any resultant wheals were measured in two perpendicular directions after 15 min. A positive response to an allergen on the SPTs was recorded in the presence of a wheal >3 mm greater in its longest measured dimension than the negative (saline) control. Participants were requested not to take any antihistamine medications for a minimum of 48 h prior to the test.

2.2.5.3 Sputum Induction

Sputum induction was performed using a protocol based on that described previously by Pavord *et al.* (417) based on the method of Pin *et al.* (405) using an ultrasonic nebuliser to deliver hypertonic saline. Due to the potential for nebulised saline to cause bronchoconstriction, subjects were pre-treated with inhaled salbutamol, their FEV_1 was closely monitored and the test was supervised by a clinical fellow at all times. Briefly, after pre-treatment with 400 µg of salbutamol inhaled via Volumatic® spacer (if subjects post-bronchodilator FEV_1 was ≥60% predicted), subjects were asked to inhale nebulized saline via a saline nebulizer (NE-U17, Omron Healthcare™, Milton Keynes, UK). Subjects inhaled 10 mL of 3% saline for 5 min, were asked to blow their nose and rinse their mouth with drinking water and were encouraged to cough to try and aid expectoration of a sputum samples. Assuming their FEV_1 did not decrease by ≥20% from the baseline measurement they went on to inhale 10 mL of 4% saline for 5 min after which they again were asked to blow their nose and rinse their mouth with water before a second attempt to try and expectorate a sample. If there was no resultant decrease in FEV_1 of ≥20% after the second attempt, the

process was repeated once more with 10 mL of 5% saline for 5 min followed by a third attempt to produce a sputum sample.

2.2.5.4 Sputum Processing

Once collected, sputum samples were stored in ice and processed at 4^0C within 2 hours of collection as described previously (417) but with some minor adaptions to produce supernatants free of dithiothreitol (DTT) for future work. Sputum plugs were isolated from saliva using curved forceps on the lid of a petri dish and then processed as summarised in Fig 2.2.

1) Sputum plugs selected

2) Weigh and incubate with 8 volumes (ml) x sputum weight (g) of phosphate buffered saline (PBS)

3) Add 150 volumes (µl) x sputum weight (g) of protein inhibitor complex (PIC)

4) Vortex sample for 15 s and rock on ice for 10 min

5) Centrifuge at 600g for 10 minutes at 4°C

6) Four volumes of the supernatant are then removed into a fresh 15ml falcon labelled *'PBS Supernatant'* and centrifuged at 1500g for 10 minutes at 4°C.

7) Other four volumes supernatant split between four cryovials labelled '*PBS Supernatant'* and store at -80°C.

8) Four volumes of 0.2% DTT added to sputum sample, ensuring a final DTT concentration of 0.1%.

9) Vortex sample for 15 s and rock on ice for 10 min

10) Filter through 48 µm nylon gauze

11) Reweigh sample and aspirate 10µl, mix well with 10µl Trypan blue. Flood a haemocytometer chamber with 10µl of the cell suspension/Trypan blue solution.

12) Count cells: Live leukocytes, dead leukocytes and squamous cells, calculate:

1. Total number of cells in sample
2. Cell concentration
3. Total number of cells per gram of sputum
4. Volume required for a concentration of $5x10^5$ cells/ml

13) Centrifuge cell suspension at 600g for 10 minutes at 4°C.

14) Split the resulting supernatant between four cryovials labelled *'DTT Supernatant'* and store at -80°C.

15) Re-suspend cell pellet in appropriate volume of d-PBS to give a concentration of $5x10^5$ cells/ml.

16) Centrifuge 75µl on one slide and 150µl on another in Shandon cytospin at 450rpm for 6 min

17) Air-dry slides, fix in methanol and stain with RappiDiff II.

18) Differential cell count of 400 cells

Figure 2.2: Protocol for sputum processing and cell count

2.2.5.5 Methacholine Challenge

AHR testing was performed using methacholine as a provocative agent and the tidal breathing method to determine the concentration of methacholine causing a 20% fall in FEV_1 (PC_{20}). The protocol was based on that described previously (545) and recommended by the ATS guidelines (434).

In brief, the subject's baseline FEV_1 was measured, followed by the inhalation of normal saline and then doubling concentrations of methacholine from 0.03 mg/mL to 16 mg/mL via a Wright's® nebuliser (Roxon, Canada) with a flow rate of 0.13 mL/min driven by dry compressed air. Each nebulisation period lasted 2 min, during which time the subject was wearing a nose clip and instructed to breathe normally through the nebuliser (which contained a two-way valve). After each nebulisation period the FEV_1 was measured after 30, 90 and 180 s. If the FEV_1 did not decrease 20% from the baseline measurement the procedure was repeated with the next highest concentration. The test ended if the FEV_1 fell ≥20% from baseline or if the highest methacholine concentration of 16 mg/mL had been administered.

Exact values for methacholine PC_{20} FEV_1 concentration were calculated by linear interpolation of the log dose response curve.

2.2.5.6 Phlebotomy

Samples for FBC were obtained using the 21 gauge BD Vacutainer® Safety-Lok™ blood collection set (BD, Plymouth, UK) into 4 mL EDTA tubes. Samples were processed in the Nottingham City Hospital biochemistry laboratory by automated cytometers.

Two extra EDTA and two serum samples (taken into 5 mL BD Vacutainer serum tubes) were also taken. EDTA tubes were immediately placed in ice and taken to the laboratory where one of these tubes was centrifuged at 1000 g for 15 min at 4^0C, whilst the other was immediately frozen at -80˚C. The supernatant from the other EDTA tube was aspirated and frozen in 10 aliquots at -80˚C for future use. The 2 serum tubes were allowed to clot in an upright position at room temperature for 45 min and were then centrifuged at 1300 g for 10 min at 25˚C. Supernatants were aspirated and frozen in 10 aliquots at -80˚C for future use.

2.2.5.7 Exhaled Nitric Oxide

Exhaled nitric oxide concentration was measured using an offline electrochemical analyser (NIOX MINO®; Aerocrine™, Tolna, Sweden). All subjects inhaled NO-free air (via an NO scrubber built into the device) to near total lung capacity and exhaled for 10 s at a flow rate of 50 mL/s to provide two approved FE_{NO} measurements. The NIOX MINO device provides visual feedback to ensure an exhalation pressure of between 12-18 cmH_2O, with a built in flow controller ensuring a resultant flow rate of 50 mL/s.

2.2.5.8 Juniper Asthma Control Questionnaire

The ACQ is a validated questionnaire, designed after international consultation with 91 'expert' asthma clinicians, which has been used to assess the adequacy of asthma control and any changes in asthma control over time (546). It contains five questions on the five symptoms judged to be the most important when assessing asthma control (night time symptoms, morning symptoms, limitation of daily activities, shortness of breath and

wheeze), one question regarding the dose of daily 'rescue' bronchodilator used and one question assessing the subjects FEV_1 as a percentage of their predicted value. Subjects are asked to recall the severity of each of the 5 symptoms and their bronchodilator use in the last week and quantify these on a 7 point scale (0=no impairment, 6=maximum impairment). The FEV_1 % predicted is recorded and also quantified on a 7 point scale. The questions all have equal weight so the ACQ is the mean score of the 7 questions ranging between 0 (totally controlled) and 6 (severely uncontrolled). The ACQ has been demonstrated to give very consistent scores in patients with stable asthma between clinic visits as well as being very sensitive in detecting changes in asthma control (546). The minimal clinically important difference (smallest change in outcome that a subject would see as important) for the ACQ is 0.5 (547). A copy of the ACQ is included in Appendix B.

2.2.5.9 Asthma Control Test

The ACT is another validated questionnaire to assess asthma control designed by a working group of 4 primary care clinicians and 7 asthma specialists from the USA (548). It contains five questions concerning symptoms of/statements with respect to asthma control (effect of asthma on daily activities, number of episodes of shortness of breath, night time or morning symptoms, frequency of 'reliever' bronchodilator use and self-rated "asthma control"). Subjects are asked to recall the severity of each of these 5 symptoms over the last 4 week period and quantify these on a 5 point scale (1=maximum impairment, 5=no impairment). The ACT test score is calculated by simply adding the scores of all of the questions to produce a total ranging between 5 (severely uncontrolled and 25 (totally controlled). The ACT has a high level of internal consistency reliability (548) and has a minimal clinically important difference of 3 points (549). A copy of the ACT is included in Appendix C.

2.2.6 Analysis

After the final visit the results of the investigations at each visit were reviewed in order to answer two questions:

1) Does the patient meet any of the internationally recognised criteria for a diagnosis of asthma?
2) Has there been any significant response to ICS treatment?

Diagnosis of asthma was based on the following criteria:

- **Reversibility of ≥12% and ≥200 mL of FEV_1 from baseline 15 min after inhaled salbutamol (217, 550)**
- **A positive test for AHR, defined as a provocative concentration of methacholine resulting in a 20% reduction in FEV_1 (PC_{20}) of ≤8 mg/mL (434)**

Response to ICS was based on a combination of *2 of any of the objective criteria or 1 objective criterion and 1 subjective criterion* from the following previously defined response criteria:

Objective:

- **Improvement in FEV_1 ≥12% with ICS (217)**

- **Improvement in PC_{20} ≥1 doubling dose shift (434)**
- **FE_{NO}: Decrease of ≥20% for baseline values >50 ppb or decrease of ≥10 ppb for baseline values ≤50 ppb (502)**

Subjective:

- **ACQ score decrease ≥0.5 (551)**
- **ACT score increase ≥3 points (549)**

Data was entered into Stata (Statacorp, Texas, USA) and a series of logical operators were used to classify subjects' asthma diagnosis and ICS response status based on the criteria above. ROC analysis was carried out in Stata and GraphPad Prism (GraphPad Software, California, USA) to produce ROC curves, ROC AUC values and values for sensitivity, specificity, PPV and NPV at selected cut-points.

Odds ratios, sensitivity, specificity, PPV and NPV of symptoms for asthma diagnosis and to predict ICS response were also calculated in Stata. Stepwise logistic regression was performed with symptoms that were significant predictors of asthma diagnosis, high FE_{NO} or ICS response ($p<0.05$) in univariate analysis included in a multiple logistic regression model. Any symptoms that were not significantly associated with asthma diagnosis, high FE_{NO} or ICS response but changed the odds ratio for any of these outcomes by 10% or more on addition to the analysis were retained in the final model.

2.3 Results

2.3.1 Recruitment

One hundred and ten subjects were referred by their general practitioners between November 2012 and November 2014. Of these 15 decided not to participate, and 18 did not meet the inclusion criteria (10 were already taking ICS, 6 had a significant respiratory comorbidity and 2 displayed acute symptoms not suggestive of asthma). Accordingly a total of 77 subjects were enrolled into the study.

2.3.2 Losses and exclusions

At visit 1, three subjects were unable to perform the required investigations and were withdrawn from the study. Seven patients were lost to follow up before visit 2 (3 of these withdrew consent and 4 were unable to be contacted despite repeated attempts) and a further 7 patients were lost before visit 3 (2 of these withdrew consent and 5 were unable to be contacted despite repeated attempts).

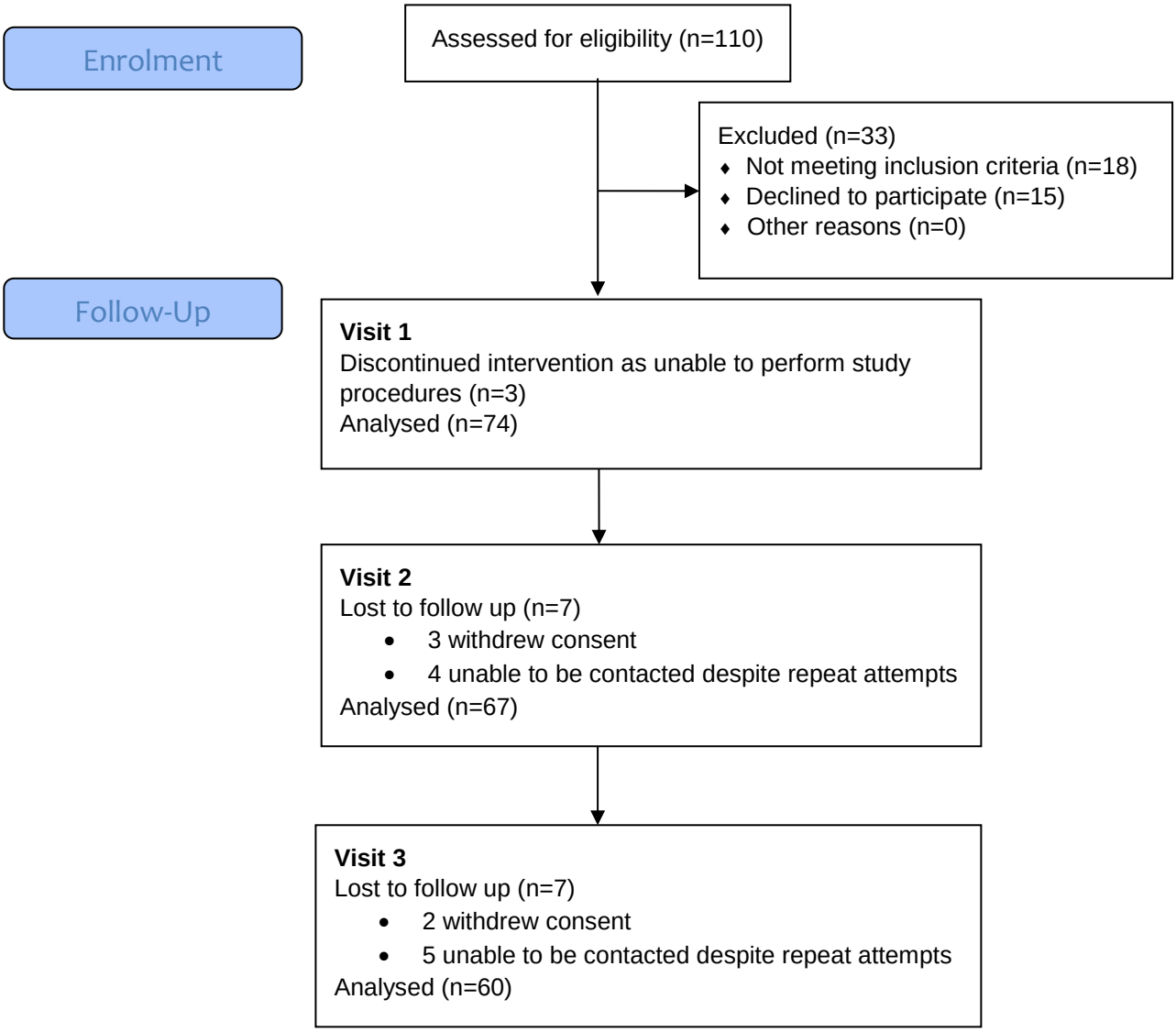


Figure 2.3: Consort diagram demonstrating losses and exclusions from study

2.3.3 Missing data

AHR could not be measured in all study participants due to low FEV_1 at baseline (n=4), subjects declining the test (n= 4) or an inability to perform the test consistently (n=1).

Also, sputum induction was attempted on the first 40 subjects recruited to the study, but only 4 of these were able to produce an adequate sample for analysis, therefore this test was abandoned and the limited data obtained were not included in the final analysis.

2.3.4 Baseline demographics

The baseline demographics and clinical features of all of the 74 participants able to complete sufficient investigations to rule in or rule out a diagnosis of asthma are shown in Table 2.1. The age distribution of the cohort is shown in Table 2.2. Table 2.3 shows the demographic information and clinical features of the subjects diagnosed with asthma and those classified as not having asthma.

There was a slight female preponderance and a large majority of the subjects were Caucasian. This was predominantly a young cohort with ~50% of patients below the age of 25. Around 31% were smokers or ex-smokers although the median pack year history was 0 (IQR 0.05 pack years, range 0-20 pack years).

The demographics of the groups categorised as having asthma or not having asthma were broadly similar although the median age of the group with asthma tended to be slightly higher (borderline significance p=0.06) and the ethnic composition of the groups was significantly different (p=0.02). The difference in ethnic composition of the groups is largely explained by the observation by that 4/28 subjects with asthma were Black/Black British whilst none of the 46 non-asthma subjects were Black/Black British.

The asthmatic group had on average a lower mean FEV_1 (p=0.005), lower mean FEV_1/FVC ratio (p=0.007), higher mean reversibility (p=0.0001) and higher median blood eosinophil count (p=0.004) and higher mean ACQ score (p=0.02) (i.e. symptoms less well controlled) (Table 2.7). However, some of these differences would be expected as FEV_1 and reversibility are included in our definition of asthma.

2.3.5 Primary outcome: ICS Response

Response to ICS, as defined by the response criteria described in Section 2.3.6, was seen in 27 out of 67 (40%) subjects after 4 weeks of ICS and 28/60 (47%) subjects after 12 weeks (Table 2.4).

Eighteen of the 32 (56%) subjects with asthma showed a response to ICS after 4 weeks, with 14 of these having sustained this response after 12 weeks. Eleven of the non-asthma subjects also demonstrated a response to ICS after 4 weeks, with 6 sustaining this response after 12 weeks. Figure 2.4 illustrates the response or non-response to ICS of subjects with asthma and subjects without asthma after 4 then 12 weeks of ICS treatment, and also lists the subjects lost to follow up in this time.

A ROC curve calculated for baseline FE_{NO} level as a predictor of ICS response after 4 weeks had an AUC of 0.89 ($p<0.0001$) (Fig 2.5).The optimal FE_{NO} cut-off point for predicting non-response to ICS was <27 ppb (NPV 93%) and for predicting response was >33 ppb (PPV 92%).

Exploratory analysis was conducted using different combinations of objective and subjective response variables as ICS response criteria after 4 weeks with baseline FE_{NO} level as a predictor of response. This produced ROC curves with AUCs between 0.7 (FEV_1 or PC_{20} response alone) and 0.91 (response in either FEV_1 or FE_{NO}) as shown in Table 2.5.

The accuracy of FE_{NO} level to predict steroid response after 12 weeks was consistent with response at 4 weeks (ROC AUC = 0.86 $p<0.0001$) although a few individuals who showed a response in objective measures after 4 weeks did not sustain this response at 12 weeks and vice versa (Fig 2.6).

2.3.6 Secondary outcomes:

2.3.6.1 FE_{NO} for asthma diagnosis

The diagnosis of asthma, according to the international consensus criteria outlined in Section 2.2.6, was made in 28 out of 74 patients. Of these 28 patients 10 were diagnosed by reversibility criteria alone and 12 were diagnosed by PC_{20} alone, with 6 being positive on both investigations.

A ROC curve was constructed to assess the utility of baseline FE_{NO} level as a diagnostic test for asthma (as diagnosed by reversibility and PC_{20}) as shown in Figure 2.7. The AUC for the curve was 0.62 ($p=0.09$).

2.3.6.2 Ability of other baseline characteristics to predict a response to ICS

When baseline PC_{20} and FEV_1 were used as predictors of response (as defined by different combinations of response variables) this produced ROC curves with AUCs between 0.02 and 0.67, shown in Table 2.6. ROC AUC values of <0.5 are negative predictors of an outcome i.e. the lower the value of the predictor variable, the greater the probability of a response. Therefore PC_{20} would appear to be an excellent predictor of response when response is defined by PC_{20} alone (ROC AUC = 0.02); PC_{20} with subjective response criteria (ROC AUC = 0.04) or ≤2 objective criteria (ROC AUC = 0.12). However, these results are misleading because 'response' using these response criteria is based solely (or largely) on a doubling dose increase in PC_{20}. These ROC AUC figures therefore simply reflect that of the 58 subjects who had MCT performed at V1 and V2, all of the 13/58 subjects who 'responded' in terms of PC_{20} had a baseline PC_{20} of ≤8 mg/mL whereas 40/45 who did not 'respond' had a baseline PC_{20} of ≥16 mg/mL (the highest concentration of methacholine used in the test). These latter subjects were all classified (correctly or incorrectly) using these response criteria as 'non-responders' as their PC_{20} could not improve due to a 'ceiling' effect, hence artificially increasing the AUC values.

The odds ratios, sensitivity, specificity, PPV and NPV of symptoms to predict ICS response and diagnose asthma were also calculated and these are shown in Table 2.8.

Cough was found to be a significant positive predictive factor for raised FE_{NO} (odds ratio (OR) 8.7; 95% CI 3.1-24.6; $p<0.0001$) and symptoms on activity were a negative predictor of

raised FE_{NO} (OR 0.3; 95% CI 0.1-0.8; p<0.02). The symptom of wheeze was found to be of borderline statistical significance as a positive predictor for raised FE_{NO} (OR 2.5; 95% CI 1-6.5; p<0.06). None of the other symptoms altered the OR for cough by >10% on addition to a stepwise regression model, suggesting there are no significant associations between these symptoms. To predict raised FE_{NO} (>27 ppb) the symptom of cough performed best overall in terms of sensitivity, specificity, PPV and NPV.

Cough was also a significant positive predictor of ICS response (OR 10.6; 95% CI 3.0-37.4; p<0.0001) with a particularly good sensitivity (0.85) and NPV (0.85) to predict ICS response according to the defined criteria used in the study. None of the other symptoms significantly predicted ICS response and did not alter the OR for cough by >10% on addition to a stepwise regression model.

2.3.6.3 Ability of other baseline characteristics to diagnose asthma

The diagnostic value of blood eosinophil count and skin prick test positivity for asthma were also assessed. Blood eosinophil count had a ROC AUC of 0.7 (p=0.005) for asthma diagnosis (Table 2.6) and a positive skin prick test (weal diameter >3mm in response to any of the allergens listed in Section 2.2.5.2) had a moderate sensitivity/NPV but low specificity/PPV for asthma diagnosis (Table 2.7)

None of the recorded symptoms were significantly associated with a diagnosis of asthma although cough (OR 2.3; 95% CI 0.9-6.0; p<0.08) and wheeze (OR 2.3; 95% CI 0.9-5.8; p<0.09) reached borderline statistical significance. In terms of the predictive value of symptoms to diagnose asthma, symptoms on waking had the highest specificity and dyspnoea, wheeze and cough produced similar values of sensitivity, specificity, PPV and NPV between 0.5 and 0.7 (Table 2.9).

	Frequency	Percentage	
Total number included for analysis	74		
Mean age (range)	32.3 (18-73)		
Sex: male	33	45	
Ethnic group: Asian Or Asian British Black Or Black British Mixed Ethnicity White Or White British	 6 4 1 63	 8.1 5.4 1.4 85.1	
Smoking history: Current Ex-smokers Non smokers	 10 13 51	 13.5 17.5 68.9	
Positive family history of asthma	34	45.9	
History/symptoms of GORD	15	20.3	
History/symptoms of eczema	9	12.2	
History/symptoms of rhinitis	17	23.0	
History/symptoms of hay fever	31	41.9	
History of NSAID allergy	2	2.7	
Positive skin prick for ≥1 allergen	43	59.7	
	Mean	**Standard deviation**	**Range**
FEV_1 % predicted	93	15.7	56-141
FEV_1/FVC ratio %	79.8	9.5	52-94
Reversibility (%)	7.0	9.7	-18-43
Blood eosinophil count ($x10^9$/L)*	0.2*	0.2*	0-0.9
Baseline ACQ score	1.67	0.89	0-4
Baseline ACT score	16.6	4.3	7-25

***Data presented are median and interquartile range as variable not normally distributed**

Table 2.1: Demographics of study population

Age group	Frequency	Percentage
<20	16	21.1
20-25	21	27.6
25-30	9	11.8
30-40	8	10.5
40-50	8	10.5
50-60	8	10.5
60+	6	7.9
Total	**76**	**100**

Table 2.2: Age distribution of study population

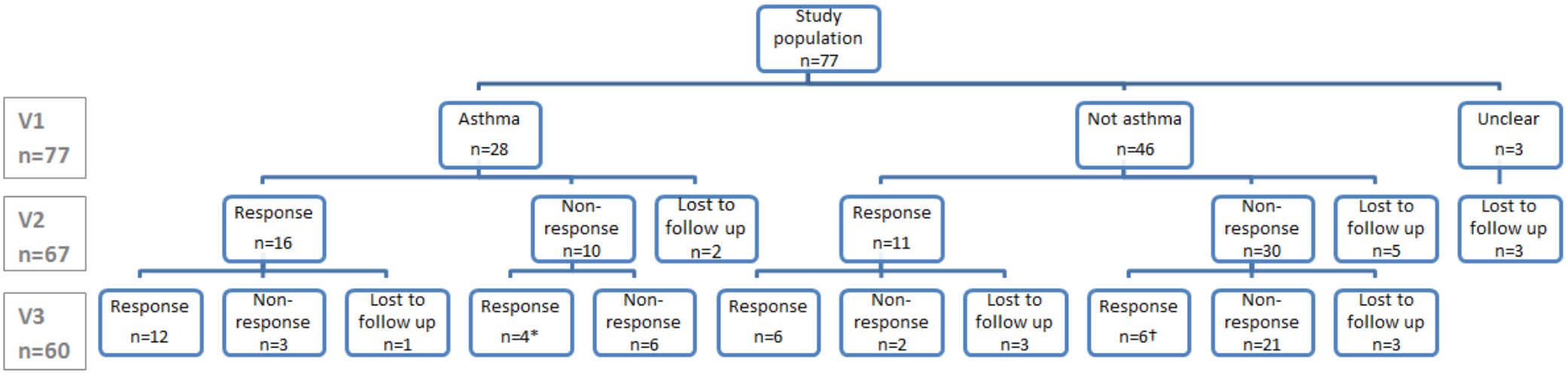
	Asthmatics	**Non asthmatics**	
	Frequency (%) (except [a])	**Frequency (%) (except [a])**	**Significance (p=)**
Total number included for analysis	28	46	
Median age (range)	29 (18-70)[a]	22 (18-73)[a]	0.06
Sex: male	11 (39)	23 (50)	0.37
Ethnic group: Asian Or Asian British Black Or Black British Mixed Ethnicity White Or White British	 1 (3.6) 4 (14.3) 1 (3.6) 22 (78.6)	 5 (10.9) 0 (0) 0 (0) 41 (89.1)	 **0.01***
Smoking history: Current Ex-smokers Non smokers	 5 (17.9) 4 (14.3) 19 (67.9)	 5 (10.9) 9 (19.6) 32 (69.6)	 0.62
Positive family history of asthma	13 (46.4)	21 (45.7)	0.95
History/symptoms of GORD	5 (17.9)	9 (19.6)	0.86
History/symptoms of eczema	4 (14.3)	6 (13.0)	1.0
History/symptoms of rhinitis	9 (32.1)	8 (17.4)	0.14
History/symptoms of hay fever	12 (42.9)	20 (43.5)	0.96
History of NSAID allergy	1 (3.6)	1 (2.2)	1.0
Positive skin prick for ≥1 allergen	17 (60.7)	26 (56.5)	0.86
	Mean (SD)	**Mean (SD)**	
FEV_1 % predicted	86.7 (14.0)	96.9 (15.6)	**0.005***
FEV_1/FVC ratio %	76 (10)	82.1 (8.4)	**0.007***
Reversibility (mL)	12.6 (11.7)	3.6 (6.3)	**0.0001***
Blood eosinophil count ($x10^9$/L)[b]	0.35 (0.4)	0.2 (0.1)	**0.004***
Baseline ACQ score	1.96 (0.81)	1.42 (0.82)	**0.02***
Baseline ACT score	15.8 (4.4)	17.2 (4.2)	0.09

[b]Data presented are median and interquartile range as variable not normally distributed
***Figures highlighted represent statistically significant differences between the two groups**

Table 2.3: Demographics and clinical characteristics of subjects with and without asthma

Responded between V1-V2:	**Frequency**
FEV_1 ↑≥12%	2
PC_{20} ↑≥ 1 doubling dose shift	5
FE_{NO} ↓20% if baseline >50 ppb or ↓≥10 ppb if baseline ≤50 ppb	13
FEV_1+PC_{20}	1
FEV_1+FE_{NO}	1
PC_{20}+FE_{NO}	5
All 3 objective criteria	2
Any objective criteria	29
Using study defined criteria	**27**
Responded between V1-V3:	
FEV_1 ↑≥12%	2
PC_{20} ↑≥ 1 doubling dose shift	5
FE_{NO} ↓20% if baseline >50 ppb or ↓≥10 ppb if baseline ≤50 ppb	12
FEV_1+PC_{20}	1
FEV_1+FE_{NO}	1
PC_{20}+FE_{NO}	8
All 3 objective criteria	1
Any objective criteria	30
Using study defined criteria	**28**

Table 2.4: Frequency of subjects responding to ICS treatment according to different criteria/combinations of criteria



*3 of these subjects had an improvement in PC_{20} and 1 in FE_{NO}

† 3 of these subjects had an improvement in FE_{NO}

Figure 2.4: Showing pathway of patients through the study

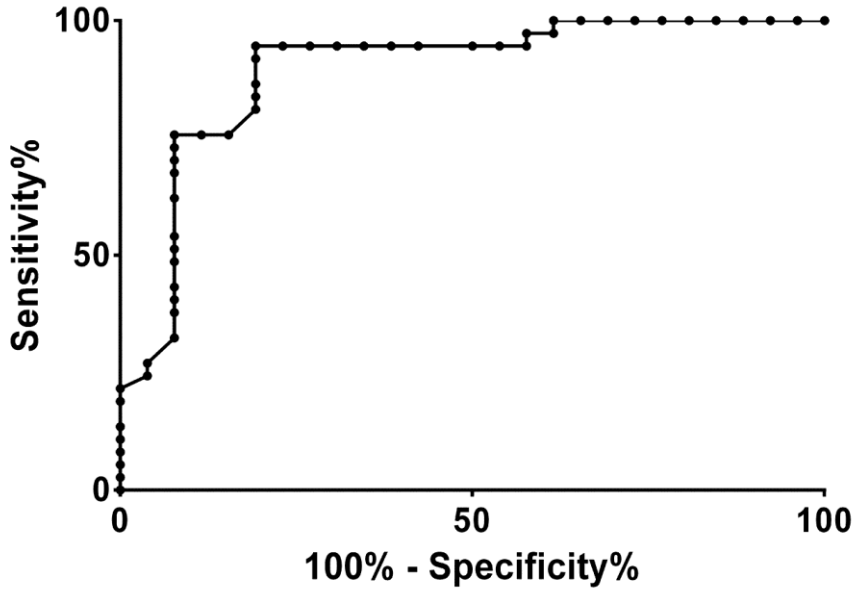


Figure 2.5: ROC curve analysis showing the sensitivity (%) and the 100 – specificity (%) of FE_{NO} levels for predicting ICS response after 4 weeks of ICS treatment

Baseline value	Response Criteria	ROC AUC	Optimal cut-off
FE_{NO}	Study defined criteria	0.89 (p<0.0001)	**For non-response: <27 ppb** • Sensitivity 92% • Specificity 75% • NPV 93% • PPV 71% **For response: >33 ppb** • Sensitivity 85% • Specificity 95% • NPV 91% • PPV 92%
FE_{NO}	Any objective criteria	0.85	
FE_{NO}	≥2 objective criteria	0.84	
FE_{NO}	Any subjective response	0.53	
FE_{NO}	FEV_1 alone	0.7	
FE_{NO}	PC_{20} alone	0.7	
FE_{NO}	FE_{NO} alone	0.89	
FE_{NO}	FEV_1 or PC_{20}	0.7	
FE_{NO}	FE_{NO} or PC_{20}	0.86	
FE_{NO}	FEV_1 or FE_{NO}	0.91	
FE_{NO}	FEV_1 + subjective response	0.7	
FE_{NO}	PC_{20} + subjective response	0.74	
FE_{NO}	FE_{NO}+ subjective response	0.88	

Table 2.5: ROC AUCs for FE_{NO} to predict ICS response as defined by different combinations of response criteria

Baseline value	Response Criteria	ROC AUC
PC_{20}	Study defined criteria	0.32
PC_{20}	Any objective criteria	0.32
PC_{20}	≥2 objective criteria	0.12
PC_{20}	Any subjective response	0.44
PC_{20}	FEV_1 alone	0.36
PC_{20}	PC_{20} alone	0.02
PC_{20}	FE_{NO} alone	0.46
PC_{20}	FEV_1 or PC_{20}	0.09
PC_{20}	FE_{NO} or PC_{20}	0.30
PC_{20}	FEV_1 or FE_{NO}	0.43
PC_{20}	FEV_1 + subjective response	0.36
PC_{20}	PC_{20} + subjective response	0.04
PC_{20}	FE_{NO}+ subjective response	0.47
FEV_1	Study defined criteria	0.58 (p=0.25)
FEV_1	Any objective criteria	0.43
FEV_1	≥2 objective criteria	0.29
FEV_1	Any subjective response	0.39
FEV_1	FEV_1 alone	0.29
FEV_1	PC_{20} alone	0.33
FEV_1	FE_{NO} alone	0.49
FEV_1	FEV_1 or PC_{20}	0.29
FEV_1	FE_{NO} or PC_{20}	0.44
FEV_1	FEV_1 or FE_{NO}	0.46
FEV_1	FEV_1 + subjective response	0.28
FEV_1	PC_{20} + subjective response	0.31
FEV_1	FE_{NO}+ subjective response	0.49
Blood eosinophils	Our defined criteria	0.67

Table 2.6: ROC AUCs for baseline PC_{20} and FEV_1 to predict ICS response as defined by different combinations of response criteria

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Skin prick positive (weal >3mm)	60.1	41.3	38.6	63.3

Table 2.7: Sensitivity, specificity, PPV and NPV of skin prick positivity (to any allergen) to diagnose asthma

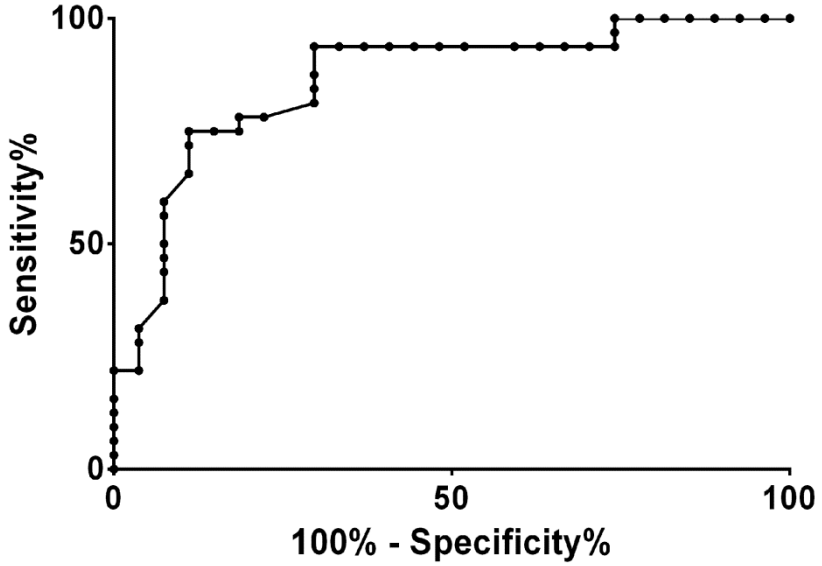


Figure 2.6: ROC curve analysis showing the sensitivity (%) and the 100 – specificity (%) of FE_{NO} levels for predicting ICS response after 12 weeks of ICS treatment

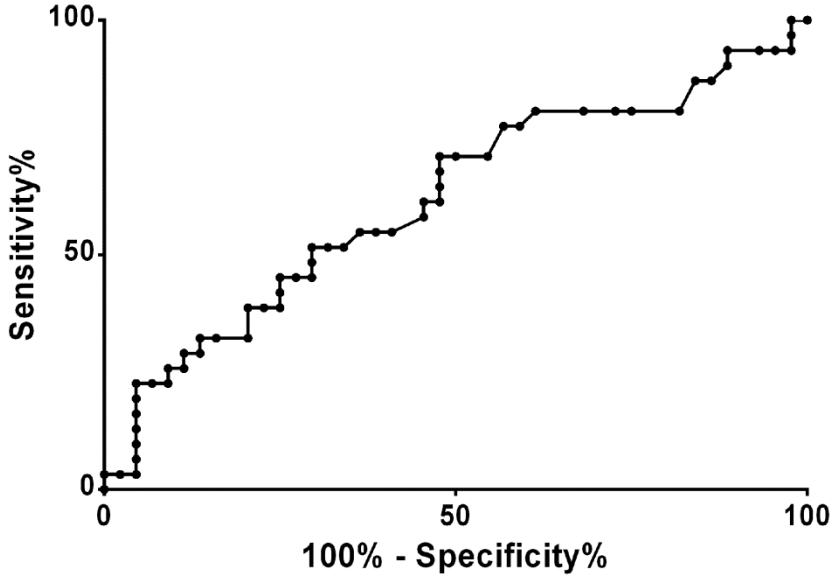


Figure 2.7: ROC curve analysis showing the sensitivity (%) and the 100 – specificity (%) of FE_{NO} levels for asthma diagnosis

a) For diagnosing asthma

Symptom	Sensitivity	Specificity	PPV	NPV
Nocturnal Sx	0.25	0.74	0.42	0.56
Sx on waking	0.22	0.81	0.47	0.58
Sx on activity	0.53	0.52	0.46	0.59
Dyspnoea	0.69	0.5	0.51	0.65
Wheeze	0.53	0.67	0.55	0.65
Cough	0.66	0.55	0.53	0.68

b) For predicting 'high FE_{NO}' (>27 ppb)

Symptom	Sensitivity	Specificity	PPV	NPV
Nocturnal Sx	0.2	0.69	0.42	0.43
Sx on waking	0.15	0.78	0.43	0.44
Sx on activity	0.38	0.34	0.39	0.32
Dyspnoea	0.55	0.4	0.51	0.44
Wheeze	0.5	0.71	0.67	0.55
Cough	0.75	0.74	0.77	0.72

c) For predicting 'high FE_{NO}' (>33 ppb)

Symptom	Sensitivity	Specificity	PPV	NPV
Nocturnal Sx	0.17	0.69	0.26	0.55
Sx on waking	0.1	0.76	0.21	0.56
Sx on activity	0.37	0.37	0.37	0.37
Dyspnoea	0.53	0.4	0.37	0.56
Wheeze	0.5	0.67	0.5	0.67
Cough	0.73	0.62	0.56	0.78

d) For predicting response to ICS

Symptom	Sensitivity	Specificity	PPV	NPV
Nocturnal Sx	0.18	0.75	0.36	0.54
Sx on waking	0.25	0.81	0.5	0.58
Sx on activity	0.5	0.44	0.41	0.53
Dyspnoea	0.53	0.44	0.43	0.55
Wheeze	0.5	0.67	0.54	0.63
Cough	0.85	0.64	0.65	0.85

Table 2.8: Sensitivity, specificity, positive (PPV) and negative (NPV) predictive values for each of the listed symptoms to a) diagnose asthma b) predict FE_{NO} >27 ppb c) predict FE_{NO} >33 ppb and d) predict response to ICS

Symptoms to predict asthma diagnosis			
	Odds ratio	**95% CI**	**p >Z**
Nocturnal Sx	0.94	0.33 - 2.70	0.91
Sx on waking	1.19	0.38-3.71	0.77
Sx on activity	1.25	0.50-3.13	0.64
Dyspnoea	2.2	0.81-5.75	0.11
Wheeze	2.27	0.88-5.83	0.09
Cough	2.31	0.89-5.97	0.08
Increased SABA use	3.64	0.86-15.4	0.08
Symptoms to predict high FE_{NO} (>27 ppb)			
	Odds ratio	**95% CI**	**p >Z**
Nocturnal Sx	0.55	0.19-1.56	0.26
Sx on waking	0.6	0.18-1.92	0.39
Sx on activity	**0.31***	**0.12-0.81**	**0.02**
Dyspnoea	0.81	0.32-2.04	0.66
Wheeze	2.5	0.96-6.53	0.06
Cough	**8.67***	**3.1-24.6**	**<0.0001**
Increased SABA use	1.37	0.35-5.30	0.65
Symptoms to predict ICS response			
	Odds ratio	**95% CI**	**p >Z**
Nocturnal Sx	0.65	0.19-2.22	0.5
Sx on waking	1.38	0.42-4.53	0.6
Sx on activity	0.8	0.30-2.15	0.66
Dyspnoea	0.92	0.34-2.49	0.87
Wheeze	2	0.73-5.52	0.18
Cough	**10.62***	**3.02-37.35**	**<0.0001**
Increased SABA use	0.74	0.16-3.42	0.7

Table 2.9: Odds ratios of symptoms to predict asthma diagnosis, high FE_{NO} (>27 ppb) and ICS response. Significant values are indicated*

2.4 Discussion

The results of this study indicate that measuring exhaled nitric oxide levels in patients presenting to primary care with symptoms suggestive of asthma is useful in predicting a response to ICS but not in diagnosing asthma. Another finding was that the symptom of cough in this patient cohort is associated with a significantly increased likelihood of response to ICS.

2.4.1 FE_{NO} for predicting ICS response

Our results suggest that FE_{NO} is a good predictor of ICS treatment response, with a high NPV and PPV for ICS non-response and response using cut-points of <27 and >33 ppb.

This supports previous findings where FE_{NO} was reported to be a useful predictor of response to ICS treatment in steroid naïve patients with symptoms suggestive of asthma (540). Similarly Little *et al.* (541) demonstrated FE_{NO} to have good predictive accuracy for oral steroid response in a group of 37 subjects, although these subjects had chronic asthma and were already treated with ICS.

The results obtained in the current study, do however, differ from those found previously (540) in terms of the optimal FE_{NO} cut-point to signify a likely 'negative' response to ICS. Smith *et al.* reported a FE_{NO} cut-point of 47 ppb to have a NPV for steroid response of 77-94% depending on the steroid response endpoint chosen, whereas these data suggest a lower value than this of 27 ppb with a NPV of 93%. This may be due to the different criteria chosen to designate ICS response as Smith *et al.* did not include decreased FE_{NO} value as a response criterion and considered a significant improvement in PC_{20} to be two or more doubling doses. Although the response criteria selected by the earlier study (540) are probably more definitive measures of ICS response, the criteria here are likely to be more sensitive to the detection of a response, and hence less likely to miss potential responders to ICS treatment. A fall in FE_{NO} was included as a criterion here which is not used routinely but was frequently the only objective change in patients with a subjective improvement in cough. This test has the advantage of being easily performed in primary care. Also, it has previously been established that a reduction in Th2 inflammation is associated with a reduced risk of exacerbations (552, 553), bringing some validity to the inclusion of FE_{NO} as a response criterion.

A selected cut-off of 33 ppb for ICS response found by this study is similar to the FE_{NO} value of 36 ppb previously determined to identify significant eosinophilic inflammation as defined by a sputum eosinophil count >3% (481).

Using investigations to determine the nature of airway inflammation underlying a patient's symptoms is attractive because it allows a prediction of response to treatment that labelling individuals with a diagnosis of 'asthma' or 'COPD' does not (159). Asthma has been shown to have eosinophilic and neutrophilic phenotypes (Sections 1.4.6 and 1.4.7) with a potentially good response to ICS being limited to the former (303). Of the 32 patients diagnosed with asthma in the current study only 18 (56%) responded to ICS, a finding in

keeping with that of Martin *et al.* (554) who found that only 54% of 72 asthma patients who had ICS withheld for 4 weeks responded when ICS were re-introduced. Likewise there is a subpopulation of patients with COPD who have eosinophilic inflammation and respond well to ICS treatment (555) and patients with cough secondary to EB who also improve with ICS (87). Our findings suggest that FE_{NO} could be a useful test in primary and secondary care but as a tool to target corticosteroid-responsive patients rather than to make or exclude a diagnosis of asthma.

2.4.2 FE_{NO} for asthma diagnosis

The poor sensitivity and specificity of FE_{NO} in diagnosing asthma may be due to the heterogeneity of the condition with different inflammatory subtypes expressing high or low levels of Th2 inflammation (552). Subjects with neutrophilic asthma, who may comprise up to 30% of all asthmatics (260, 296), by definition have no active eosinophilic/Th2 airway inflammation and therefore are likely to have normal FE_{NO} levels. The finding that only 57% of subjects with confirmed asthma responded to ICS further highlights the limitations of this diagnostic label.

Previous studies investigating the role of FE_{NO} for asthma diagnosis have produced mixed results, with widely varying values of sensitivity and specificity for a range of different defined optimal cut-points and a recent meta-analysis concluded the sensitivity and specificity of FE_{NO} was insufficient for accurate diagnosis (556). Some of this variation may be explained by differences in study methodology and FE_{NO} devices, which even using standardised flow rates may produce significantly different FE_{NO} readings (557). Measured FE_{NO} levels can be affected by a number of other factors including coexistent atopy (508) and respiratory tract infection (558) which increase FE_{NO} levels, whilst decreased FE_{NO} levels may be caused by smoking (558) and certain medications.

Owing to the variety of factors that can affect FE_{NO} levels, and the significant crossover in values between healthy and asthmatic populations the proposed NICE guidelines have advocated an approach to using FE_{NO} to help 'rule in' or 'rule out' asthma diagnosis, with subjects with 'intermediate' FE_{NO} levels requiring further investigation (543) (Section 2.2.4.5). However, the results of this study do not support this strategy.

2.4.3 Symptoms for predicting asthma diagnosis and ICS response

None of the symptoms assessed were significant independent predictors of objectively defined asthma. This is consistent with the results of the studies presented in Section 1.3. A literature review conducted as part of the draft NICE guidelines for asthma diagnosis (539) also concluded that the sensitivities and specificities of individual symptoms for asthma diagnosis were moderate or low, and as such the diagnosis of asthma based on symptomatology could not be recommended.

The symptom of cough was found to be an independent predictor of a FE_{NO} value >27 ppb, which was the optimal cut-off point derived for non-response to ICS in the first part of the study. The presence of cough made it ~9 times more likely that the subject would have a FE_{NO} value >27 ppb. It is not surprising then, given the results of the first part of the study, that cough was also a significant predictor of ICS response, with the presence of cough

signalling a 10 fold increase in likelihood of ICS response. Cough also had a high sensitivity and NPV for the prediction of ICS response but only moderate specificity and PPV.

The ability of symptoms to predict ICS response has not previously been assessed so this is a novel finding. The most likely explanation for this result is that Th2 high inflammation is more likely to cause the symptom of cough than other typical symptoms of airways disease such as dyspnoea and wheeze. A cough, which is usually dry, is described as the characteristic symptom of EB (88). Therefore if the sole or main underlying pathophysiological feature of a subjects' airway disease is Th2 high inflammation rather than other pathology such as AHR and airway obstruction, the most likely resultant clinical picture may be of a subject with a dry cough, rather than predominant dyspnoea or wheeze.

The value of high FE_{NO} and the symptom of cough in predicting ICS response could both be used in future to improve targeting of ICS treatment to subjects who are more likely to respond.

2.4.4 Study limitations

This study had several limitations which need to be addressed.

Firstly, criteria had to be selected on which to base a diagnosis of asthma whilst recognising there is no gold standard. The diagnostic criteria selected are standard criteria from international consensus guidelines and subject was classified as asthmatic if any one of the two chosen criteria were positive. Despite the lack of gold standard these objective tests were deemed to be the best on which to classify asthma diagnosis and limiting the criteria to positive MCT (considered the best 'rule-in' 'rule-out' test available) alone did not significantly alter the ROC AUC value for asthma diagnosis.

Secondly there was no formal measure of ICS adherence during the study although patients were questioned about their ICS usage at each visit. Therefore failure to respond to ICS may have been due to lack of adherence rather than a true negative response to treatment. This may also explain why three asthmatic patients who responded to ICS at 4 weeks did not show a sustained response at week 12 (Fig. 2.6). However, it is very unlikely that the degree of adherence with ICS would have varied markedly and consistently enough between those with higher and lower FE_{NO} baseline values to explain these findings, especially as patients were blinded to their FE_{NO} results.

Thirdly, this was an open label trial of ICS which did not include a placebo arm meaning at least part of the ICS treatment response was likely to be a placebo effect. However, when examining an individual's response to ICS a placebo treatment cannot be included unless a crossover study design is used. This was deemed unethical because it would have meant delaying patients' treatment as prescribed by their GPs. Further, several different objective measures of airway function were measured before and after treatment and subjects were blinded to the results, limiting the likelihood of bias.

Finally, the selected criteria for ICS response included a reduction in FE_{NO} levels which has not been used by others. Although it seems reasonable to assume that a decrease in FE_{NO} will translate into a clinical benefit this is, as yet, unproven. As with the other selected ICS

response criteria, a reduction in FE_{NO} alone was not considered significant unless accompanied by an improvement in another objective or subjective criterion. The nine subjects who had a response to ICS limited to a reduction in FE_{NO} with symptomatic improvement all presented with cough as their predominant symptom and none of them met the diagnostic criteria for asthma. Including a response measure relating to the level of eosinophilic airway inflammation was deemed important as suppression of eosinophilic inflammation is the main mechanism by which ICS have a beneficial treatment effect (543). A direct and therefore superior method of measuring eosinophilic airway inflammation is sputum induction to determine sputum eosinophil count. This was also attempted in 40/74 of our subjects but only 4 of these produced an adequate sample for analysis, making this an unsuitable investigation for use in this patient cohort.

2.5 Conclusion

In conclusion, in this group of patients presenting to primary care with symptoms suggestive of asthma, FE_{NO} is not a useful test for asthma diagnosis but is accurate at predicting ICS treatment response and non-response. The symptom of cough, which was predictive of raised FE_{NO} levels, also appears to be sensitive at predicting ICS response and could help to identify patients more likely to respond to ICS. We propose that FE_{NO} measurement in patients with symptoms suggestive of airways disease (shortness of breath, chest tightness and cough) could be used to identify patients in whom ICS response is highly unlikely. This would avoid unnecessary treatment with inhaled steroids and encourage further investigation of the cause of the symptoms and more effective treatment. This study has provided pilot data for the design of a multicentre placebo-controlled clinical trial to assess the value and safety of this approach.

Chapter 3: Chronic Productive Cough and the use of Macrolides in Airways Disease

3.1 Background

3.1.1 Summary of background (Chapter 1.6)

A cohort of adult patients presenting with chronic productive cough which improves with antibiotic treatment but quickly relapses has been described (1). A number of conditions that result in the symptom of chronic productive cough have been described but this cohort cannot be accurately described using any of these diagnostic labels, although the clinical course of the disease and response to antibiotics seems similar to that of the paediatric condition 'protracted bacterial bronchitis' (PBB). Many subjects in this cohort have been given a diagnostic label of asthma and are being treated with inhaled corticosteroids. (1).

A marked, and often sustained, improvement in symptoms has been observed in these patients following a 3 month course of low dose azithromycin. Azithromycin is a macrolide antibiotic which has demonstrated efficacy in the treatment of respiratory conditions including diffuse panbronchiolitis (DPB) (2), chronic obstructive pulmonary disease (COPD) (3) and bronchiectasis (4). In addition to antibiotic effects, azithromycin has demonstrated immunomodulatory and anti-inflammatory effects (5) which may be more pronounced in subjects with underlying neutrophilic airway inflammation (6).

3.1.2 Rationale for study

Although this cohort of patients seems to be recognised by clinicians in the respiratory clinic these patients are not described in the scientific literature. Hence the purpose of this study is to try and describe the clinical and pathological features of this condition and assess the response of these patients to an open label treatment trial of azithromycin.

3.1.3 Hypothesis and Aims

3.1.3.1 Hypothesis

There is a cohort of patients with chronic productive cough whose underlying pathology cannot be described by existing labels and who respond to treatment with azithromycin.

3.1.3.2 Aims

1) To describe the clinical, pathological and radiological features of this cohort of patients

2) To determine the response to a 12 week course of low dose azithromycin therapy and assess if any of the baseline characteristics measured could predict response to azithromycin.

3.2 Methods

3.2.1 Purpose and Design

3.2.1.1 Purpose

1) The primary objectives of the study were to describe the clinical and pathological features of a cohort of patients who present with chronic productive cough (with no evidence of bronchiectasis, smoking-related chronic bronchitis or immunodeficiency) and determine if treatment of these patients with 12 weeks of low dose azithromycin is both effective and safe.
2) The secondary objectives of the study were to determine the effect of 12 weeks azithromycin treatment on selected clinical measures and biomarkers and to describe the features of responders and non-responders to azithromycin

3.2.1.2 Study Design

This was a single centre open label clinical trial with an in-depth description of baseline clinicopathological features.

3.2.1.3 Ethical Approval

This study was approved by the National Research Ethics Committee Yorkshire & The Humber – Leeds West (Ref 13/YH/0245) and Nottingham University Hospitals NHS Trust Research and Innovation department (Ref 13RM015).

3.2.2 Study Population:

3.2.2.1 Eligibility criteria

Patients were recruited according to the following eligibility criteria:

Inclusion criteria

- Age 18 or above
- Male or female
- Non-smokers for 10 years and <20 pack year equivalents in total
- Persistent productive cough for >3 months in duration
- Use of effective contraception:
    - Acceptable contraceptive methods include: established use of oral, injected or implanted hormonal methods; placement of an intrauterine device (IUD) or intrauterine system (IUS); condom or occlusive cap (diaphragm or cervical/vault caps) with spermicide; true abstinence (when this is in line with the preferred and usual lifestyle of the participant); or vasectomised partner

Exclusion criteria

- History of obvious inhaled irritant exposure
- Evidence of primary or secondary immunodeficiency

- Clinically important bronchiectasis on HRCT scan
- Prolonged QT interval on baseline or 1 month electrocardiogram (ECG) or significant cardiac pathology prior to commencing azithromycin
- Pregnancy or intent to become pregnant during course of study
- Contra-indication to bronchoscopy (as per BTS Guidelines (747))
- Abnormal liver function tests (LFTs) (greater than 2x upper limit of normal)
- Hypersensitivity to azithromycin or any macrolide/ketolide antibiotic

3.2.2.2 Study setting and participant recruitment

This study was conducted at the Nottingham Respiratory Research Unit (Nottingham City Hospital UK). Subjects with symptoms of chronic productive cough without bronchiectasis, smoking-related chronic bronchitis or immunodeficiency were prospectively identified from outpatient respiratory clinics according to the eligibility criteria.

Interested subjects were provided with full written information from their respiratory consultant regarding the study and given the contact information for the study team (Appendix D). Upon contacting the study team by telephone they were screened to ensure they met the inclusion and exclusion criteria.

3.2.3 Outcome measures

3.2.3.1 Primary endpoints

- A description of the baseline clinicopathological features of the cohort including FEV_1, FE_{NO}, LCQ score, sputum differential cell count, 24 hour sputum volume, HRCT scan features and histological analysis of bronchial biopsy samples
- The effect of 12 weeks of azithromycin treatment on LCQ score

3.2.3.2 Secondary endpoints

- The effect of 12 weeks of azithromycin on sputum colour and 24 h sputum collection volume
- The effect of 12 weeks of azithromycin on sputum cell counts and FE_{NO} level
- The effect of 12 weeks of azithromycin on FEV_1
- The effect of 12 weeks of azithromycin treatment on sputum microbiology
- The effect of 12 weeks of azithromycin treatment on the levels of IL-8, IL-1β, IL-17A and TNFα in pre and post treatment sputum supernatant
- Comparison of the baseline clinical features of responders and non-responders to azithromycin

3.2.3.3 Sample size calculation

A power calculation was performed using a common standard deviation of 1.2 in LCQ score from a previous successful interventional study in patients with chronic cough, using LCQ score as a primary outcome (748). This determined that 30 patients would need to complete

the trial in order to give a 90% power at a 5% two-sided significance level to detect a drop in our primary outcome of the LCQ of at least 1.3 points, which is the minimum clinically important difference in LCQ (749).

3.2.4 Summary of study protocol

3.2.4.1 Visit 1

All patients meeting the entrance criteria for the study were invited to attend the first study visit where eligibility was rechecked and written informed consent obtained prior to any study-related interventions. FE_{NO}, exhaled carbon monoxide (ECO), spirometry, LCQ, sputum induction, electrocardiogram (ECG), liver function tests (LFTs) and pregnancy tests (if applicable) were carried out as outlined in Fig. 3.1 below.

The subject was asked to score the colour of their sputum according to a sputum colour chart. If an adequate sputum sample was produced by induction sputum colour was also visually assessed by the investigator and scored according to the sputum colour chart.

Subjects were given a universal sample container and instructed to collect all of the sputum produced by coughing in the 24 h period prior to their next study visit. Any subjects who opted out of having a bronchoscopy were instructed to begin the course of azithromycin following completion of 24 h sputum collection.

3.2.4.2 Visit 2

Subjects then attended a bronchoscopy visit as soon as possible after Visit 1, unless they had specifically opted out of this procedure. Bronchial biopsies and washes were taken. The 24 h sputum volume was also measured. Following bronchoscopy subjects were instructed to begin the course of azithromycin.

3.2.4.3 Visit 3

Visit 3 was a safety visit after 6 weeks of azithromycin treatment in which any adverse effects of the azithromycin were recorded. An ECG and LFTs were performed and use of medication was confirmed. Subjects were again given a universal sample container and instructed to collect all of the sputum produced by coughing in the 24 h period prior to their next study visit.

3.2.4.4. Visit 4

Visit 4 was the post-treatment visit (following 12 weeks of treatment) and use of medication was confirmed. Exhaled nitric oxide, spirometry and reversibility, LCQ and sputum induction were carried out (Fig. 3.1) and the 24 h sputum volume was also measured. Subjects were asked to score the colour of their sputum according to the sputum colour chart. If an adequate sputum sample was produced by induction this was visually assessed by the investigator and scored according to the sputum colour chart. If an adequate sputum sample was not produced an objective sputum colour score was determined from the 24 h sputum volume sample.

3.2.4.5 Visit 5

Visit 5 was the follow up visit 4 weeks after stopping treatment. The LCQ was performed and subjects were asked to score the colour of their sputum according to the sputum colour chart.

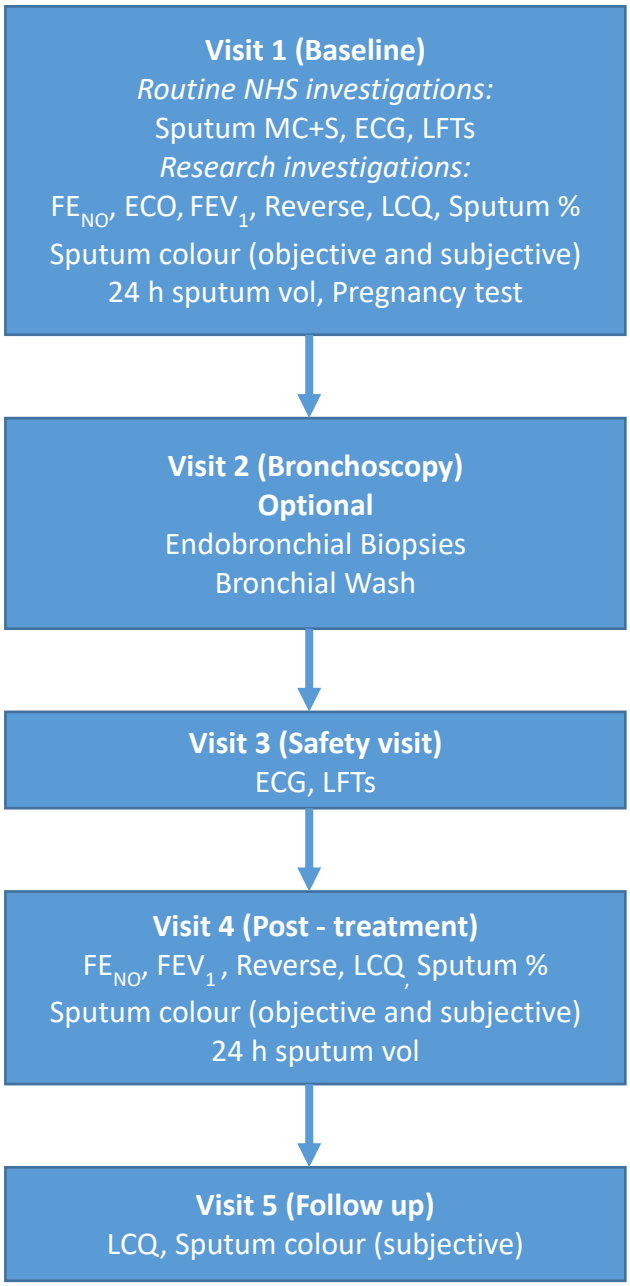


Figure 3.1: Investigations performed at each study visit.

Sputum MC+S = sputum microscopy, culture and sensitivity, ECG = electrocardiogram, LFTs = liver function tests, FE$_{NO}$ = Fractional exhaled nitric oxide level, ECO = Exhaled carbon monoxide, FEV$_1$ = spirometry, Reverse = reversibility, LCQ = Leicester cough questionnaire, Sputum % = Sputum differential cell count, Sputum colour = Sputum colour chart assessment, 24 h sputum vol = 24 hour sputum collection volume

3.2.5 Clinical Measurements

3.2.5.1 Leicester Cough Questionnaire

The LCQ is a validated questionnaire designed to assess the health related quality of life in patients with chronic cough (750). A copy is included in Appendix E.

It consists of 19 questions which are divided into 3 different domains: physical, psychological and social. Subjects are asked to provide a rated response to each question, all of which are designed to assess the impact of cough on the subject's life over the preceding 2 weeks. The total score ranges from 3 to 21, with a higher score corresponding to a better health related quality of life.

The LCQ has been validated for use in subjects with a number of different conditions leading to cough. It has been demonstrated to have a good level of internal consistency and reliability (751) and the minimal clinically important difference (MCID) is 1.3 (749).

3.2.5.2 24 hour sputum volume

The 24 h sputum collection volume is frequently used as an outcome measure in interventional studies in patients with chronic productive cough secondary to bronchiectasis (752, 753).

Subjects were asked to collect all sputum expectorated over a 24 h period in a universal sample container before visit 2 (bronchoscopy). Subjects who did not have bronchoscopy were asked to collect all sputum expectorated in a 24 h period at visit 1 and to return this to study staff prior to starting azithromycin treatment.

24 h sputum volume was measured by transfer of sputum from the universal sample container via a pipette to a measuring cylinder with 0.1 mL graduations. Any obvious salivary portion of the sample was discarded before final measurement.

3.2.5.3 Sputum Colour Chart

Sputum colour was assessed using a commercially available sputum colour chart (BronkoTest®, Heredilab Inc., Salt Lake City, UT, USA). This is a 5 point colour chart based on a 9 point colour chart demonstrated by Stockley *et al*. to correlate well with ongoing airway inflammation (610, 754). Colours 1 and 2 on the chart are regarded as non-purulent and colours 3–5 as purulent. More recently, Simpson *et al.* (755) found a BronkoTest® score of ≥3 to be a good predictor of ongoing neutrophilic bronchitis.

Subjects were asked to subjectively score their sputum colour based on the chart and this was also assessed objectively by study staff.

3.2.5.4 Exhaled Nitric Oxide

FE_{NO} concentration was measured using the Bedfont NOBreath offline electrochemical analyser (Bedfont Scientific Ltd, Harrietsham, UK).

All subjects first inhaled ambient air to near total lung capacity and then exhaled for 16 s at a constant flow rate through a mouthpiece into the device to provide two approved FE_{NO} measurements. The NOBreath device provides visual feedback to ensure an exhalation pressure of between 10-20 cmH_2O, regulated by the device to ensure a resultant flow rate of 50 mL/s.

3.2.5.5 Electrocardiogram (ECG)

12 lead ECGs were obtained according to American Heart Association (AHA) Guidelines (756). Patients were positioned in a semi-recumbent position at approximately 45 degrees to the horizontal. ECG electrodes were positioned in accordance with AHA guidelines (756) and 2 ECGs were recorded for each subject with the best quality of the two used for analysis of the QT interval. The QT interval was calculated as per AHA guidelines (757).

3.2.5.6 Liver Function Tests (LFTs)

Serum samples for LFTs were obtained using the 21 gauge BD Vacutainer® Safety-Lok™ blood collection set (BD, Plymouth, UK) into 5 mL serum tubes. Samples were processed in the Nottingham City Hospital biochemistry laboratory by automated cytometers.

3.2.5.7 Exhaled Carbon Monoxide

Exhaled carbon monoxide (ECO) was measured using an electrochemical CO monitor (CO Monitor, Clement Clarke Intl., Essex UK). An ECO value of <10 ppm was used to confirm subjects non-smoking status.

3.2.5.8 Spirometry and Reversibility

Performed as described in Section 2.2.5.1.

3.2.5.9 Sputum Induction

Performed as described in Section 2.2.5.3.

3.2.6 Bronchoscopy

3.2.6.1 Bronchoscopic Technique

Bronchoscopies were performed in the Nottingham City Hospital Endoscopy Centre by the clinical fellow with appropriate supervision by a named consultant and assisted by at least two endoscopy nurses. A clinical scientist was also present to aid with the initial sample capture in the appropriate storage media (see below). All bronchoscopies were conducted in accordance with BTS guidelines (747) and local research protocols. Subjects were nil by mouth for 4 h prior to the procedure and patients with a diagnosis of asthma had spirometry assessed prior to the procedure and premedication with 400 µg of salbutamol inhaled via Volumatic® spacer if necessary.

An intravenous cannula was inserted and all procedures performed under light sedation with midazolam (2.5 – 5 mg as necessary) and alfentanyl (250-500 µg as necessary).

Subjects' oxygen saturations were continuously monitored throughout the procedure by pulse oximeter and supplemental oxygen was delivered nasally. Local anaesthesia of the naso- and oro-pharynx and vocal cords to achieve suppression of gag and cough reflexes was achieved using 5-7.5 mL Instillagel® (CliniMed, Bucks, UK) nasally, 4-5 sprays (40-50 mg) of 10% lidocaine orally and 10 mL 4% followed by 10 mL 2% lidocaine to the vocal cords. Further 10 mL volumes of 2% lidocaine were also administered in the right and left main bronchi with further doses given as necessary.

Following a brief systematic inspection of the subjects' bronchial anatomy, bronchial washes were performed in the right upper lobe to provide a minimal wash volume of 20 mL. This was divided into four 5 mL volumes, with one volume being sent to the Nottingham City Hospital Microbiology laboratory for microscopy, culture and sensitivity and three 5 mL volumes being sent to the Nottingham Respiratory Research Unit (NRRU) laboratory for processing as described below. Between 4-8 bronchial biopsies were then taken from the right bronchus intermedius using 1.8 mm width alligator forceps (Radial Jaw®4, Boston Scientific, Costa Rica). At least two of these were placed in universal specimen pots containing 5 mL 4% formaldehyde in phosphate buffered saline (PBS) and transported at room temperature to the Nottingham City Hospital Histopathology department for specimen processing, paraffin embedding and staining. The remaining samples were transported to the NRRU laboratory for processing and cell culture. Finally, two bronchial brushings were taken at the sub-carina/right bronchus intermedius into a 3 mL Falcon tube containing bronchial epithelial cell growth medium with 1% penicillin-streptomycin-fungizone (BEGM + 1% PSF) and also transported to the NRRU laboratory for cell culture. After the procedure subjects were monitored for a 30 min period and asthmatic subjects underwent spirometry again, if clinically indicated.

3.2.6.2 Processing of Bronchial Wash samples

The full protocol describing this process is included in the Appendix F. Briefly:

- 1 x 5 mL sample was sent for differential cell count. This sample was centrifuged at 600 g for 10 min at 4^0C. The resultant pellet was re-suspended at approximately 5 x 10^5 cells/mL whilst the supernatant was divided into aliquots and frozen at -80^0C for future work including cytokine profiling. 75 µl of the re-suspended pellet was added to a cytospin funnel attached to a glass slide which was centrifuged and stained (See Section 2.3.5.4). A differential cell count was performed as in Section 2.3.5.4.
- 1 x 5 mL sample was centrifuged at 200 g for 5 min at 4^0C. The supernatant was transferred to a fresh tube and centrifuged at top speed (4147 g) for 15 min at 4^0C. The pellets from the first and second spins were labelled with the anonymised study subject number and stored at -80^0C for future analysis of bacterial DNA.

3.2.6.3 Processing of Bronchial Biopsy samples

Biopsies were removed from sample containers with blunt forceps and embedded using a standard paraffin wax embedding centre. The resultant embedded sample was cut with a microtome into 4-5 µm thick slices ensuring 4-8 biopsy slices per slide. All sections were

mounted on poly-L-lysine coated slides (Fisher Scientific UK Ltd.) and stained with haematoxylin and eosin (+/- alcian blue).

3.2.6.4 Radiological review of HRCT scans

The HRCT scans of study subjects were reviewed by a consultant radiologist blinded to subjects' response to azithromycin. After an initial review to look for any commonly occurring radiological features a checklist detailing the presence and absence of certain features and allowing a semi-quantitative assessment of certain important features was developed (See Appendix G). Scans were then reviewed in detail by the same consultant radiologist and the checklist completed for each subject's scan.

3.2.6.5 Cytokine profiling of baseline sputum supernatant/bronchial wash samples

The induced sputum and bronchial wash supernatant levels of IL-8, IL-1β, IL-17A and TNFα in all available samples from the study were quantified using a multiplex suspension immunoassay system (Bio-Plex, Bio-Rad, Hemel Hempstead, UK). Briefly, samples were added to microplate wells containing beads conjugated with capture antibodies specific to the cytokines listed above. Following binding of the capture antibodies to the target protein the plates were washed and then incubated with biotinylated detector antibodies. The plates were then washed a second time and a reporter streptavidin-phycoerythrin (SA-PE) conjugate added. Labelled beads were then passed through an array reader which quantified the fluorescence of bound SA-PE. Fluorescence values were compared to a standard curve, allowing quantification of the target cytokine levels.

In order to optimise the assay a test run was performed using aliquots of selected samples to establish an expected range of values. The assay was then performed again in triplicate on all available samples with appropriate dilution of samples where necessary based on the results of the test run.

3.2.7 Analysis

3.2.7.1 Statistical software

Microsoft Excel was used for data cleaning. Data were then imported into Stata v11.0 (Statacorp, Texas, USA) and GraphPad Prism Version 6 (GraphPad Software, California, USA) for statistical analysis. The demographics and baseline clinical measures of the cohort were determined.

3.2.7.2 Primary endpoints

a) Histological review of bronchial biopsy samples

Following completion of the study bronchial biopsy slides (Section 3.2.6.3) were reviewed by a consultant histopathologist under light microscopy with settings for histological colour images. This individual was blinded to subjects' response to azithromycin and was specifically asked to determine; (1) if there were any histological features common to this cohort of patients and (2) if there were significant differences in the biopsies of responders

and non-responders to azithromycin. Following a primary review for any notable features, slides were again reviewed and the presence or absence of these features was recorded.

b) Radiological review of HRCT scans

The sensitivity, specificity, positive and negative predictive values of HRCT scan features to predict treatment response to azithromycin were calculated.

c) Effect of azithromycin on LCQ score

Median LCQ scores pre- and post- 12 weeks of azithromycin treatment were calculated and pre- and post-LCQ scores compared using the Wilcoxon signed-rank test as data were not normally distributed and could not be transformed to normality.

3.2.7.3 Secondary endpoints

a) Effect of azithromycin on other clinical measures and sputum supernatant cytokine levels

The secondary endpoints of sputum volume and FE_{NO} were non-normally distributed and could not be transformed to normality. Hence median values were calculated for both variables pre- and post- azithromycin and the Wilcoxon signed-rank test was used to compare pre- and post-values. FEV_1 was normally distributed and mean FEV_1 values pre- and post- treatment were calculated with the paired T-test used to compare pre- and post-treatment values. Frequency tables for objective and subjective sputum colour score pre- and post-treatment were constructed and the Wilcoxon signed-rank test used to compare pre- and post-treatment scores.

The median concentration of the cytokines IL-8, IL-1β, IL-17A and TNF-α in pre and post treatment sputum samples and bronchial wash samples were compared using the Wilcoxon signed-rank test if they could not be transformed to normality. Pre and post treatment IL-1β levels were transformed to normality using a logarithmic transformation and pre and post geometric means were calculated and compared using a paired T-test.

b) Sub-group assessment: Responders vs non-responders and asthma vs non-asthma

Subjects were divided into two groups of "responders" or "non-responders". Responders were defined as subjects whose LCQ score had increased by greater than the MCID of the LCQ of 1.3 points (749). The analyses for the primary and secondary endpoints stated above were repeated in these two groups in order to try and determine any differences between these groups. Subjects' sputum inflammatory type was classified accordingly:

- *eosinophilic* subjects had a sputum differential eosinophil count (from V1) or bronchial wash differential cell count of ≥3%
- *neutrophilic* subjects had a sputum differential neutrophil count (from V1) or bronchial wash differential cell count of ≥61%
- *mixed granulocytic* subjects had a sputum differential eosinophil count (from V1) or bronchial wash differential cell count of ≥3% and a sputum differential neutrophil count (from pre- or post-treatment visits) or bronchial wash differential cell count of ≥61%

- *paucigranulocytic* subjects had a sputum differential eosinophil count (from V1) or bronchial wash differential cell count of <3% and a sputum differential neutrophil count (from pre- or post-treatment visits) or bronchial wash differential cell count of <61%
- *missing sample* subjects did not have a differential cell count on any sputum or bronchial wash samples due to either poor toleration of sputum induction or failure to produce an adequate or viable sample and either declining or being unsuitable for the bronchoscopy procedure

Subgroup analyses were also performed on subjects with and without asthma.

3.3 Results

3.3.1 Recruitment

Between January 2014 and January 2016 274 subjects presenting to one of eight different outpatient respiratory clinics were identified in screening with symptoms of chronic productive cough of unknown cause. Following further investigations, including standard workup for this symptom 120 subjects were identified as being in the cohort of interest for the study. Of these 120 subjects, 75 were unable to participate in the study for the reasons listed in Table 3.1 leaving 45 patients who were eligible and invited to participate in the study. Fifteen of these declined to participate, and 30 agreed and were recruited to the study.

Reason	**Number**
Already taking or had previously taken long term azithromycin	52
Already taking other long term antibiotic treatment	5
Documented macrolide allergy	2
Symptoms eventually improved/seasonal	7
Deranged liver function tests	2
Did not attend planned appointments following investigation	6
Declined CT scan	1
Total	**75**

Table 3.1: Reasons for non-eligibility for study in subjects identified with chronic productive cough of unknown cause

3.3.2 Losses and exclusions

One subject was withdrawn from the study following an adverse event (periorbital oedema) after taking the first dose of azithromycin. All of the other 29 participants completed the full 12 weeks of azithromycin treatment and contributed data for the primary analysis. Thirteen subjects did not have a bronchoscopy at visit 2 as 2 were unsuitable for the procedure, 5 had already had bronchoscopy procedures as part of their routine work-up and 6 declined bronchoscopy. Three participants did not attend the four week follow-up visit (V5).

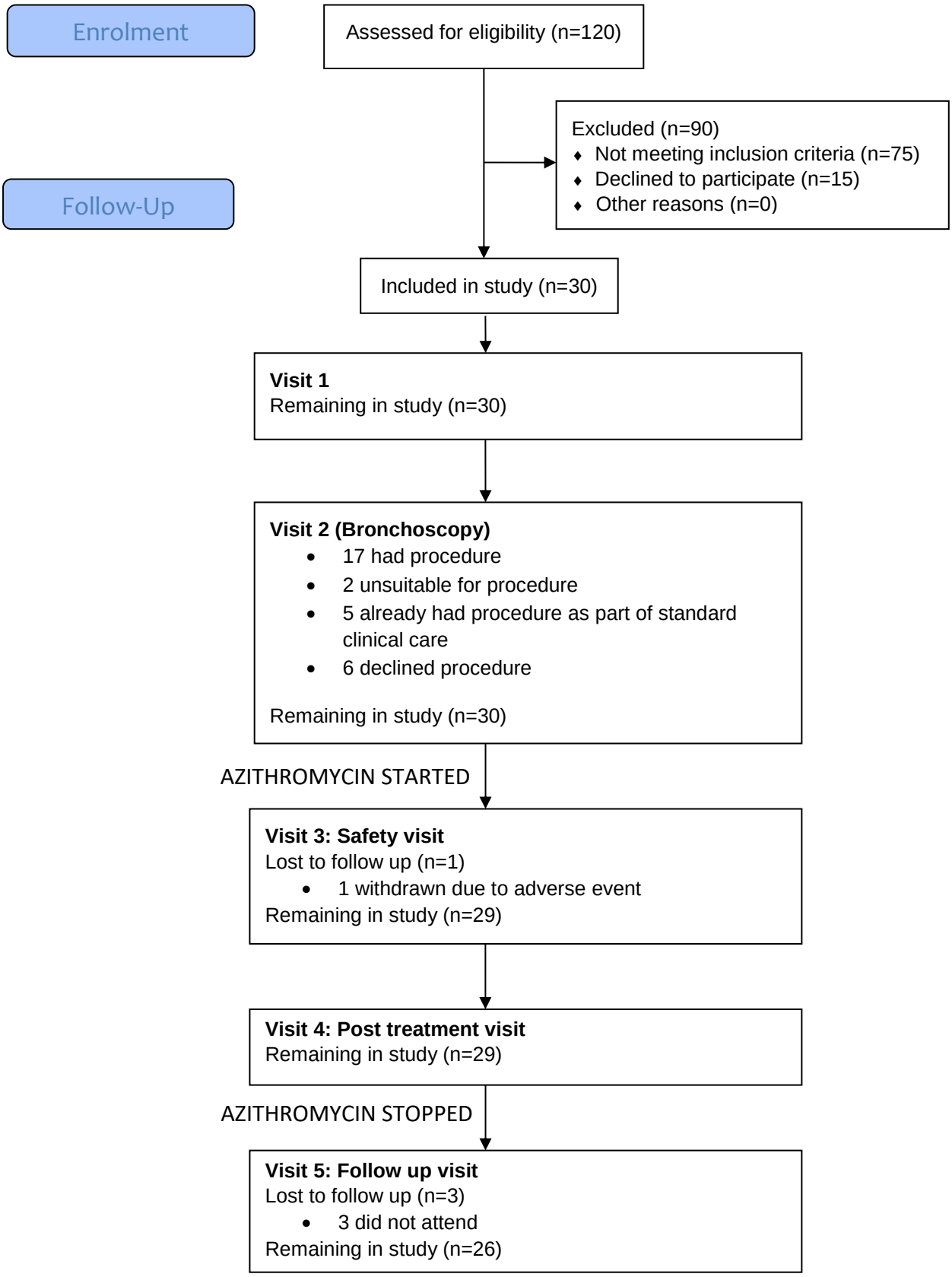


Figure 3.2: Consort diagram demonstrating losses and exclusions from study

3.3.3 Missing data

As AZCC03 was withdrawn from the study following the first dose of azithromycin, only baseline (V1) data and data from the bronchoscopy visit (V2) were collected from this subject.

Thirteen subjects were not suitable for or declined bronchoscopy meaning histological review of bronchial biopsy specimens and analysis of bronchial wash samples were not undertaken for these subjects.

One of the 30 subjects had an HRCT scan performed in another hospital and although the report was available to check the eligibility of this subject for the study the images were not accessible for subsequent radiological review on completion of the study.

Three subjects did not attend the final post-treatment visit (V5) and therefore V5 LCQ scores were not available for these subjects.

In terms of sputum samples for differential cell count; 7 subjects produced pre-treatment samples that were uncountable and 1 subject was unable to tolerate sputum induction. Post treatment samples were missing for 13 subjects; 8 of whom did not produce adequate samples post treatment; 3 produced samples that were uncountable; 1 could not tolerate sputum induction and 1 sputum induction had to be stopped for safety reasons. Three values for V4 subjective sputum colour were missing as these subjects were no longer producing sputum. Eight values for V4 objective sputum colour were missing as subjects did not produce sputum samples. Eight values for V5 subjective sputum colour were missing as these subjects were no longer producing sputum.

3.3.4 Primary outcomes

3.3.4.1 Baseline features of cohort

The baseline demographics and clinical features of the 30 participants in the study are shown in Table 3.2. The age distribution of the cohort is shown in Table 3.3. Overall there was a slight female preponderance and a large majority of the subjects were Caucasian. The age of the cohort ranged considerably from 25-77 years with a mean age of 57.3 years. Most of the cohort were overweight or obese with a median BMI of 29.9. Twelve subjects (40%) were ex-smokers (all of whom had not smoked for the preceding 10 years as stated in the inclusion criteria) and the mean pack year history was 6.8 pack years (SD 3.7 pack years, range 0.15-15 pack years). Seventeen of the 30 subjects had a diagnosis of asthma of whom all were taking ICS.

Histopathological examination of bronchial biopsies obtained from 17 of the 30 subjects revealed changes of chronic airway inflammation in 15 out of 17 of the subjects (Figs 3.3-3.6; Table 3.4). Inflammatory infiltrates were lymphocytic or plasmocytic in nature with no eosinophils seen and the severity of inflammation ranged from mild to severe. Basement membrane thickening was noted in 9/17 subjects but this did not correlate with asthma

status. Both subjects with no airway inflammation did not respond to azithromycin and the other 4 non-responders had changes consistent with mild airway inflammation only. In contrast the severity of airway inflammation in responders (n=10) varied from mild (n=5) to moderate (n=3) to severe (n=2), suggesting that the greater the burden of inflammatory changes, the more likely the response to azithromycin. The presence of moderate or severe airway inflammation had a reasonable sensitivity (0.7) and very high specificity (1.0) to predict response to azithromycin (Table 3.5).

The three most frequently identified abnormalities in the HRCT scans of 29 of the study subjects were; 1) airway dilatation graded 0=none (n=8), 1=mild dilatation (n=16) and 2=minor/borderline radiological bronchiectasis (n=5), 2) bronchial wall thickening graded 0=none (n=16), 1=some (n=11) and 2=prominent (n=2) and 3) atelectasis graded 0=none (n=9), 1=< 3 areas (n=11), 2=>3 areas (n=8) and 3=large bands (n=1). Airway dilatation (grade 1 or 2) had a good sensitivity (0.86) to predict azithromycin treatment response, but only a moderate specificity (0.56) whilst bronchial wall thickening (grade 1 or 2) had a low sensitivity and specificity (Table 3.6). Atelectasis had a reasonable sensitivity (0.62) for treatment response but no specificity. The other radiological features assessed (mosaic perfusion, lymphadenopathy, pleural thickening, patulous oesophagus, collapsible airways, endobronchial mucus, ground glass changes and tree in bud changes) were each present in only a few subjects and therefore sensitivity analysis was not performed (See Appendix H for data).

3.3.4.2 Effect of 12 weeks azithromycin treatment on LCQ score

Treatment with 12 weeks of azithromycin resulted in a significant overall improvement in the primary outcome measure of LCQ score (pre-treatment median 11.5 vs post-treatment median 17.8 $p<0.00001$) (Table 3.7). Twenty-two out of 29 subjects (76%) demonstrated a significant increase in LCQ score above the MCID of 1.3 points.

This improvement was largely sustained at 4 weeks post-treatment, with a follow-up median LCQ score of 15.9 ($p=0.0006$) (Table 3.7). Seventeen out of 26 (65%) subjects still reported LCQ scores greater than the MCID above baseline. However, 4 subjects (15%) did report worsening of their symptoms following the end of the azithromycin treatment course with visit 5 LCQ scores that were within the MCID from baseline or lower than the baseline LCQ score.

	Frequency	Percentage	
Total number included for analysis	30		
Mean age (range)	57.3 (25-77)		
Sex: male	13	43.3	
Ethnic group: Black Or Black British White Or White British	 1 29	 3.3 96.7	
Smoking history: Ex-smokers Non smokers	 12 18	 40 60	
Diagnosis of asthma	17	56.7	
On inhaled steroid treatment	17	56.7	
History/symptoms of GO reflux	6	20	
History/symptoms of PNDS	6	20	
	Mean	**Standard deviation**	**Range**
ICS dose (BDP equivalent µg)*	800	1000	0-4000
FEV_1 % predicted	96.4	22.0	49-131
FEV_1/FVC ratio %	76	8.5	60-90
	Median	**Interquartile range**	**Range**
Baseline (V1) sputum % neutrophils	65.6	41.3	4.5-99.25
Baseline (V1) median sputum % eosinophils	0.68	1.5	0-58
LCQ score	11.5	3.0	7.8-18.2
FE_{NO} (ppb)	19	20.5	0.5-52.5
Sputum volume (ml)	8.1	5.5	3-31.1

*Figures shown are median and interquartile range

Table 3.2: Demographics of all study subjects

Age group	Frequency	Percentage
20-30	2	6.7
30-40	3	10
40-50	4	13.3
50-60	7	23.3
60-70	9	30
70-80	5	16.7
Total	**30**	**100**

Table 3.3: Age distribution of study population

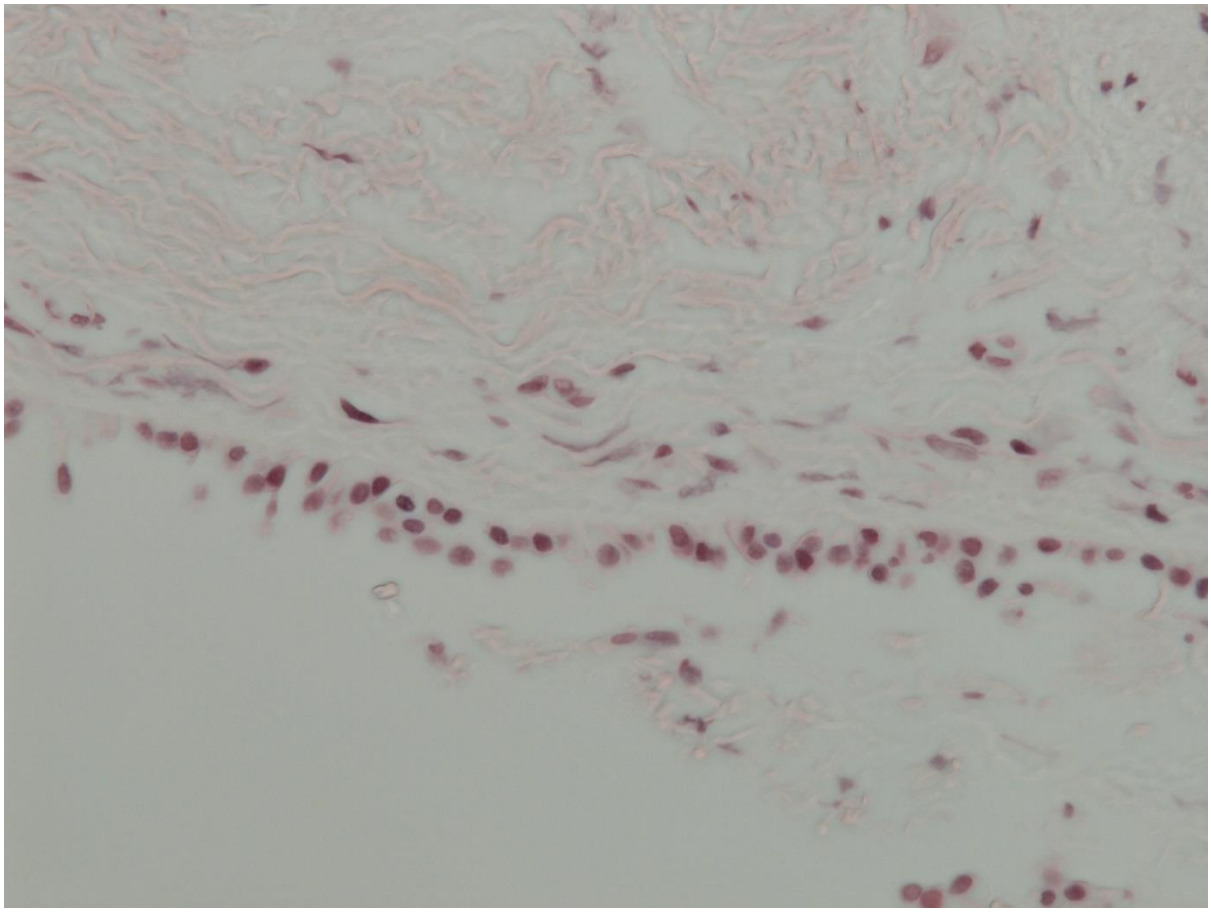


Figure 3.3: Representative image of bronchial biopsy from study patient AZCC06 (non-responder) showing no inflammation (Hematoxylin and eosin (H&E) stain, original magnification x200)

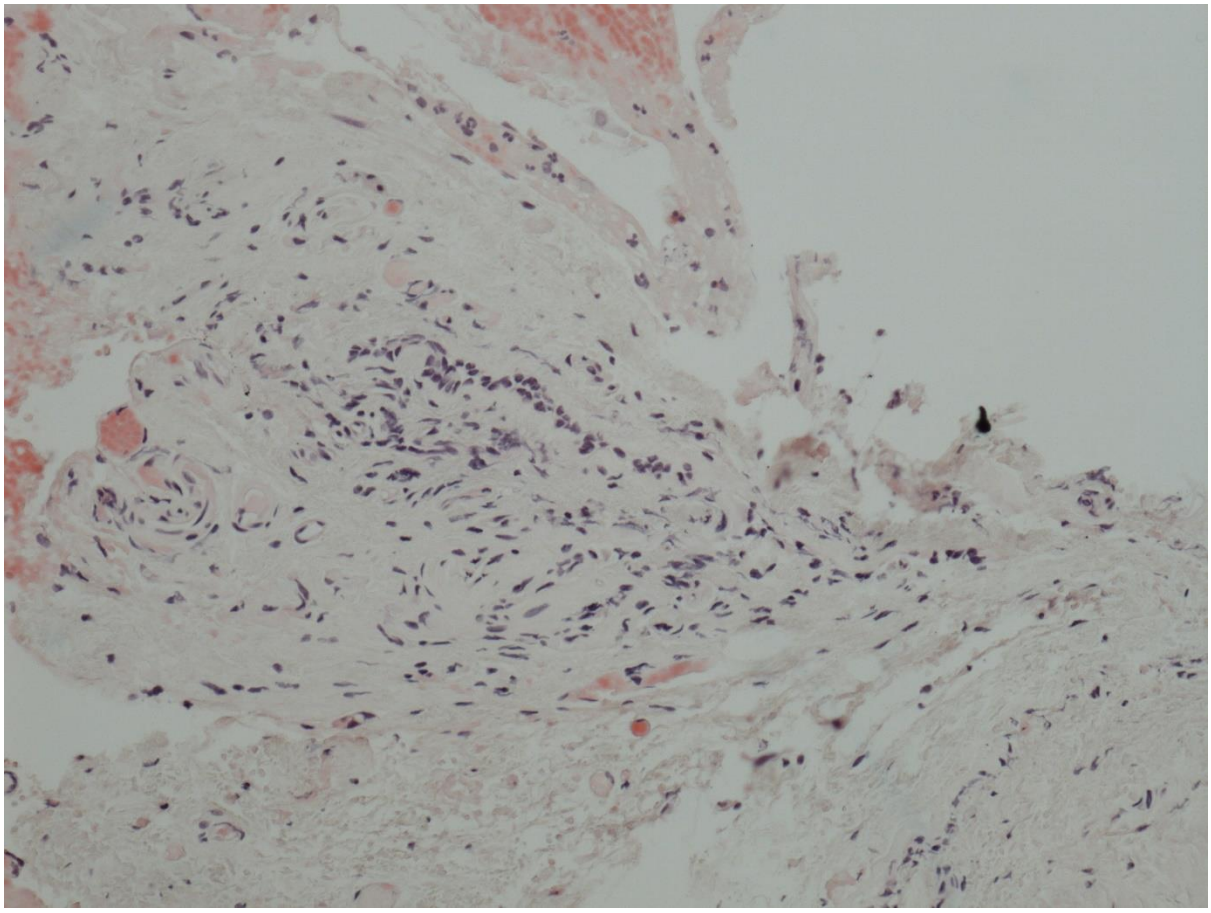


Figure 3.4: Representative image of bronchial biopsy from study patient AZCC21 (non-responder) showing mild inflammation with a lymphocytic infiltrate (H&E stain, original magnification x200)

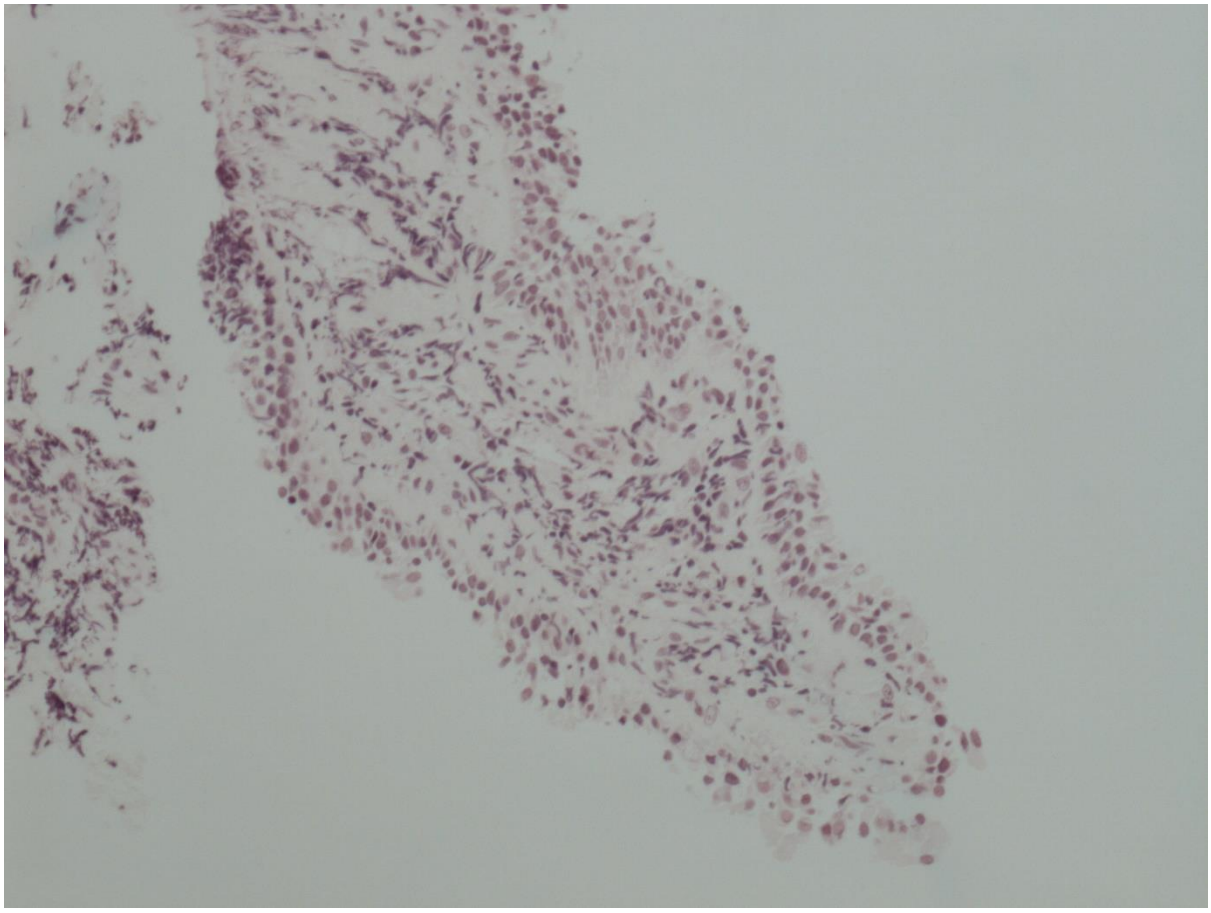


Figure 3.5: Representative image of bronchial biopsy from study patient AZCC10 (responder) showing moderate inflammation with a lymphocytic infiltrate (H&E stain, original magnification x200)

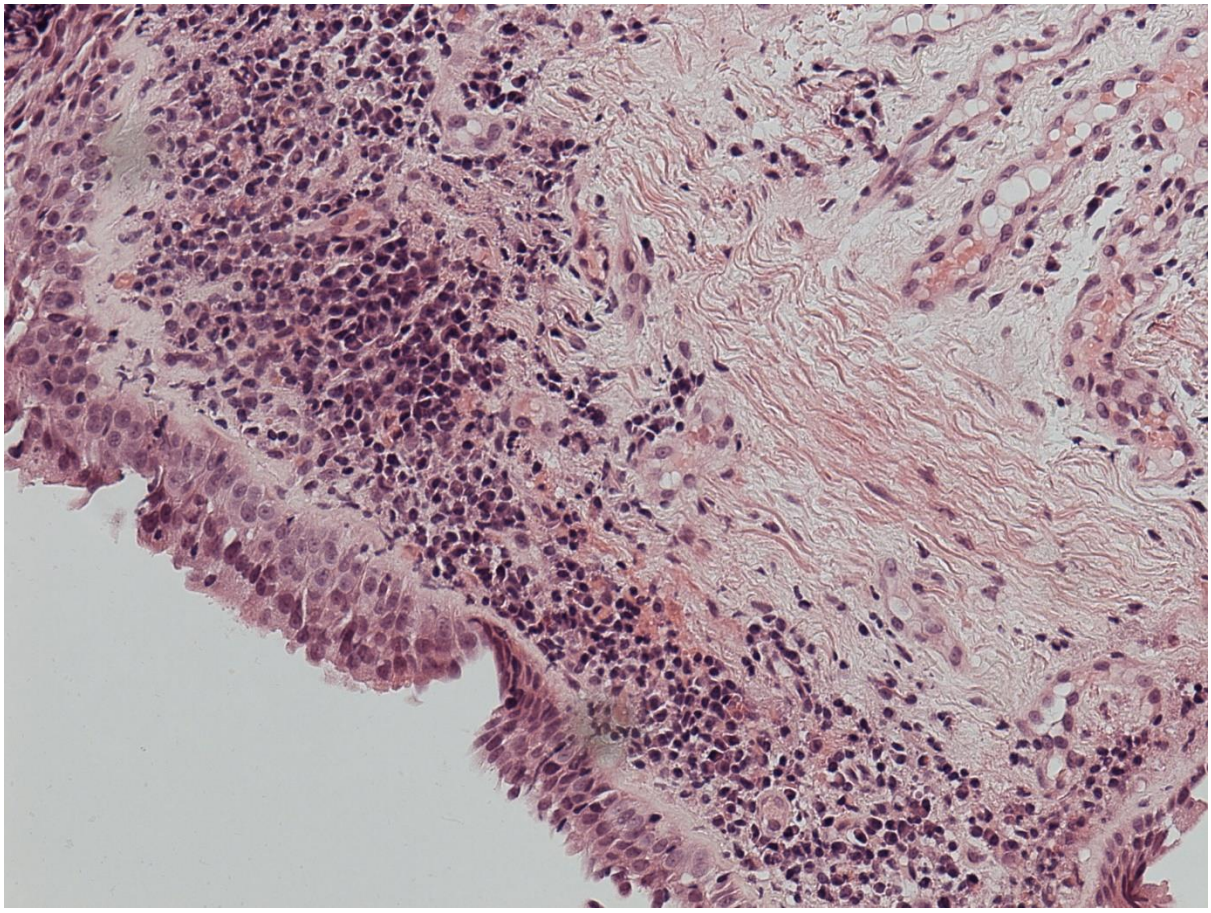


Figure 3.6: Representative image of bronchial biopsy from study patient AZCC03 showing severe inflammation with a lymphocytic infiltrate with prominent neutrophils and slight thickening of the basement membrane (H&E stain, original magnification x200)

Study No	Inflammation	Inflammatory infiltrate	Basement membrane thickening	Other features	Asthma	Response
AZCC01	Y Mild chronic	Lymphocytic	+	N	Y	Y
AZCC03	Y Severe chronic	Plasmocytic/ lymphocytic	+	N	N	dropped out
AZCC04	Y Severe chronic	Plasmocytic	Normal	Squamous metaplasia	N	Y
AZCC06	N	NA	+	N	Y	N
AZCC07	Y Mild chronic	Lymphocytic	Normal	Slightly oedematous	Y	Y
AZCC10	Y Mod chronic	Lymphocytic	++	N	Y	Y
AZCC12	Y Mild chronic	Lymphocytic	Normal	N	Y	N
AZCC14	Y Very Mild	N ?artefact of biopsy	Normal	N	Y	Y
AZCC15	Y Mild chronic	Lymphocytic	Normal	N	Y	Y
AZCC16	Y Severe chronic	Plasmocytic	++	N	N	Y
AZCC18	Y Mild chronic	Lymphocytic	+	N	Y	N
AZCC21	Y Mild chronic	Lymphocytic	Normal	N	N	N
AZCC22	Y Mild chronic	Lymphocytic	+	N	N	N
AZCC23	Y Mod chronic	Plasmocytic/ lymphocytic	+	N	Y	Y
AZCC24	N	NA	+	N	Y	N
AZCC25	Y Mild chronic	N	Normal	N	N	Y
AZCC26	Y Mod chronic	Lymphocytic	Normal	N	N	Y

Table 3.4: Histological features of bronchial biopsy samples

Feature	Sensitivity	Specificity	PPV	NPV
Moderate to severe airway inflammation	0.7	1.0	1.0	0.67

Table 3.5: Predictive values of moderate to severe airway inflammation on bronchial biopsy for azithromycin treatment response

Feature	Sensitivity	Specificity	PPV	NPV
Airway dilatation	0.86	0.57	0.86	0.57
Bronchial wall thickening	0.33	0.14	0.54	0.07

Table 3.6: Predictive value of HRCT abnormalities for azithromycin treatment response

	V1	V4	V1-V4 difference	Significance (p=)	V5	V1-V5 difference	Significance (p=)
LCQ score	11.5 (3)	17.8 (5.9)	6.3	**<0.00001***	15.9 (8.3)	4.4	**0.0006***
24 hour sputum volume (ml)	7.9 (5.5)	2.1 (7.2)	-5.8	**0.0001***			
FE_{NO} level (ppb)	19 (19.5)	12.5 (12)	-6.5	0.14			
FEV_1 (l) †	2.77 (0.99)	2.75 (1.0)	-0.02	0.78			

Figures shown are Median (IQR) except FEV_1† which is Mean (SD)

Table 3.7: Changes in primary and secondary outcome measures for whole cohort (n=29) with azithromycin treatment

	V1 (n=)	V4 (n=)	V1-V4 difference (p=)	V5 (n=)	V1-V5 difference (p=)	V4-V5 difference (p=)
Subjective sputum colour:						
1 (non-purulent)	4	4	0.44	5	0.76	0.33
2	7	8		3		
3	13	11		9		
4	3	2		3		
5 (purulent)	3	4		1		
Objective sputum colour:						
1 (non-purulent)	6	9	**0.003***			
2	6	7				
3	9	3				
4	5	2				
5 (purulent)	3	0				

Table 3.8: Changes in subjective and objective sputum colour for whole cohort (n=29) with azithromycin treatment

3.3.5 Secondary Outcomes:

3.3.5.1 Effect of treatment on other outcome measures

Treatment with 12 weeks of azithromycin also resulted in significant improvements in the secondary outcome measures of 24 h sputum volume (pre-treatment median 7.9 mL vs post-treatment median 2.1 mL, p=0.0003) (Table 3.7) and objective sputum colour (p=0.003) post-treatment (Table 3.8).

There were no statistically significant differences in FEV_1, FE_{NO} or subjective sputum colour score (Tables 3.7 & 3.8). Ten subjects produced paired pre and post treatment sputum samples. There was a significant decrease in the sputum differential neutrophil count (pre-treatment median 86.1% vs post-treatment median 69.4%, p=0.049) but no significant change in the sputum differential eosinophil count (Table 3.9).

Adequate sputum samples for cytokine analysis were obtained from 28 subjects at visit 1 and 15 of these subjects also produced adequate samples for analysis at visit 4. In these 15 subjects there was no significant difference in the sputum concentration of IL-17, TNF-α or IL-8 after azithromycin treatment, although sputum IL-1β concentration decreased significantly (p=0.02) following azithromycin treatment (Table 3.10).

	V1	V4	V1-V4 difference	Significance (p=)
% Sputum differential neutrophil count (IQR)	86.1 (33.5)	69.4 (18.6)	-16.7	**0.049**
% Sputum differential eosinophil count (IQR)	0.75 (1.5)	0.5 (7)	-0.25	0.64

Table 3.9: Changes in sputum differential neutrophil and eosinophil counts in subjects with pre and post treatment sputum samples (n=10)

Cytokine	Median concentration pre – treatment pg/ml (IQR)	Median concentration post treatment pg/ml (IQR)	Difference in median	p value
IL-17	15.2 (6.4)	11.8 (5.7)	-3.4	0.82
TNF-α	52.2 (38.7)	38.3 (18)	-13.9	0.33
IL-8	14146.1 (3904.3)	14324 (5326.3)	177.9	0.46
*IL-1β**	943.8	372.4	-571.4	0.02

Table 3.10: Sputum concentrations of measured cytokines pre and post azithromycin treatment (v1 n=28, v4 n=15) *Data presented for IL-1β are geometric means as variable logarithmically transformed to normality

3.3.5.2 Responders vs non-responders

Table 3.11 shows the demographic information and clinical features of the subjects who responded to azithromycin and those who did not. The demographics of the group who responded to azithromycin (n=22) and those who did not respond (n=7) were compared. There were no significant differences in the composition of these groups in terms of age, gender, ethnicity, smoking status or diagnoses of asthma, PNDS or GORD. The majority of the responder group had underlying neutrophilic inflammation (63.6%) in sputum or BAL whereas most of the non-response group had underlying eosinophilic inflammation (71.4%). The responder group also had significantly higher FEV_1 values and FEV_1/FVC ratios than the non-responder group.

When considered separately the difference between the pre and post treatment median LCQ score in the response group was 7 ($p<0.0001$) (Table 3.12). Significant improvements were also seen in median 24 h sputum volume (pre-treatment 6.9 mL vs post-treatment 2.0 mL $p<0.0001$), subjective sputum colour between V1 and V5 ($p=0.01$) and objective sputum colour ($p=0.001$) (Tables 3.12 & 3.13). There was also a significant decrease in median FE_{NO} level (pre-treatment 18 ppb vs post-treatment 12 ppb $p=0.009$) (Table 3.12). There were no significant changes in any of these measures for the non-response group, except for the subjective sputum colour becoming more purulent between V1 and V5 ($p=0.02$) (Tables 3.14 & 3.15).

3.3.5.3 Sub-group analysis based on asthma diagnosis

The demographics of the groups categorised as having a diagnosis of asthma or not having asthma were also compared (Table 3.16). There were no statistically significant differences in the composition of these groups and no significant differences in their average spirometric values.

Seventeen subjects (57%) had a diagnosis of asthma. Twelve of these (71%) showed improvements in LCQ score above the MCID following 12 weeks of azithromycin treatment and the median LCQ score improved from 12 to 16.5 following treatment ($p=0.008$) (Table 3.17). The median 24 h sputum volume also significantly decreased from 9.5 mL to 2.1 mL ($p=0.005$) and objective sputum colour improved significantly ($p=0.02$) (Tables 3.17 & 3.18). No significant changes were noted in FEV_1, FE_{NO} or subjective sputum colour (Tables 3.17 & 3.18).

Of the 12 subjects without an asthma diagnosis, 10 (83%) had a significant improvement in LCQ score and the median LCQ score improved from 11.4 to 18.8 ($p=0.002$) (Table 3.19). The median 24 h sputum volume significantly decreased from 6.8 mL to 0 mL ($p=0.02$) and there were significant changes in subjective sputum colour between visits 1 and 5 ($p=0.05$), although not between visits 1 and 4 (Tables 3.19 & 3.20). Objective sputum colour also improved significantly between visits 1 and 4 ($p=0.05$) (Table 3.20). There were no significant changes in FEV_1 or FE_{NO} (Table 3.19).

	Responders	**Non responders**	
	Frequency (%) (except [a])	**Frequency (%) (except [a])**	**Significance (p=)**
Total number included for analysis	22	7	
Mean age (range)	55.5 (25-77)	63.9 (55-70)	0.20
Sex: male	7 (31.8)	5 (71.4)	0.09
Ethnic group: Black Or Black British White Or White British	 1 (4.6) 21 (95.4)	 0 (0) 7 (100)	 1.0
Smoking history: Ex-smokers Non smokers	 7 (31.8) 15 (68.2)	 4 (57.1) 3 (42.9)	 0.38
Diagnosis of asthma	11 (50)	6 (85.7)	0.19
On inhaled steroid treatment	11 (50)	6 (85.7)	0.19
History/symptoms of GO reflux	4 (18.2)	1 (14.3)	1.0
History/symptoms of PNDS	4 (18.2)	2 (28.6)	0.61
Sputum/bronch inflammatory type			
Neutrophilic (>61%)	14 (63.6)	1 (14.3)	
Eosinophilic (>3%)	0 (0)	5 (71.4)	
Paucigranulocytic	5 (22.7)	1 (14.3)	
Missing sample	3 (13.6)	0 (0)	**<0.001**
	Mean (SD)	**Mean (SD)**	**Range**
ICS dose (BDP equivalent µg)*	800 (800)	900 (800)	0.12
FEV_1 % predicted	103.6 (18.8)	73.6 (17.3)	**0.0009**
FEV_1/FVC ratio %	78.4 (7)	67.6 (8)	**0.0019**
Baseline (V1) sputum % neutrophils	73.2 (21.9)	46.8 (34.2)	0.06
Baseline (V1) sputum % eosinophils	0.5 (0.75)	13.7 (24.8)	**0.03**

**Figures shown are median and IQR*

Table 3.11: Demographics of azithromycin responders vs non-responders

	V1	V4	V1-V4 difference	Significance (p=)	V5	V1-V5 difference	Significance (p=)
LCQ score	11.5 (2.9)	18.5 (3.2)	7.0	**<0.0001***	19.2 (6.3)	7.7	**0.0003***
24 hour sputum volume (ml)	6.9 (4.9)	2.0 (3.5)	-4.9	**<0.0001***			
FE_{NO} level (ppb)	18 (17)	12 (9)	-6	**0.009***			
FEV_1 (l) †	2.91 (0.98)	2.96 (1.0)	0.05	0.23			

Figures shown are Median (IQR) except FEV_1† which is Mean (SD)

Table 3.12: Changes in primary and secondary outcome measures for azithromycin responders (n=22) with azithromycin treatment

	V1 (n=)	V4 (n=)	V1-V4 difference (p=)	V5 (n=)	V1-V5 difference (p=)	V4-V5 difference (p=)
Subjective sputum colour:						
1 (non-purulent)	1	4	0.09	5	**0.01***	0.59
2	6	6		2		
3	10	8		7		
4	3	1		0		
5 (purulent)	2	0		0		
Objective sputum colour:						
1 (non-purulent)	3	6	**0.001***			
2	6	6				
3	5	2				
4	5	0				
5 (purulent)	3	0				

Table 3.13: Changes in subjective and objective sputum colour for azithromycin responders (n=22) with azithromycin treatment

	V1	V4	V1-V4 difference	Significance (p=)	V5	V1-V5 difference	Significance (p=)
LCQ score	12.0 (4.0)	10.8 (4.2)	-1.2	0.5	11.1 (2.7)	-0.9	0.45
24 hour sputum volume (ml)	11.5 (5.9)	13.5 (8.3)	2	0.61			
FE_{NO} level (ppb)	19 (37.5)	35.5 (70)	16.5	0.13			
FEV_1 (l) †	2.31 (0.94)	2.09 (0.93)	0.23	0.23			

Figures shown are Median (IQR) except FEV_1† which is Mean (SD)

Table 3.14: Changes in primary and secondary outcome measures for azithromycin non-responders (n=7) with azithromycin treatment

	V1 (n=)	V4 (n=)	V1-V4 difference (p=)	V5 (n=)	V1-V5 difference (p=)	V4-V5 difference (p=)
Subjective sputum colour:						
1 (non-purulent)	3	1	0.16	0	**0.02**	0.03
2	1	2		1		
3	3	3		2		
4	0	1		3		
5 (purulent)	0	0		1		
Objective sputum colour:						
1 (non-purulent)	3	3	0.56			
2	0	1				
3	4	1				
4	0	2				
5 (purulent)	0	0				

Table 3.15: Changes in subjective and objective sputum colour for azithromycin non-responders (n=7) with azithromycin treatment

	Asthma	Non asthma	
	Frequency (%) (except [a])	**Frequency (%) (except [a])**	**Significance (p=)**
Total number included for analysis	17	13	
Mean age (range)	55 (25-75)	59 (30-77)	0.45
Sex: male	6 (35.3)	7 (53.9)	0.43
Ethnic group: Black Or Black British White Or White British	 0 (0) 17 (100)	 1 (7.7) 12 (92.3)	 0.41
Smoking history: Ex-smokers Non smokers	 5 (29.4) 12 (70.6)	 7 (53.9) 6 (46.1)	 0.26
On inhaled steroid treatment	17 (100)	0 (0)	
History/symptoms of GO reflux	2 (11.8)	4 (30.8)	0.62
History/symptoms of PNDS	3 (17.7)	3 (23.1)	0.67
Sputum/bronch inflammatory type			
Neutrophilic (>61%)	9	6	
Eosinophilic (>3%)	5	0	
Paucigranulocytic	2	4	
Missing sample	1	2	0.11
	Median (IQR)	**Median (IQR)**	
ICS dose (BDP equivalent µg)	800 (200)	0 (0)	
FEV_1 % predicted	89 (36)	100.5 (24)	0.71
FEV_1/FVC ratio %*	76 (10.1)	76.1 (6.2)	0.86

*Figures shown are mean and standard deviation

Table 3.16: Demographics of sub-group with asthma diagnosis vs those without asthma diagnosis

	V1	V4	V1-V4 difference	Significance (p=)	V5	V1-V5 difference	Significance (p=)
LCQ score	12 (3.8)	16.5 (4.8)	4.5	0.008	13.6 (5.7)	1.6	0.09
24 hour sputum volume (ml)	9.5 (7.0)	3.5 (8.3)	6.0	0.002			
FE_{NO} level (ppb)	19 (17)	12 (11.5)	7	0.36			
FEV_1 (l) †	2.67 (0.96)	2.59 (1.02)	-0.08	0.37			

Figures shown are Median (IQR) except FEV1† which is Mean (SD)

Table 3.17: Changes in primary and secondary outcome measures for subjects with a diagnosis of asthma (n=17) with azithromycin treatment

	V1 (n=)	V4 (n=)	V1-V4 difference (p=)	V5 (n=)	V1-V5 difference (p=)	V4-V5 difference (p=)
Subjective sputum colour:						
1 (non-purulent)	2	3	0.69	3	0.23	0.12
2	5	5		0		
3	8	6		6		
4	2	2		3		
5 (purulent)	0	0		1		
Objective sputum colour:						
1 (non-purulent)	4	8	0.02			
2	2	3				
3	7	2				
4	2	2				
5 (purulent)	2	0				

Table 3.18: Changes in subjective and objective sputum colour for subjects with a diagnosis of asthma (n=17) with azithromycin treatment

	V1	V4	V1-V4 difference	Significance (p=)	V5	V1-V5 difference	Significance (p=)
LCQ score	11.4 (2.2)	18.8 (3.0)	7.4	0.002	19.7 (4.9)	8.3	0.005
24 hour sputum volume (ml)	6.8 (4.3)	1.05 (5.25)	-5.75	0.01			
FE_{NO} level (ppb)	15.25 (17.25)	13.5 (11.25)	-1.75	0.48			
FEV_1 (l) †	2.90 (1.06)	2.98 (1.07)	0.08	0.28			

Figures shown are Median (IQR) except FEV_1† which is Mean (SD)

Table 3.19: Changes in primary and secondary outcome measures for subjects without a diagnosis of asthma (n=12) with azithromycin treatment

	V1 (n=)	V4 (n=)	V1-V4 difference (p=)	V5 (n=)	V1-V5 difference (p=)	V4-V5 difference (p=)
Subjective sputum colour:						
1 (non-purulent)	2	2	0.45	2	0.05	0.47
2	2	3		3		
3	5	5		3		
4	1	0		0		
5 (purulent)	2	0		0		
Objective sputum colour:						
1 (non-purulent)	2	1	0.05			
2	4	4				
3	2	1				
4	3	0				
5 (purulent)	1	0				

Table 3.20: Changes in subjective and objective sputum colour for subjects without a diagnosis of asthma (n=12) with azithromycin treatment

3.4 Discussion

The results of this study support previous observations that there is a cohort of patients with chronic productive cough of unknown cause whose symptoms frequently respond well to prolonged low dose azithromycin treatment (560). There was a marked improvement in the primary outcome of LCQ score, a subjective measure of cough-related quality of life, as well as significant improvements in the objective secondary outcome measures of 24 h sputum volume and sputum colour as assessed objectively.

3.4.1 Primary Outcome: Description of cohort

Although some further work needs to be done to carefully delineate the underlying pathophysiology in this cohort of subjects several key features of this previously undescribed phenotype of airways disease have been recognised.

Firstly, although the overall number of subjects (n=30) recruited to the study in a 24 month period seems relatively few, which would suggest a low incidence of subjects with this phenotypic characteristic, the number of subjects screened who met the entrance criteria for this study was much higher. The majority of screen positive subjects could not be included in the study as they were already being treated with prolonged low-dose azithromycin, suggesting recognition of this patient group and the response of their symptoms to azithromycin may already be widespread amongst consultant respiratory physicians in the respiratory clinic.

Secondly, the symptoms experienced by this cohort do not appear to be related to some of the most frequent recognised causes of chronic cough. Only 6 subjects (20%) had a diagnosis of GORD and/or were receiving treatment for the condition but none described active symptoms. Entirely eliminating clinically silent reflux as a cause of these symptoms would be very challenging, but the diagnosis of GORD alone as an explanation of these symptoms seems insufficient given the significant symptom burden displayed by most of these patients. Similarly, only 6 subjects (20%) had the clinical features or diagnosis of post nasal drip syndrome (PNDS), and none of these had responded symptomatically to a lengthy period of conventional treatment for this condition. Equally, these subjects' symptoms are unlikely to be due to chronic bronchitis secondary to cigarette smoking or other noxious stimuli. None of the patients described in the study had smoked in the last 10 years, and all of them had total smoking pack year histories of less than 20 pack years. None of the study subjects had a significantly raised ECO on entering the study, which would be consistent with a non-smoking population.

The relationship between asthma and this cohort of patients is harder to discern. The majority of patients (n=17, 56.7%) had an asthma diagnosis, but this was usually a historic diagnosis on the basis of symptoms with little or no supporting objective evidence. Ten (33%) patients had evidence of airways obstruction (9 of whom had diagnoses of asthma) and 4 of these (40%) had evidence of ongoing eosinophilic inflammation. These 4 patients

seemed to fit more suitably into a 'classical' eosinophilic asthma phenotype than the rest of the cohort, and their productive cough was likely secondary to ongoing airway eosinophilia and chronic mucus hypersecretion, which have previously been described as features of uncontrolled severe asthma (621).

Unfortunately as the study did not include measures of reversibility or AHR further supporting or opposing objective evidence of asthma in this cohort was not available. A significant proportion of this cohort did not have an asthma diagnosis and it is clear that the prominent symptom in all of these subjects was cough, with very few describing other symptoms of airways disease such as significant dyspnoea, wheeze or episodes of asthma exacerbation. It is possible therefore that some of the cases in the described cohort may represent neutrophilic asthma. It is also possible that some of the subjects may have originally had eosinophilic asthma with suppressed eosinophilic inflammation due to ICS treatment and these subjects are now displaying neutrophilic inflammation as a result of other factors, for example, bacterial airway colonisation. However, this does not explain the significant proportion of subjects with no clinical features of asthma who were not taking ICS treatment and had ongoing neutrophilic inflammation.

The two most frequent radiological features of disease noted in this cohort were airway dilatation and bronchial wall thickening. A small proportion of the cohort (n=5) exhibited a minor degree of bronchiectasis on detailed review of their HRCT scans but with a disparity between their low burden of disease and prominent symptoms. Airway dilatation was a sensitive but non-specific predictor of azithromycin treatment response. Bronchial wall thickening is a common radiological feature of airways disease especially recognised in subjects with neutrophilic asthma (758) but this was not sensitive or specific at predicting treatment response.

The changes of airway dilatation, in combination with histological changes of chronic inflammation and cytokine profiling demonstrating high levels of Th1 and Th17 cytokines would all be compatible with a diagnosis of bronchiectasis. This cohort may represent subjects with "pre" - bronchiectasis, who have sustained an initial airway insult and have features of persisting neutrophilic inflammation and excessive airway secretions, but whose disease has not yet progressed to macroscopic airway destruction.

3.4.2 Primary Outcome: Effect of 12 weeks azithromycin treatment on LCQ score

LCQ improved significantly with azithromycin treatment in this group of patients. LCQ was selected as the primary outcome measure as this is a well validated quality of life measure relating to cough. The LCQ has been validated in separate distinct conditions such as bronchiectasis (759), but the symptoms in this cohort would seem to be sufficiently similar for this measure to retain validity. This measure was chosen as azithromycin has previously been demonstrated to improve symptom or quality of life scores in studies of subjects with neutrophilic asthma, COPD and bronchiectasis (615, 722, 760). The outcome of disease exacerbation rate, which has also been demonstrated to improve with azithromycin

treatment (723-725) was not suitable to use in this study as most subjects reported a constant level of symptomatology rather than 'exacerbations' of disease activity.

3.4.3 Secondary Outcome: Effect of azithromycin on clinical measures/biomarkers

Evidence of an objective response to azithromycin treatment was observed with a reduction in sputum amount and purulence. As expected, azithromycin treatment did not result in any improvement in lung function, a finding consistent with multiple previous RCTs demonstrating no significant change in lung function with azithromycin treatment (719, 723, 725, 728, 730). There was also no significant change in FE_{NO} level.

Ten subjects produced paired pre and post treatment sputum samples. There was a significant decrease in the sputum differential neutrophil count (pre-treatment median 86.1% vs post-treatment median 69.4%, p=0.049) but no significant change in the sputum differential eosinophil count. This suggests azithromycin treatment may lead to symptomatic improvement by decreasing levels of neutrophilic inflammation. This finding is consistent with previous studies showing significant decreases in neutrophilic airway inflammation with azithromycin treatment (671, 672, 760).

Levels of IL-8, IL-1β, IL-17A and TNFα were assessed in sputum and bronchial wash samples as increased levels of these cytokines have previously been noted in neutrophilic airways disease (674, 761). In comparison with previous investigations that utilised the same multiplex immunoassay system to quantify sputum cytokine levels in patients with asthma and COPD, the sputum concentrations of IL-8, IL-1β, IL-17 and TNFα were generally found to be much higher in our patients (762, 763). The sputum concentrations of IL-8 and TNFα were comparable to those detected in sputa from subjects with bronchiectasis using ELISA (764). IL-1β is a potent pro-inflammatory cytokine found at high levels in the sputum and lung tissue of COPD patients (765) and sputum in more severe phenotypes of bronchiectasis (766). Expression of IL-1β is induced by transcription factor NF-κB which is released by innate immune cells after exposure to *alarmins* (endogenous molecules released by tissue damage which cause activation of the immune system) (767) and high IL-1β levels have been demonstrated to induce pulmonary neutrophil airway inflammation and airway damage in mice (674). Sputum levels of IL-1β decreased significantly with azithromycin treatment which is consistent with findings from previous studies (673, 768) and may suggest a mechanism for decreased neutrophilic inflammation.

3.4.4 Responders vs non-responders

The improvement in LCQ was significant across the cohort as a whole, but separate consideration of azithromycin responders and non-responders revealed that none of the subjects with underlying eosinophilic airway inflammation (n=5) responded symptomatically to azithromycin. This is consistent with previous RCT data demonstrating no significant treatment response to azithromycin in subjects with asthma, a significant proportion of whom would be expected to have underlying eosinophilic airway inflammation (636, 742, 744). However, one previous study (769) investigating clarithromycin treatment in asthmatic

subjects reported an improvement in symptoms, AHR and sputum eosinophilia after 8 weeks of clarithromycin treatment, although the number of subjects was small (n=17).

The response of subjects with neutrophilic airways inflammation to azithromycin is consistent with the clinical improvement seen in other cohorts of patients with proven sputum neutrophilia (760, 770) or likely neutrophilic predominant airway disease such as bronchiectasis and COPD (615, 723-725). When considered separately from the neutrophilic subgroup the improvement seen in the paucigranulocytic group is less marked, with a lesser degree of improvement in LCQ score and no improvement in any other variable. The reason for this improvement in LCQ score with azithromycin in the paucigranulocytic group is less clear. Potential mechanisms for this observed improvement include immunomodulatory or antibiotic effects of azithromycin as well as the possibility of a placebo response.

Recognition of the heterogeneity and complexity of airways disease has led to proposals for a different system of classifying disease, based not on archetypal disease labels but on the recognition of phenotypic or biological markers of disease (so-called 'treatable traits') that enable targeted treatment (160). The results of this study, irrespective of the exact underlying airway pathology in this cohort, indicate that the symptom of chronic productive cough, especially when combined with evidence of ongoing neutrophilic airway inflammation, may represent a trait which could in future be used to target prolonged macrolide therapy.

3.4.5 Study limitations

The main limitations of this study include its relatively small size and the lack of a placebo group.

A significant number of subjects who were eligible according to our criteria were already using low dose azithromycin (Figure 3.2) and hence were not suitable for the trial. As suggested above this means the prevalence of individuals with this phenotype is much higher than the study suggests. Although the final number completing the trial was small (n=29) the improvement in the primary outcome measure of LCQ was very highly significant owing to a large reported improvement in symptoms by the majority of participants.

The true magnitude of this effect is difficult to discern firstly because of the subjective nature of the LCQ as an outcome in comparison to objective measures and secondly because of the potential of a placebo response in these subjects. However, placebo response alone in these subjects is less likely owing to the concurrent improvement noted in more objective measures such as 24 hr sputum collection volume and sputum colour (as assessed objectively) as well as the significant decreases in the sputum differential neutrophil count and sputum IL-1β concentrations.

A placebo controlled arm was not included in the study as the underlying pathophysiology in these subjects was still obscure and may have been attributable to an existing disease label

that had already been demonstrated to be responsive to azithromycin treatment such as bronchiectasis or asthma. On conclusion of the study there is now further evidence to suggest these patients may represent a distinct phenotype, or at least exhibit features that are not adequately described by an existing disease label.

3.4.6 Further work

The findings of this study need confirmation via a placebo controlled trial of azithromycin in subjects selected using similar eligibility criteria. Further work that would help to establish the nature of disease in this cohort of patients would include a longitudinal cohort study of such patients to clarify the natural history of disease progression in these subjects. In view of the increasing evidence suggesting that disturbances in the airway microbiota (dysbiosis) may be associated with neutrophilic airway inflammation further studies assessing the airway microbiota of these subjects are also warranted.

3.5 Conclusion

This study describes a cohort of patients with chronic productive cough not adequately described by existing disease labels whose symptoms responded well to low dose azithromycin. Patients who demonstrated the most significant symptomatic response to azithromycin primarily had neutrophilic airway inflammation while those with paucigranulocytic airway inflammation also improved albeit less markedly. Good predictors of treatment response also included moderate to severe inflammatory changes on bronchial biopsy and airway dilatation on HRCT scan. Possible mechanisms of response to azithromycin include reduction in airway neutrophilia and IL-1β levels. Further studies, especially longitudinal studies of this cohort, are required to validate these initial findings and determine the prognosis and progression of disease in this patient group.

Chapter 4: Microbiota in Asthma

4.1 Background

4.1.1 Summary of background (Chapter 1.7)

Analysis of the human microbiota is a promising and rapidly expanding field. Studies to date of the lung microbiota have led to new insights into the pathogenesis and progression of lung disease. Generally, findings so far in most respiratory conditions indicate that reducing bacterial diversity and dysbiosis of the microbiota with dominance of certain pathogenic species correlate with worsening disease severity and outcomes. Studies of the bacterial microbiota in asthma have revealed differences in its composition to that of healthy controls, suggesting that an increased abundance of potentially pathogenic species such as *Proteobacteria* are associated with asthma, particularly the neutrophilic asthma subgroup, and with certain clinical measures.

4.1.2 Rationale for study

It has not been possible to establish whether the presence of these organisms in the airways of asthmatic subjects is due to disease itself, or whether this may represent a treatment effect of high dose inhaled steroids. This study aims to examine, in unprecedented detail, the effects of ICS dose and type on the microbiota composition in subjects with asthma.

4.1.3 Hypothesis and Aims

4.1.3.1 Hypothesis

It is hypothesised that the use of high dose ICS alters the microbiota in asthma, acting as a selective pressure that favours the establishment of colonising species of potentially pathogenic species such as *H. influenzae* (See Fig 1.10).

It is further speculated that the type of ICS used by an individual may have an effect on the composition of the airway microbiota in asthma.

4.1.3.2 Aims

1) To compare the microbiota composition in sputum samples from subjects with mild (BTS Step 2) and moderate/severe asthma (BTS Step 4)

2) To compare microbiota composition between samples from subjects with asthma using the inhaled steroid fluticasone and those using budesonide.

Other aims of the study include assessment of the reproducibility of the induced sputum method for assessing the lung microbiota, assessment of the longitudinal stability of the bacterial population and comparison of the bacterial load of two common respiratory pathogens (*H. influenzae* and *S. pneumoniae*) in the BTS Step 2 and 4 groups.

4.2 Methods

4.2.1 Purpose and Design

4.2.1.1 Purpose

1) To determine if there is any relationship between the diversity of the bacterial microbiota of the lung (as sampled by induced sputum) and clinical features of asthma and severity

2) To determine if there is any difference in lung microbiota diversity between patients using inhaled fluticasone and those using inhaled budesonide

3) To assess the reproducibility and stability of the composition of the microbiota in sputum samples induced at multiple time points.

4.2.1.2 Study Design

This was a single centre non-interventional study.

4.2.1.3 Ethical Approval

This study was approved by the National Research Ethics Committee East Midlands – Derby 1 (Ref 14/EM/0091) and Nottingham University Hospitals NHS Trust Research and Innovation department (Ref 14RM006).

4.2.2 Study Population

4.2.2.1 Eligibility criteria

Patients were recruited according to the following eligibility criteria:

Inclusion criteria

- Age 18 or above
- Male or female
- Diagnosis of asthma (previous physician diagnosis)
- Non-smokers for 10 years and <10 pack year equivalents in total
- BTS Step 2 patients must have been using inhaled steroids at a dose of BDP ≤400 µg/day, FP ≤200 µg/day or BUD ≤400 µg/day for at least 1 year (535)
- BTS Step 4 patients must have been using inhaled steroids at a dose of FP ≥500 µg/day or BUD ≥800 µg/day for at least 1 year as a separate steroid or inhaled steroid/long acting beta agonist combination (535)

Exclusion criteria

- Respiratory infection or antibiotics within last month
- Pregnancy or intent to become pregnant during course of study
- Other respiratory diagnosis
- Post bronchodilator FEV_1 of <60% (417)

4.2.2.2 Study setting and participant recruitment

This study was conducted at the Nottingham Respiratory Research Unit (Nottingham City Hospital UK). Subjects with asthma were identified from an existing research subject database (Ref 09/H0405/27) or prospectively identified from outpatient respiratory clinics according to the eligibility criteria.

Interested subjects were provided with full written information regarding the study and given the contact information for the study team (Appendix L). Upon contacting the study team by telephone or e-mail they were screened to ensure they met the inclusion and exclusion criteria.

4.2.3 Outcome measures

4.2.3.1 Primary endpoint

The difference in sputum microbiota diversity/composition and quantitation between the BTS Step 2 and BTS Step 4 groups

4.2.3.2 Secondary endpoints

The difference in sputum microbiota diversity/composition and quantitation between:

- The BTS Step 4 group using inhaled fluticasone and the BTS Step 4 group using inhaled budesonide
- Sputum samples taken at baseline and those taken at 24 hours to assess the repeatability of the sampling technique
- Sputum samples taken at baseline and those taken at 14 days to assess the stability of the sampled microbiota

Other planned analyses included:

- Sputum microbiota diversity/composition and quantitation vs FEV_1
- Sputum microbiota diversity/composition and quantitation vs FE_{NO} level
- Sputum microbiota diversity/composition and quantitation vs PC_{20}
- Sputum microbiota diversity/composition and quantitation vs LCQ score
- Sputum microbiota diversity/composition and quantitation vs ACQ score
- Sputum microbiota diversity/composition and quantitation vs ICS dose (BDP equivalent)
- Abundance of known respiratory pathogens (*H. influenzae and S. pneumoniae*) in BTS Step 2 and BTS Step 4 BUD/FLU groups

4.2.3.3 Sample size calculation

As published data in this field is so limited, a formal power calculation to determine the necessary sample size was not possible.

Based on the numbers of patients required in previous studies of the human microbiota to determine differences between subject groups we specified a target of 50 subjects divided into the following groups (Fig 4.1):

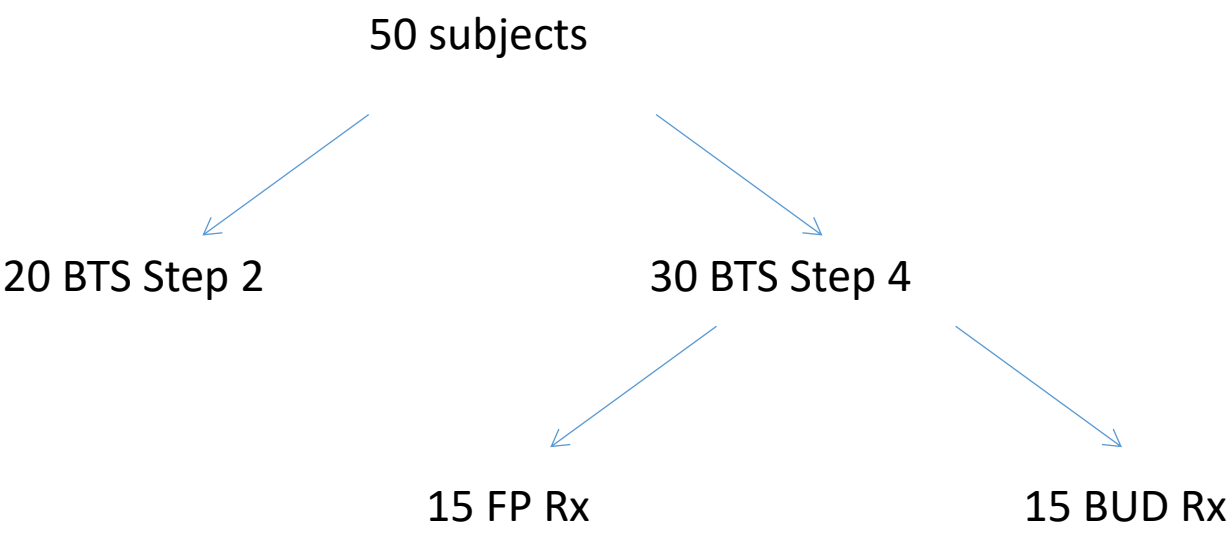


Figure 4.1: Demonstrating target patient recruitment numbers for each subgroup

Where FP Rx = Fluticasone therapy
And BUD Rx = Budesonide therapy

A sub-group of 20 patients who were available for visits 2 and 3 and successfully produced a sputum sample at visit 1 were invited to attend two further research visits. We attempted to select a sub-group of patients representative of the whole population, but the composition of this sub-group was largely determined by the subject availability for further visits.

4.2.4 Summary of study protocol

4.2.4.1 Visit 1

All patients meeting the entrance criteria for the study were invited to attend the first study visit where eligibility was rechecked and written informed consent obtained prior to any study-related interventions. The tests performed at visit 1 are listed in Figure 4.2 below:

4.2.4.2 Visits 2 and 3

Patients who were able to attend the two optional follow up visits and who successfully produced a sputum sample at Visit 1 also attended Visit 2 (within 24 h of Visit 1) and Visit 3 (within 2 weeks of Visit 1) for further sputum induction.

Figure 4.2: Demonstrating investigations performed at each study visit

ACQ = asthma control questionnaire, LCQ=Leicester cough questionnaire, FE_{NO} = Fractional exhaled nitric oxide level, FEV_1 = spirometry, BHR = methacholine challenge, Sputum = sputum samples taken for (1) microbiota analysis then (2) for differential sputum cell count if sufficient remaining then (3) for microscopy, culture and sensitivity count if sufficient remaining

4.2.5 Clinical Measurements

4.2.5.1 Spirometry

Performed as described in Section 2.2.5.1 (537).

4.2.5.2 Methacholine challenge

Performed as described in Section 2.2.5.5 (434).

4.2.5.3 Exhaled Nitric Oxide

Exhaled nitric oxide concentration was measured using an offline electrochemical analyser (Bedfont Scientific Ltd, Harrietsham, UK) as described in Section 3.2.5.4.

4.2.5.4 Leicester Cough Questionnaire

Performed as described in Section 3.2.5.1 (750).

4.2.5.5 Juniper Asthma Control Questionnaire

Performed as described in Section 2.2.5.8 (546).

4.2.5.6 Sputum Induction

Sputum induction was performed largely as described in Section 2.2.5.4 (405). However, slight alterations to this protocol were introduced to attempt to minimise oropharyngeal and environmental bacterial contamination of the samples.

These alterations consisted of:

1) Subjects were asked to rinse their mouths with 10 mL of 0.9% saline solution prior to nebulisation of saline to minimise oral contamination

2) Samples were collected into sterile specimen containers rather than standard universal collection containers

4.2.6 Protocol for processing induced sputum samples

All sputum samples were processed within 2 hours of induction. In summary, the steps according to the protocol were:

1) Isolate a 50 mg sputum plug (saliva free).
2) Add 4 x 0.1% DTT per mg sputum.
3) Vortex for 30 s to allow thorough mixing.
4) Transfer homogenate to a pre-labelled cryovial and store at -80^{o}C for future transfer to King's College London
5) If there is remaining sputum send ≥50 µL Homogenised Sputum/DTT for Microbiology.
6) If there is any remaining sample perform cell count and cytospin as per Sputum processing protocol (Section 2.2.5.4)
7) If any remaining sample divide into 4 x Equal aliquots of Homogenised Sputum/DTT ≤500 µL. (Extra equal aliquots can be used for large samples).

Transfer of samples to King's College London

Samples were transported by courier in one batch on dry ice to King's College London and were stored again at -80^{o}C prior to DNA extraction.

4.2.7 DNA extraction

DNA extraction was conducted using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich Co. Ltd., Dorset, UK) according to specific instructions for Gram-positive bacteria, with the following modifications. Sputum samples (100 µL) were initially mixed with lysozyme (200 µL; 45 mg/mL, Sigma-Aldrich Co. Ltd., Dorset, UK) suspended in Gram-Positive Lysis Solution (included in the kit), prior to incubation at 37 ^{o}C for 30 min (832). Cell disruption was then achieved by insertion of tungsten carbide and glass beads (Qiagen, Crawley, UK), followed by agitation in a Fastprep-24 Instrument (MP Biomedicals Europe, Illkirch, France) at 6.5 m/s for 60 sec (833). Further steps remained unchanged, and the DNA was resuspended in 50 µL of Elution Solution (included in the kit). DNA concentrations were quantified using the Picodrop Microlitre Spectrophotometer (GRI, Braintree, UK).

4.2.8 Quantitative PCR (qPCR) assays

4.2.8.1 Total Bacterial Load (TBL) qPCR

Total bacterial load was estimated by using the SYBR Green dye, using the primers EubF 5'-TCCTACGGGAGGCAGCAGT-3' and EubR 5'-GGACTACCAGGGTATCTA ATCCTGTT-3' (Sigma-Aldrich Co. Ltd., Dorset, UK) which amplified a 466-bp region between positions 331 to 797 of the *Escherichia coli* 16S rRNA gene (834). The assay was performed as described in (835). All PCR reactions were carried out in a total volume of 20 µl containing primers at a concentration of 500 nM each, 1 µl of template and Rotor-Gene SYBR Green PCR Master Mix (Qiagen, Crawley, UK) at 1x final concentration. Quantitative PCR assay was performed

using Rotor-Gene Q real-time thermocycler (Qiagen, Crawley, UK) with a temperature profile of 95 °C for 5 min, followed by 50 cycles at 95 °C for 15 s and 58 °C for 50 s. Gain optimisation was set manually at 5.33 on the green channel (Cycling A. Green). Melt-curve analysis was then conducted between 58°C to 99°C with 1 °C steps, to detect non-specific amplifications.

4.2.8.2 *H. influenzae* (HI) qPCR

H. influenzae densities were estimated by a TaqMan assay, using the primers HelSF 5'-CCGGGTGCGGTAGAATTTAATAA-3', HelSR 5'-CTGATTTTTCAGTGCTGTCTTTGC-3' (Eurofins Genomics, Ebersberg, Germany) and probe HelSPr 5'-FAM-ACAGCCACAACGGTA AAGTGTTCTACG-TAMRA-3' (Eurofins Genomics, Ebersberg, Germany) which amplify a 90-bp region between positions 518 to 608 of the *H. influenzae* hel gene (836). All PCR reactions were carried out in a total volume of 20 µl containing primers and probe at a concentration of 500:500:250 nM (HelSF: HelSR; HelSPr), 1 µl of template and LightCycler 480 Probes Master (Roche Diagnostics GmbH, Mannheim, Germany) at 1x final concentration. Quantitative PCR assays were carried out using the Rotor-Gene Q (Qiagen, Crawley, UK) with a temperature profile of 95 °C for 5 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 60 s. Gain setting on the green channel was optimized manually to 4.00 for each run.

4.2.8.3 *S. pneumoniae* (SPN) qPCR

The assay was performed using a TaqMan based probe, lytA-CDCPr 5'-FAM-TGCCGAAAACGCTTGATACAGGGAG- BHQ1-3' (Eurofins Genomics, Ebersberg, Germany) and primers lytA-CDCF 5'-ACGCAATCTAGCAGATGAAGCA-3', lytA-CDCR 5'-TCGTGCGTTTTAATTCCAGCT-3' (Eurofins Genomics, Ebersberg, Germany). A 53-bp region was amplified between positions 1840961 to 1841014 of the *S. pneumoniae* genome specifically targeting a region of the lytA gene (837). Primers and probe concentrations were optimised to produce an assay with a final 25 µL reaction volume as follows: Primers and probe at a concentration of 500:500:250 nM (*lytA*-CDCF: *lytA*-CDCR: *lytA*-CDCPr), 1 µL of template and LightCycler 480 Probes Master (Roche Diagnostics GmbH, Mannheim, Germany) at 1x final concentration. Quantification of DNA copies was performed using the Rotor-Gene Q (Qiagen, Crawley, UK) with a temperature profile of 95 °C for 5 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 60 s. Gain setting on the green channel was set manually to 4.00 for each run.

4.2.9 16S rRNA gene sequencing

The DNA samples were transported to Public Health England for 16S rRNA gene sequencing. The V3-V4 region of the 16S rRNA gene was amplified using a 16S Amplicon Forward primer (5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3') and 16S Amplicon Reverse primer (5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3') (838). Library preparation was carried out according to the 16S Metagenomic sequencing library preparation manual (Illumina, USA). The size of the library was measured using the Agilent High Sensitivity DNA kit (Agilent, Germany) and quantified using ABI Viaa7 and KAPA Library Quantification Kit Illumina® platforms (KAPABiosystems). The sequencing was then performed on the MiSeq platform (Illumina, USA) using the MiSeq reagent kit V2 (500 cycles) according to the manufacturer's instructions.

The raw sequence data obtained from the Illumina MiSeq sequencer were then filtered to remove any chimeric sequences from the input sequences, which could present at a low level due to premature amplicon termination during the library preparation step. The paired end reads were rarefied to 9311 reads followed by analysis based on Operational Taxonomic Unit (OTU) approach. This was determined using the QIIME version 1.9.1 pipeline to cluster the 16S rRNA gene sequences based on their similarity. Within these data, a total of 5615037 sequencing reads were clustered into a final 167 OTUs, where OTUs less than 0.01% relative abundance across all samples sets were discarded. One sample was removed from the whole OTU analysis due to low sequence reads (4693 reads).

4.2.10 Analysis

4.2.10.1 Demographic and clinical measurement data

Data were exported from the electronic study database to Microsoft Excel for data cleaning. Data were then imported into Stata v11.0 (Statacorp, Texas, USA) for statistical analysis. The demographics and baseline clinical measures of the cohort were determined. qPCR data was analysed using SPSS version 21.0 (IBM Corp, 2012). The 16S rRNA gene sequencing data was analysed using the R statistical framework version 2.11 with version 2.0–7 of the R package "vegan".

4.2.10.2 qPCR and 16S rRNA gene sequencing data

For qPCR data the mean bacterial load in colony forming units (cfu)/ml were calculated for each sample along with the mean loads of *H. influenzae* and *S. pneumoniae*. The mean overall bacterial loads and loads of *H. influenzae* and *S. pneumoniae* of the following groups were then compared using either an independent T test or Mann-Whitney U test depending on whether data were normally or non-normally distributed:

1. BTS Step 2 vs BTS Step 4
2. BTS Step 2 : BUD vs FLU
3. BTS Step 2 : BUD vs BEC
4. BTS Step 2 : FLU vs BEC
5. BTS Step 4 : BUD vs FLU
6. BUD : BTS 2 vs BTS 4
7. FLU : BTS 2 vs BTS 4
8. BUD vs FLU

For the 16S rRNA gene sequencing data OTUs were calculated in cfu/ml by multiplying their percentage abundance by the bacterial load from 16S qPCR data. Version 2.0–7 of the R package "vegan" was used to generate *richness*, *Shannon's* and *Simpson's* diversity indices. Richness and Simpson's indices were compared between the BTS 2 vs BTS 4 groups and the BTS 4 FLU vs BTS 4 BUD groups by analysis of variance (ANOVA). As Shannon's index is not a scaled vector a Kruskal-Wallis rank sum test was used to compare between the BTS 2 vs BTS 4 groups and BTS 4 FLU vs BTS 4 BUD groups.

For further analysis of similarity between the microbiota composition of the different severity and steroid types the analysis of similarity (ANOSIM) test was used. This is a non-parametric multidimensional scaling (NMDS) technique that assesses for differences between graphical representations of community composition.

Differences in OTU abundance between BTS 2 vs 4 and BTS 4 FLU vs BTS 4 BUD were assessed for using a Wilcoxon rank sum test.

For comparison of baseline alpha-diversity indices (richness, Shannon's and Simpson's) and bacterial load with these measures after 24 hours and then 2 weeks later in the subgroup of subjects who had multiple samples taken (n=19) repeated measure ANOVA tests were used. In order to assess the test-retest reliability of the total bacterial load as determined by qPCR from these 19 subjects after 24 hours and then 2 weeks, the intraclass coefficient for this measure was calculated using a two-way mixed effects model. The intraclass coefficient measure reflects both the degree of correlation and agreement between measurements.

Finally to investigate the correlation of alpha-diversity indices (richness, Shannon's and Simpson's) and microbiota composition (plotting NMDS axis 1) with clinical measurements (FEV_1 % predicted, FE_{NO}, PC_{20}, LCQ, ACQ or ICS dose) Spearman's rank correlation coefficients were calculated.

4.3 Results

4.3.1 Recruitment

One hundred and five subjects were identified on the research database who met the eligibility criteria for the study. They were all contacted between May 2014 and May 2015 by post, e-mail or telephone regarding taking part in the study. Of these 72 patients agreed to participate in the study.

4.3.2 Losses and exclusions

Whilst there were no losses or exclusions for this study *per se*, 18 participants were unable to produce an adequate sputum sample for analysis, and hence their demographic and clinical data will not be used in the final analysis (See Fig 4.3).

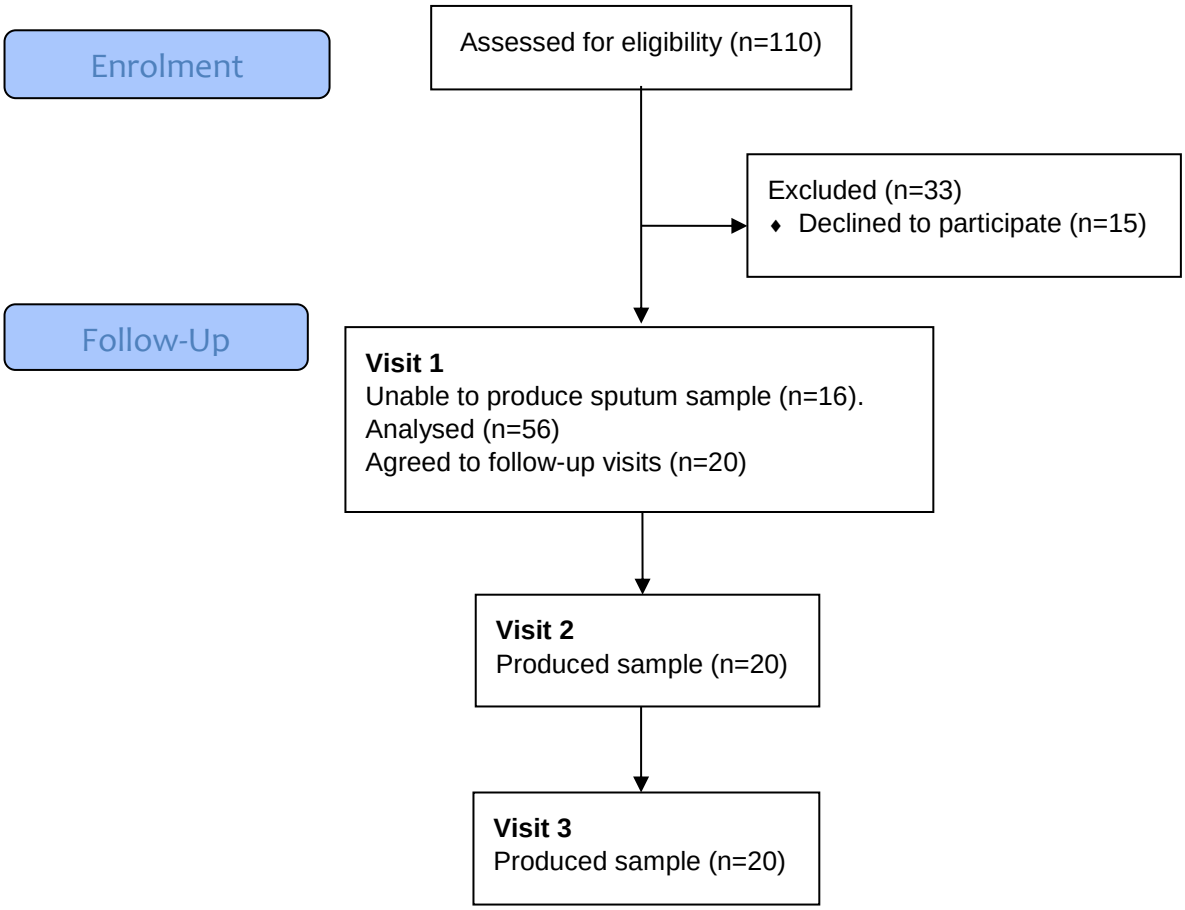


Figure 4.3: Consort diagram demonstrating losses and exclusions from study

4.3.3 Missing data

AHR was not measured in 3 subjects as two declined and one had symptoms of chest pain during the test. As stated above, 16 participants failed to produce sputum samples. MIA 048 attended V1, V2 and V3 and provided sputum samples but a subsequent sputum culture was positive for *S. pneumoniae*. This subject was treated with antibiotics and their microbiota results were excluded from analyses.

4.3.4 Baseline demographics

The baseline demographics and clinical features of the 72 participants who took part in the study (Table 4.1) and the 56 participants who produced sputum samples that were used in the study (Table 4.2) are shown. Table 4.4 shows the participants who provided samples divided into those who were BTS Step 2 and BTS Step 4.

In the overall cohort there was a slight female preponderance and a large majority of the subjects were Caucasian. The mean age of the cohort was 56, with a range of ages from 21-80. Around 32% were ex-smokers who all had pack year histories of <10 pack years and had not smoked in the previous 10 years as per the inclusion criteria. The group were fairly well controlled symptomatically with an average ACQ score of 1.1 and LCQ score of 18.3. The median bacterial load was $9.6x10^6$ cfu/mL.

There were no statistically significant differences in the demographic composition or baseline clinical measures of the BTS Step 2 and BTS Step 4 groups (Table 4.3). Also, there were no statistically significant differences between the BTS Step 4 fluticasone and budesonide groups (Table 4.4).

Twenty-four subjects produced sufficient sputum to also obtain sputum cell counts (Table 4.5). The majority of these had ongoing neutrophilic inflammation (n= 14; 58%) with 6 subjects (25%) displaying paucigranulocytic counts, 3 (12.5%) with mixed granulocytic counts and only 1 (4%) with isolated ongoing eosinophilic inflammation.

4.3.5 Primary Outcomes:

4.3.5.1 Sputum microbiota diversity/composition and quantitation between the BTS Step 2 and BTS Step 4 groups

No significant differences in sputum bacterial load were seen between the BTS 2 and 4 groups (Tables 4.3 & 4.7, Figs 4.4 & 4.5, Appendix M). There were also no significant differences in the relative abundance of the respiratory pathogens *H. influenzae or S. pneumoniae* between the two groups (Tables 4.6 & 4.7).

There was no significant difference in the alpha diversity measures of species richness (Table 4.8), Simpson's (Table 4.9) and Shannon's (Table 4.10) indices between BTS2 and BTS 4. The groups also showed no significant difference in community composition when compared with ANOSIM (Fig 4.8, Table 4.11).

On comparison of the abundance of individual OTUs in BTS Step 2 and 4 subjects, 39 OTUs were found to have significantly different abundances in the two groups (Table 4.16), with 14 of these belonging to the phylum *Firmicutes*.

4.3.5.2 Sputum microbiota diversity/composition and quantitation between the BTS Step 4 fluticasone and BTS Step 4 budesonide groups

No significant differences in sputum bacterial load were seen between the BTS 4 fluticasone and BTS 4 budesonide groups (Table 4.6, Figs 4.6 & 4.7). There were also no significant differences in the relative abundance of the respiratory pathogens *H. influenzae or S. pneumoniae* between the two groups. *H. influenzae* was more abundant than *S. pneumoniae* in all groups (Table 4.7).

There was no significant difference in the alpha diversity measures of species richness (Table 4.12), Simpson's (Table 4.13) and Shannon's (Table 4.14) indices between BTS 4 fluticasone and BTS 4 budesonide groups. The groups also showed no significant difference in community composition when compared with ANOSIM (Table 4.15, Fig 4.9).

The abundance of OTUs in BTS 4 fluticasone and BTS 4 budesonide patients was also compared. There were significant differences in the abundance of 13 OTUs between the groups (Table 4.17).

4.3.6 Secondary Outcomes

4.3.6.1 Sputum microbiota diversity/composition at baseline vs 24 h and 14 days

There was no significant difference in sputum bacterial load or in alpha diversity measures (richness, Simpson's or Shannon's indices) in baseline samples and those taken at 24 h (n=20) or at 14 days (Table 4.22 and Table 4.23). This finding did not alter when these 20 subjects were divided into BTS 2 (n=8) and BTS 4 (n=12) groups (Table 4.18, Figs 4.10-4.13).

The test-retest reliability of the qPCR bacterial load measurements appeared to be poor with an intraclass coefficient value of 0.17 (Figures 4.13 & 4.14; Table 4.20). However, this result was not statistically significant which was likely owing to the relatively low number of subjects.

4.3.6.2 Sputum microbiota diversity/composition at baseline vs clinical features/measures

No significant correlation was found between alpha diversity measures (richness, Simpson's or Shannon's indices) or microbiota composition (plotting NMDS axis 1) with any of the clinical measurements (FEV_1 % predicted, FE_{NO}, PC_{20}, LCQ, ACQ or ICS dose) (Table 4.21).

	Frequency	Percentage
Total number included for analysis	72	
Mean age (range)	55.3 (21-80)	
Sex: male	34	47.2
Ethnic group: Asian Or Asian British Black Or Black British White Or White British	 1 3 68	 1.4 4.2 94.4
Smoking history: Ex-smokers Non smokers	 22 50	 30.6 69.4
Current eczema	9	12.5
Current hay fever	29	40.3
ACQ score Median + IQR	 6 (6)	
LCQ score Median + IQR	18.3 (3.0)	
FEV_1 mean (SD)	92.3 (24.1)	
FEV_1/FVC ratio	70.4 (10.9)	
FE_{NO} concentration (ppb) Geometric mean and 95% CI	13.6 (11.0 – 16.9)	

Table 4.1: Demographics and clinical characteristics of all subjects recruited to study

	Frequency	Percentage
Total number included for analysis	56	
Mean age (range)	56.0 (21-80)	
Sex: male	26	46.4
Ethnic group: Asian Or Asian British Black Or Black British White Or White British	 1 2 53	 1.8 3.6 94.6
Smoking history: Ex-smokers Non smokers	 18 38	 32.1 67.9
Current eczema	7	12.5
Current hay fever	23	41.1
ACQ score Mean + SD	1.10 (0.74)	
LCQ score Median + IQR	18.28 (2.71)	
FEV_1 mean (SD)	93.2 (24.6)	
FEV_1/FVC ratio	70.3 (10.9)	
FE_{NO} concentration (ppb) Geometric mean and 95% CI	14.5 (11.3-18.5)	
Sputum bacterial load (cfu/mL) (Median + IQR)	$9.63x10^6$ ($4.28x10^7$)	

Table 4.2: Demographics and clinical characteristics of all subjects who produced samples in the study

	STEP 2	STEP 4	
	Frequency (%)	**Frequency (%)**	**Significance (p=)**
Total number included for analysis	22	34 (16 BUD / 18 FLU)	
Mean age (range)	58.9 (14.4) (21-72)	54.1 (14.3) (25-80)	0.22
Sex: male	12 (54.6)	14 (41.2)	0.33
Ethnic group: Asian Or Asian British Black Or Black British White Or White British	 0 0 22 (100)	 1 (2.9) 2 (5.9) 31 (91.2)	 0.70
Smoking history: Ex-smokers Non smokers	 7 (31.8) 15 (68.2)	 11 (32.4) 22 (67.7)	 0.97
Current eczema	4 (18.2)	3 (8.8)	0.42
Current hay fever	10 (45.5)	13 (38.2)	0.59
ICS dose (BDP equivalent) (Median + IQR)	400 (200)	1000 (200)	
ACQ score Mean + SD	0.9 (0.6)	1.2 (0.8)	0.12
LCQ score Median + IQR	19.0 (2.0)	17.8 (3.5)	0.08
FEV_1 mean (SD)	93.5 (28.0)	93.1 (22.5)	0.95
FEV_1/FVC ratio	68.8 (10.1)	71.2 (11.4)	0.43
FE_{NO} concentration (ppb) Geometric mean and 95% CI	17.2 (12.8-23.1)	13.0 (9.0-18.6)	0.27*
Sputum bacterial load (cfu/mL) (Median + IQR)	$1.35x10^7$ ($9.89x10^7$)	$8.86x10^6$ ($2.81x10^7$)	0.27

*T-test comparing log FE_{NO}

Table 4.3: Demographics and clinical characteristics of BTS Step 2 and BTS Step 4 groups that produced samples in the study

	STEP 4 BUD	**STEP 4 FLU**	
	Frequency (%)	**Frequency (%)**	**Significance (p=)**
Total number included for analysis	16	18	
Mean age (range)	52.9 (25-80)	55.2 (39-71)	0.65
Sex: male	7 (43.8)	7 (38.9)	0.77
Ethnic group: Asian Or Asian British Black Or Black British White Or White British	 0 2 (12.5) 14 (87.5)	 1 (5.6) 0 15 (94.4)	 0.21
Smoking history: Ex-smokers Non smokers	 8 (50.0) 8 (50.0)	 3 (16.7) 15 (83.3)	 1.0
Current eczema	1 (6.3)	2 (11.1)	1.0
Current hay fever	6 (37.5)	7 (38.9)	0.59
ICS dose (BDP equivalent) (Median + IQR)	800 (0) *difficult due to common doses for each preparation – “2 medians” for each	1000 (100)	
ACQ score Mean + SD	1.3 (0.9)	1.1 (0.8)	0.41
LCQ score Median + IQR	19.4 (2.3)	17.6 (6.2)	0.25
FEV_1 mean (SD)	86.5 (22.6)	98.9 (21.3)	0.11
FEV_1/FVC ratio	67.9 (10.6)	74.1 (11.6)	0.12
FE_{NO} concentration (ppb) Geometric mean and 95% CI	16.0 (8.9-28.6)	10.8 (6.7-17.5)	0.28
Sputum bacterial load (cfu/mL) (Median + IQR)	$1.08x10^7$ ($2.71x10^7$)	$8.23x10^6$ ($3.80x10^7$)	0.59

Table 4.4: Demographics and clinical characteristics of BTS Step 4 budesonide and BTS Step 4 fluticasone groups that produced samples in the study

Sputum inflammatory type	**BTS Step 2**	**BTS Step 4 BUD**	**BTS Step 4 FLU**	**Totals**
Neutrophilic (>61%)	5	3	6	**14**
Eosinophilic (>3%)	0	1	0	**1**
Mixed granulocytic (N>61% and E>3%)	1	1	1	**3**
Paucigranulocytic	2	2	2	**6**
Totals	**8**	**7**	**8**	**24**

Table 4.5: Sputum inflammatory types of a subgroup of 24 subjects who produced sufficient sputum for cell counts

No.	Group 1	Group 2	Group 1 Mean total bacterial load (cfu/mL)	Group 2 Mean total bacterial load (cfu/mL)	p-value	Group 1 Mean *H. influenzae* load (cfu/mL)	Group 2 Mean *H. influenzae* load (cfu/mL)	p-value	Group 1 Mean *S. pneumoniae* load (cfu/mL)	Group 2 Mean *S. pneumoniae* load (cfu/mL)	p-value
1	**BTS 2**	**BTS 4**	1.25E+08	4.81E+07	0.272	6.35E+03	1.31E+06	0.705	8.57E+01	7.83E+00	1.00
2	**BTS 2 / BUD**	**BTS 2 / FLU**	4.26E+07	1.64E+08	0.973	5.35E+02	8.04E+02	0.152	4.64E+01	2.67E+02	1.00
3	**BTS 2 / BUD**	**BTS 2 / BEC**	4.26E+07	1.62E+08	0.493	5.35E+02	1.32E+04	0.583	4.64E+01	2.28E+01	0.340
4	**BTS 2 / FLU**	**BTS 2 / BEC**	1.64E+08	1.62E+08	0.563	8.04E+02	1.32E+04	0.407	2.67E+02	2.28E+01	0.535
5	**BTS 4 / BUD**	**BTS 4 / FLU**	2.73E+07	6.77E+07	0.589	1.79E+04	2.52E+06	0.650	7.13E+00	8.50E+00	0.743
6	**BTS 2 / BUD**	**BTS 4 / BUD**	4.26E+07	2.73E+07	0.919	5.35E+02	1.79E+04	0.630	4.64E+01	7.13E+00	0.488
7	**BTS 2 / FLU**	**BTS 4 / FLU**	1.64E+08	6.77E+07	0.784	8.04E+02	2.52E+06	0.218	2.67E+02	8.50E+00	0.957
8	**BUD**	**FLU**	3.19E+07	8.96E+07	0.944	1.26E+04	1.95E+06	0.748	1.91E+01	6.72E+01	0.925
9	**FLU**	**BUD + BEC**	8.96E+07	7.14E+07	0.236	1.95E+06	1.28E+04	0.829	6.72E+01	2.02E+01	0.703

Table 4.6: Comparisons of mean total bacterial load and abundance of *H. influenzae* and *S. pneumoniae* in groups as stated

No.	Group	Mean *H. influenzae* load (cfu/mL)	Mean *S. pneumoniae* load (cfu/mL)	p-value
1	BTS 2	6.35E+03	8.57E+01	0.053
2	BTS 4	1.31E+06	7.83E+00	**0.001**
3	BUD	1.26E+04	1.91E+01	**0.005**
4	FLU	1.95E+06	6.72E+01	**0.028**
5	FLU/BTS 4	2.52E+06	8.50E+00	**0.017**

Table 4.7: Demonstrating comparison of abundance of *H. influenzae* and *S. pneumoniae* in groups as stated.

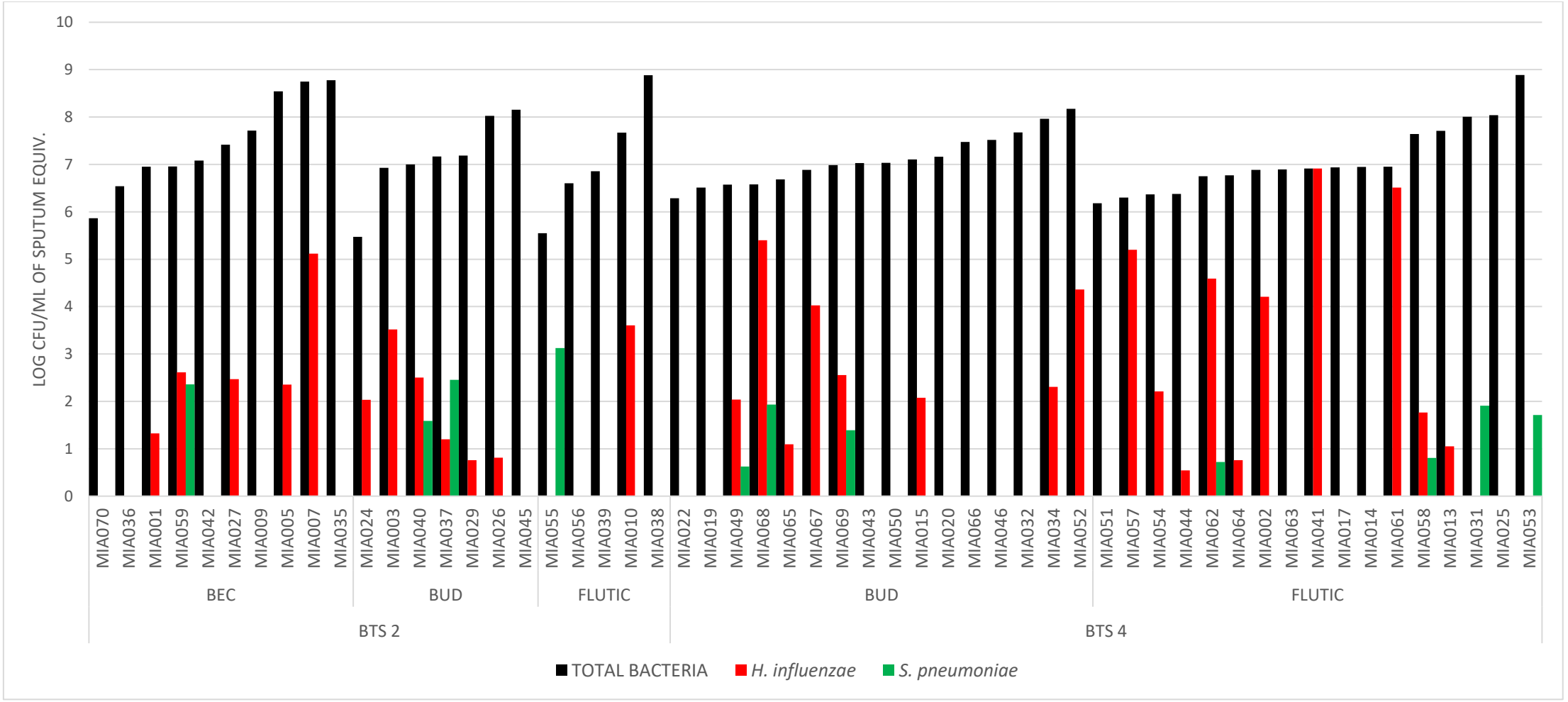


Figure 4.4: Bacterial load (cfu/mL of sputum equivalent) in MIA patients

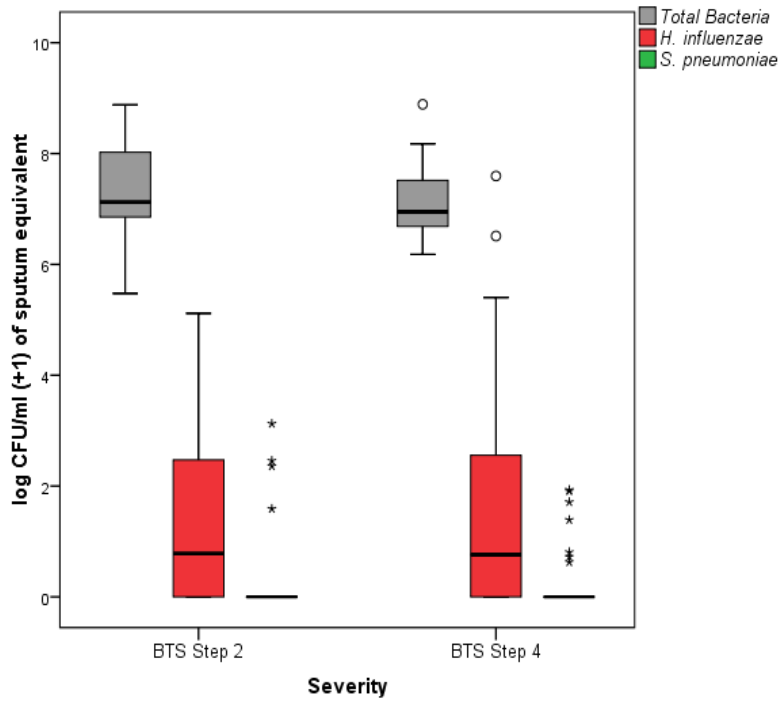


Figure 4.5: Bacterial load (log cfu/mL of sputum equiv.) in BTS 2 and 4 groups

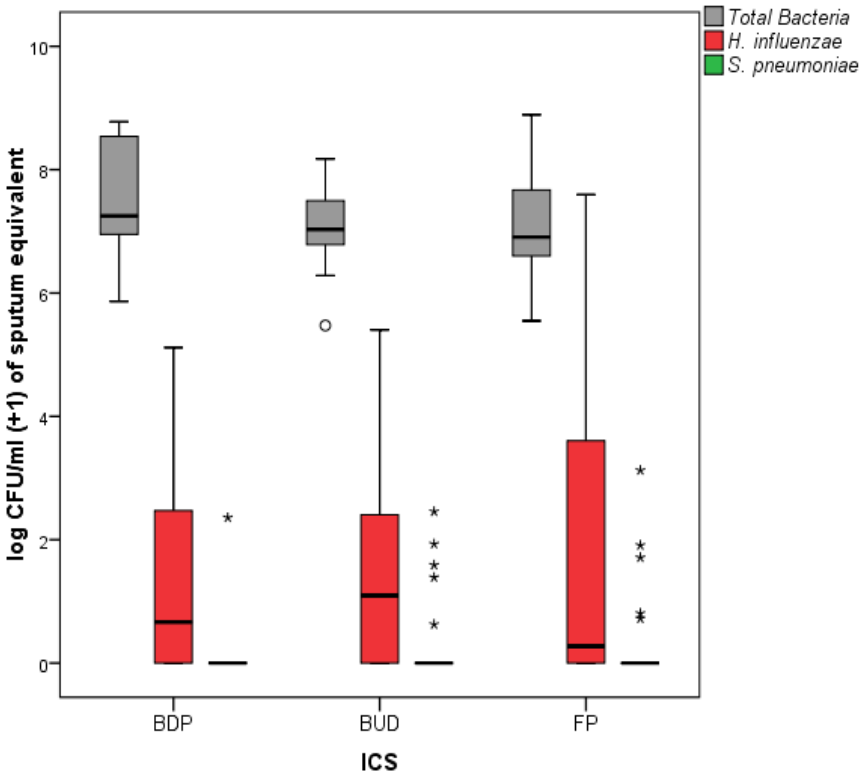


Figure 4.6: Bacterial load (log cfu/mL of sputum equiv.) based on patient's inhaled steroid

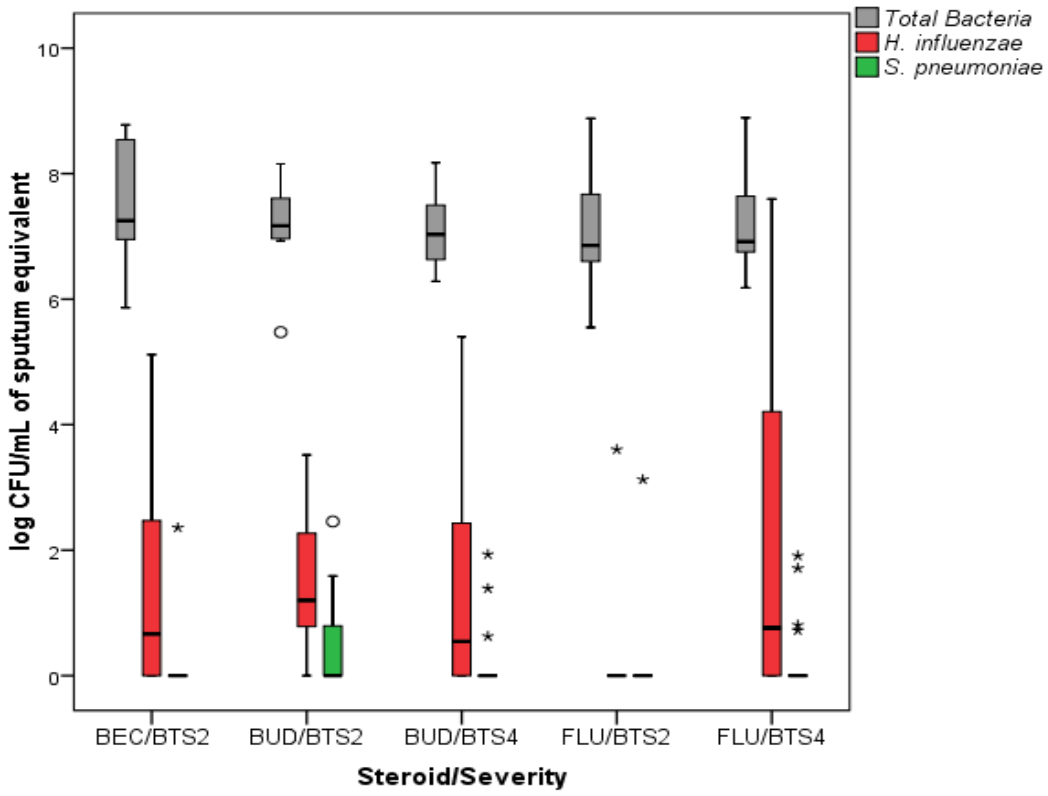


Figure 4.7: Bacterial load (log cfu/mL of sputum equiv.) in BTS 2 and 4 groups based on patient's inhaled steroid

	Sum Sq	Df	Mean Sq	F value	Pr (>F)
Factor (severity)	460.7	1	460.67	1.4972	0.2266
Residuals	16000.1	52	307.70		

Table 4.8: ANOVA table for the effect of severity on bacterial richness

	Sum Sq	Df	Mean Sq	F value	Pr (>F)
Factor (severity)	0.00002458	1	2.4578e-05	2.321	0.1337
Residuals	0.00055065	52	1.0589e-05		

Table 4.9: ANOVA table for effect of severity on Simpson's index

	Chi-square	Df	Pr (>Chi-Square)
Factor (severity)	1.291	1	0.2559

Table 4.10: Kruskal-Wallis test for effect of severity on Shannon's index

	R	p
BTS 2 vs BTS 4	-0.02035	0.676

Number of permutations: 999

Table 4.11: Results from ANOSIM between BTS 2 Group and BTS 4 Group (Bray-Curtis dissimilarity)

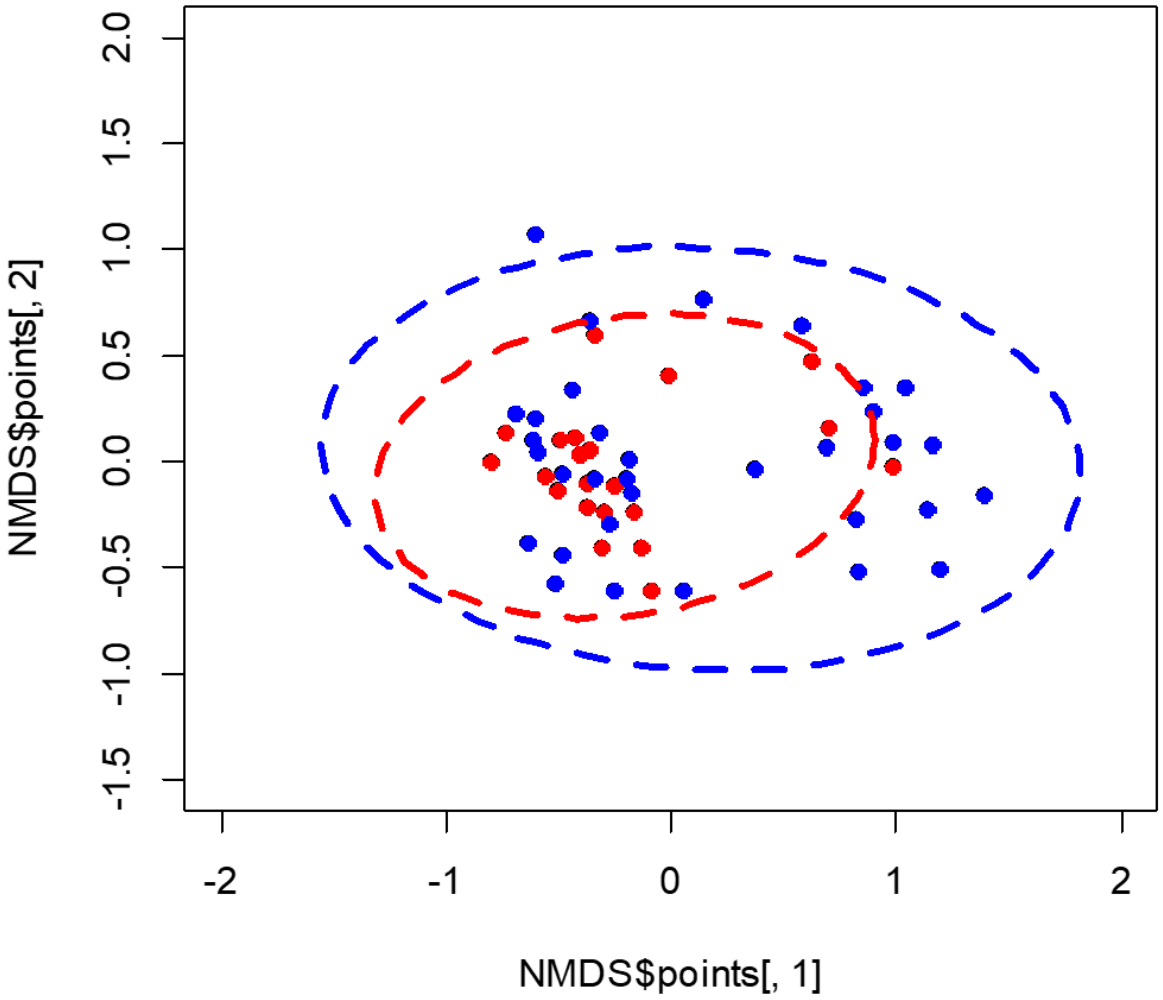


Figure 4.8: Non-multidimensional scaling plot of the bacterial compositions of each samples, grouped by severity;
Red = BTS Step 2, Blue = BTS Step 4. Both x and y axes are arbitrary scales. The distance between points in the figure represents the degree of similarity of the bacterial composition between samples. The closer the points are together, the more similar the bacterial composition. This figure demonstrates that the composition of the BTS Step 2 and 4 groups are similar as the clustering of points on the plot for both groups is similar.

	Sum Sq	Df	Mean Sq	F value	Pr (>F)
Factor (steroid)	6.1	1	6.1	0.016	0.9001
Residuals	11796.8	31	380.54		

Table 4.12: ANOVA table for the effect of steroid type on richness

	Sum Sq	Df	Mean Sq	F value	Pr (>F)
Factor (steroid)	0.00000031	1	3.0530e-07	0.0204	0.8873
Residuals	0.00046327	31	1.4944e-05		

Table 4.13: Steroid effect on Simpsons index

	Chi-square	Df	Pr (>Chi-Square)
Factor (steroid)	0.0731	1	0.7868

Table 4.14: Kruskal-Wallis test for effect of severity on Shannon's index

	R	p
BTS 4 Bud vs BTS 4 Flu	0.008473	0.345

Number of permutations: 999

Table 4.15: Results from ANOSIM between BTS 2 Group and BTS 4 Group (Bray-Curtis dissimilarity)

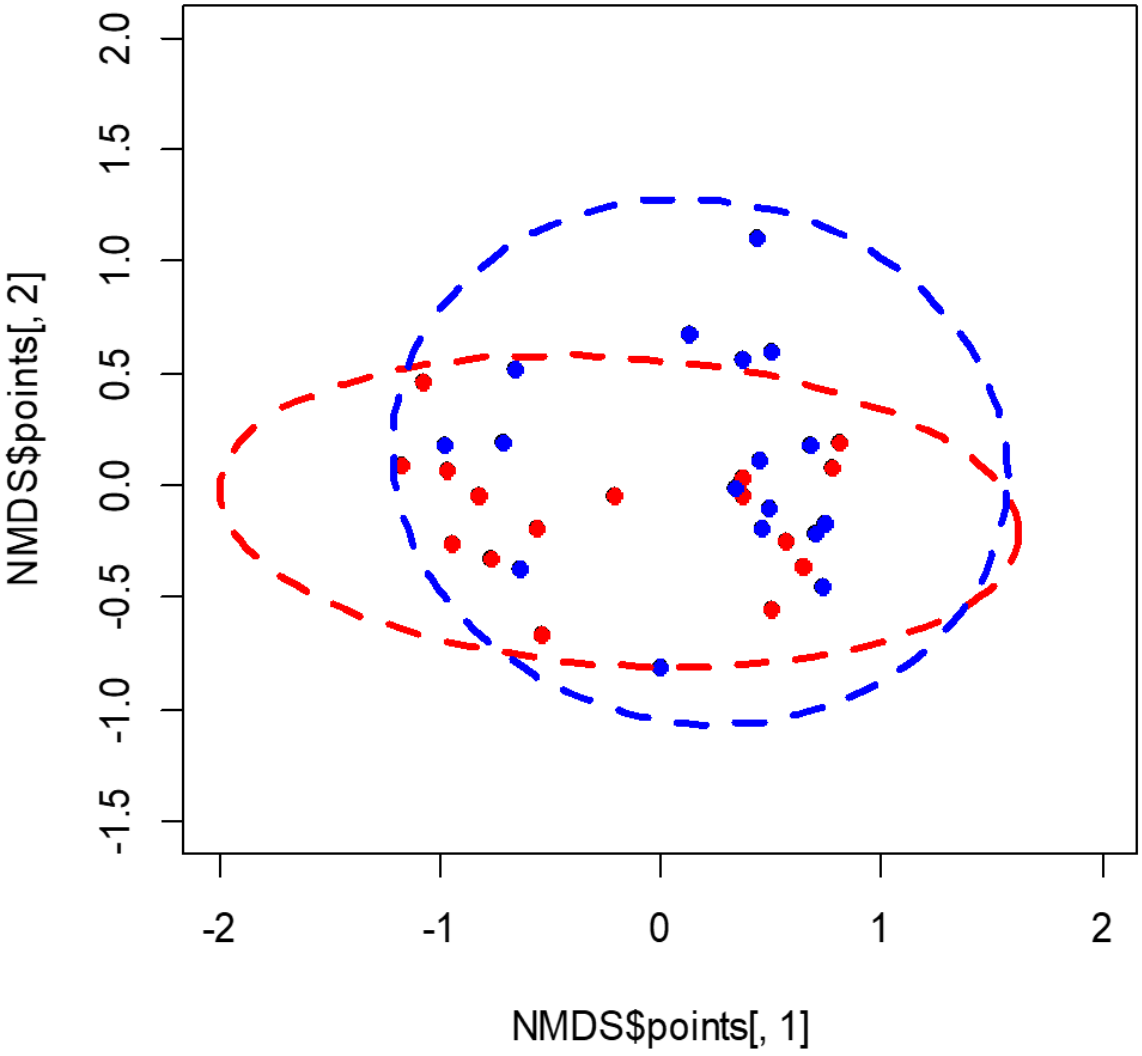


Figure 4.9: NMDS of steroid effect on composition.

Red = BUD, Blue = FLUTIC. Both x and y axes are arbitrary scales. This figure demonstrates that the composition of the BTS Step 4 Budesonide and Step 4 Fluticasone groups are similar as the clustering of points on the plot for both groups is similar

		BTS2		BTS4	
OUT.ID	**p value**	**Mean (cfu/mL)**	**SD (cfu/mL)**	**Mean (cfu/mL)**	**SD (cfu/mL)**
Cerasicoccaceae	0.045	0	0	48136.46	242558.4
Prevotella.tannerae	0.031	567421.5	2166916	37186.51	171202.7
Weeksellaceae	0.002	57953.84	187283.1	4621.297	17242.01
Acidocella.spp.	0.011	37739.59	122368.7	3285.693	7715.95
Actinomyces.spp.	0.020	2402551	4704338	178311.9	681813.9
Aggregatibacter.segnis	0.043	405902.9	996459.4	23549.74	62863.32
Atopobium.spp.	0.006	614218.9	957477.6	81613.48	230382.3
Bacillaceae1	0.047	22683.45	96642.81	261821.9	1010540
Bacillaceae2	0.031	17273.54	73086.08	181728.9	571889.1
Campylobacter.spp.	0.048	713264.1	1223965	225809.4	716058
Capnocytophaga.spp.	0.005	408979.6	1067674	12787.48	35474.43
Cardiobacterium.spp.	0.019	30024.16	89067.01	649.9432	1367.122
Catonella.spp.	0.048	145950.7	325845	25571.6	84832.2
Corynebacterium.durum	0.031	25080.47	65504.5	6904.183	26225.17
Dialister.spp.	0.016	735403.8	2545526	138994.5	525555.5
Dysgonomonas.spp.	0.008	572.3388	2577.694	53199.23	189629
Enterobacteriaceae1	0.032	18.93667	86.77871	6524.605	23041.53
Granulicatella.spp.	0.015	1227271	3155185	64060.74	140362.6
Lautropia.spp.	0.039	740902.1	2328397	20944.79	104157.1
Leptotrichia.spp.	0.003	10814168	28385555	2188100	7752618
Megasphaera.spp.	0.009	382720.4	795826.2	57274.12	159554.3
Microbacteriaceae1	0.012	896604.2	2419294	912765.1	4027665
Moryella.spp.	0.001	801977.1	1531354	60022.51	187047.1
Neisseriaceae	0.007	242214.6	532659.2	5252.301	9597.861
Oribacterium.spp.	0.006	787499.9	1707436	160294.7	572340.5
Parvimonas.spp.	0.005	502481.3	1465182	7597336	44172428
Prevotella. melaninogenica	0.016	10674788	20711212	1571315	4412534
Prevotella.nigrescens	0.008	153201.7	310928.7	4767.503	9067.782
Prevotella.pallens	0.029	737098.2	1449476	177312.4	646154.3
Rothia.aeria	0.005	616580.9	1447615	19857.93	45271.42
Rothia.dentocariosa	0.010	844303.4	1743688	63093.43	129531.7
Rothia.mucilaginosa	0.021	14034074	34188166	609291.8	1428920
Selenomonas.noxia	0.028	29108.5	82901.73	520.7932	964.6912
Selenomonas.spp.	0.006	682271.2	1904229	310393.3	1462465
Streptococcus.anginosus	0.002	95150.47	266841	11707.6	47283.08
Streptococcus.spp.	0.002	25992589	55531721	1715461	2891616
TM7	0.002	567835.6	1790638	14413.45	31237.25
TM_7.Rs_045	0.035	137999.3	363084.4	1342.991	3414.309
Veillonella.dispar	0.013	8799488	15574777	1091461	3438765

Table 4.16: OTUs demonstrating significantly different abundance in BTS Step 2 and 4 groups

		FLUTIC		BUD	
OUT.ID	**p value**	**Mean (cfu/mL)**	**SD (cfu/mL)**	**Mean (cfu/mL)**	**SD (cfu/mL)**
Anaerobacillus.spp.	0.026	99984.27	420897.1	82621.61	194653.4
Capnocytophaga.ochracea	0.038	10745.07	39826.14	1224.851	3779.901
Dysgonomonas.spp.	0.035	53319.58	225535.9	53063.83	146497.4
Exiguobacterium	0.026	312.1798	1324.467	4431.924	11758.82
Fluviicola.spp.	0.043	13.83541	58.69869	1256.938	2695.984
Haemophilus. parainfluenzae	0.047	963546.1	2920793	144552.9	309026.3
Lactobacillus.reuteri	0.027	28511.36	91197.13	0	0
Paracoccus.spp.	0.016	89993.13	380919.2	195139.2	570462.6
Peptococcus.spp.	0.014	18608.36	75010.17	69.26747	277.0699
Porphyromonas. endodontalis	0.009	64019.62	172897.6	6009.521	21444.89
Rhodobaca.spp.	0.049	17989.71	75130.93	19048.09	42095.53
Veillonellaceae2	0.048	11768.51	29602.59	5.81175	23.247
Xanthomonadaceae1	0.019	71839.09	197731.9	5312.727	11613.61

Table 4.17: Abundances of OTUs that change significantly in BTS 4 patients treated with different steroids

	A) Mean total bacterial load (cfu/mL)				
Group	**Baseline**	**After 24 hours (V1 – V2)**	**p value**	**After 2 weeks (V1 – V3)**	**p value**
BTS 2	1.48E+08	1.45E+08	0.345	3.12E+08	0.679
BTS 4	9.67E+07	9.35E+06	0.158	2.44E+07	0.651
BUD / BTS 4	6.97E+06	4.18E+06	0.231	1.09E+07	0.334
FLU / BTS 4	1.48E+08	1.23E+07	0.284	3.20E+07	0.349
Combined groups	1.18E+08	6.63E+07	0.085	1.46E+08	0.545

	B) *H. influenzae* load (cfu/mL)				
Group	**Baseline**	**After 24 hours (V1 – V2)**	**p value**	**After 2 weeks (V1 – V3)**	**p value**
BTS 2	3.16E+01	3.73E+00	0.416	8.83E+01	0.269
BTS 4	1.55E+04	5.07E+02	0.269	1.78E+03	0.845
BUD / BTS 4	2.70E+03	7.61E+02	0.761	1.59E+02	0.584
FLU / BTS 4	2.27E+04	3.62E+02	0.072	2.70E+03	0.712
Combined groups	8.96E+03	2.95E+02	0.153	1.07E+03	0.323

	C) *S. pneumoniae* load (cfu/mL)				
Group	**Baseline**	**After 24 hours (V1 – V2)**	**p value**	**After 2 weeks (V1 – V3)**	**p value**
BTS 2	0.00E+00	4.75E+00	0.351	9.08E+00	0.351
BTS 4	1.30E+01	3.76E+02	0.770	7.95E+00	0.384
BUD / BTS 4	1.06E+00	0.00E+00	0.391	0.00E+00	0.391
FLU / BTS 4	1.99E+01	5.91E+02	0.873	1.25E+01	0.497
Combined groups	7.55E+00	2.20E+02	0.977	8.43E+00	0.755

Table 4.18: Demonstrating A) Mean total bacterial load B) *H. influenzae* load and C) *S. pneumoniae load* in groups stated at baseline and after 24 hours and 2 weeks (total n=19). Comparisons between values made using repeated measure ANOVA tests.

	Richness	Shannon's	Simpsons
All subjects	0.131	0.113	0.106

Table 4.19: Repeated measure ANOVA p-values for alpha-diversity measures for subjects that had repeat visits (n=19) after 24 hours and 2 weeks

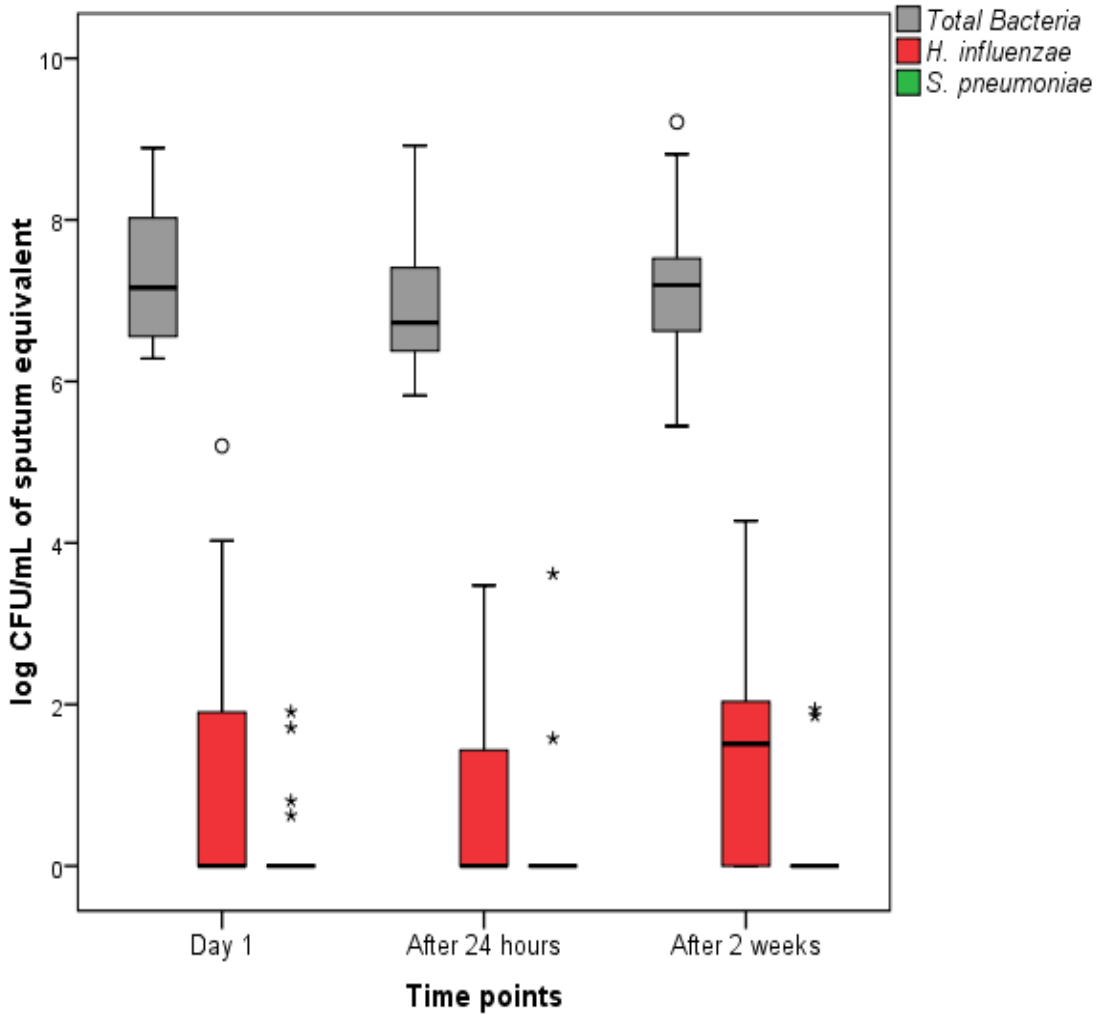


Figure 4.10: Bacterial load (log cfu/mL of sputum equiv.) in subjects (n=19) after 24 hours and 2 weeks.

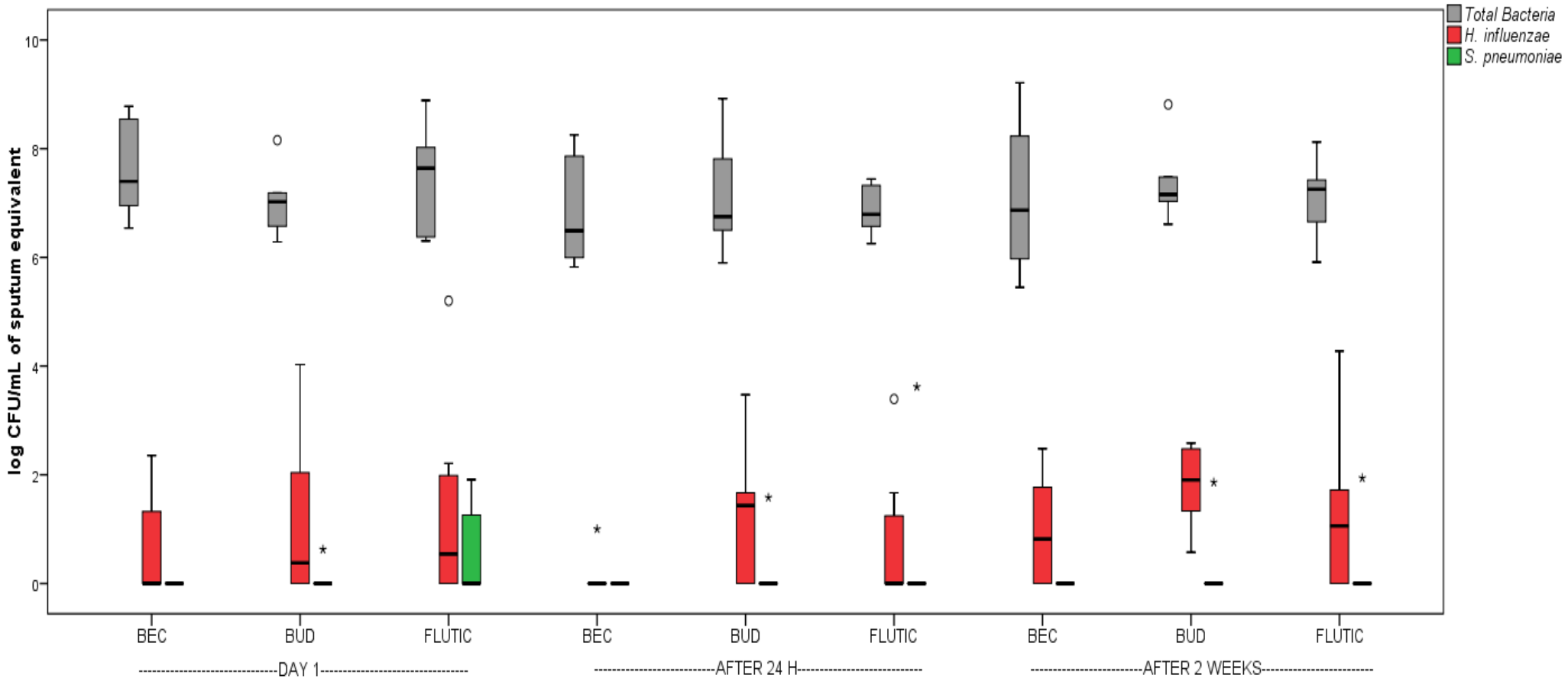


Figure 4.11: Bacterial load (log cfu/mL of sputum equiv.) in subjects (n=19) based on inhaled steroid groups after 24 hours and 2 weeks.

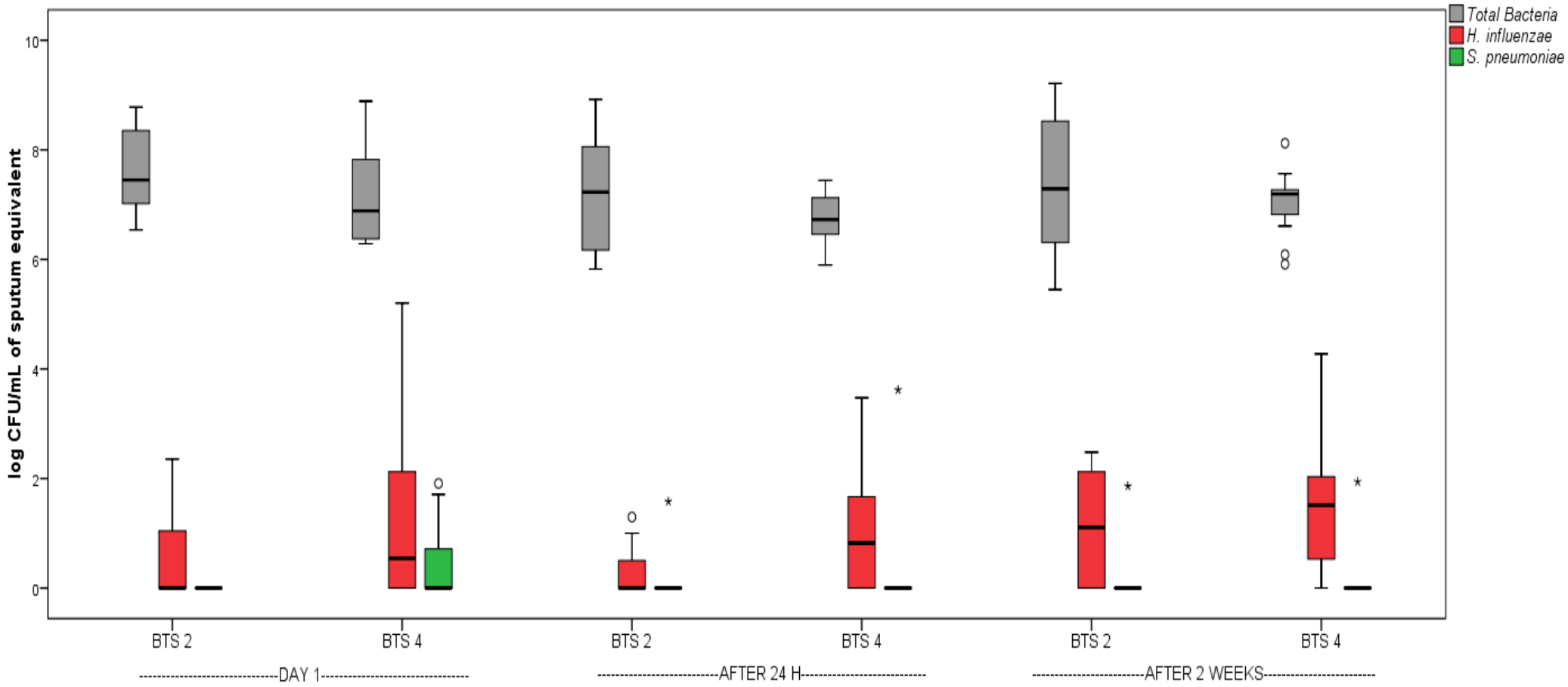


Figure 4.12: Bacterial load (log cfu/mL of sputum equiv.) in BTS groups 2 and 4 (total n=19) after 24 hours and 2 weeks.

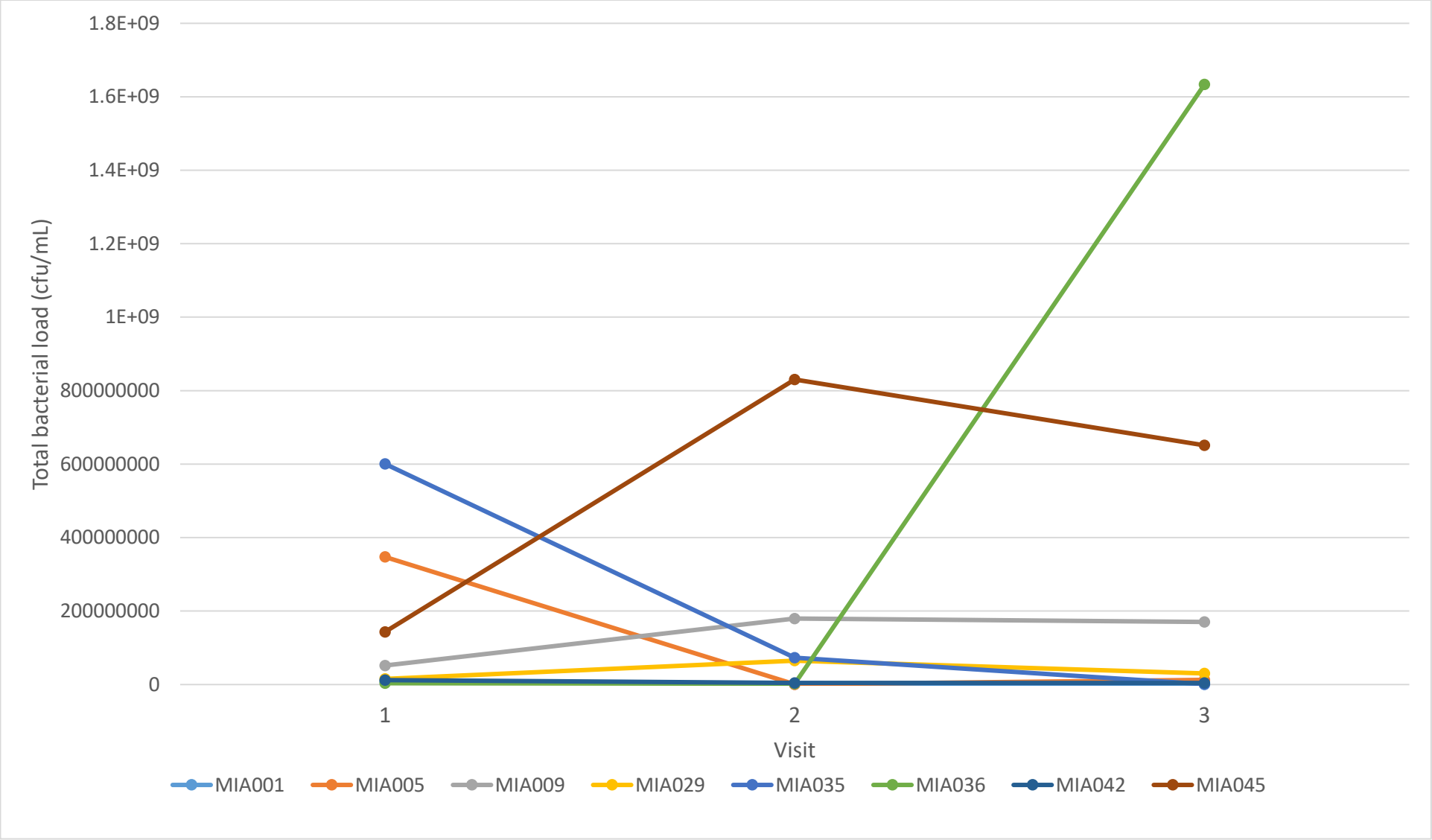


Figure 4.13: Individual bacterial loads (cfu/mL) of BTS Step 2 subjects (total n=8) after 24 hours and 2 weeks

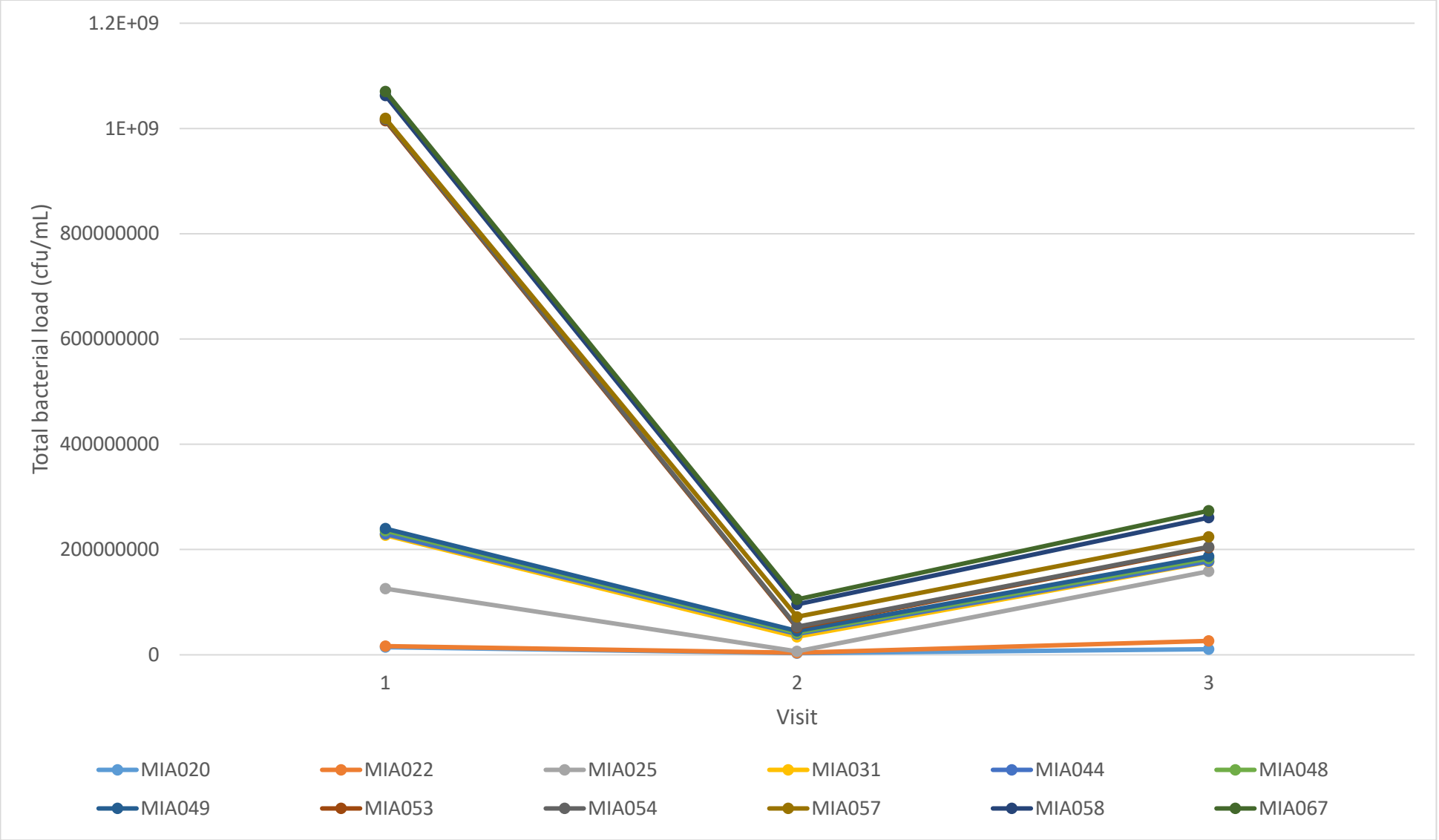


Figure 4.14: Individual bacterial loads (cfu/mL) of BTS Step 4 subjects (total n=12) after 24 hours and 2 weeks

	Intraclass coefficient	95% CI	p value
Total bacterial load	0.17	-0.74 – 0.65	0.3

Table 4.20: Intraclass coefficient for repeated qPCR measures of total bacterial load from all subjects who made 3 visits (n=20)

Measure	Diversity	p value	Composition	p value
FEV_1 (%predicted)	-0.122	0.3731	0.118	0.3891
FE_{NO}	-0.153	0.2648	-0.113	0.4124
PC_{20}	-0.051	0.7129	-0.149	0.282
LCQ	-0.218	0.1102	-0.241	0.0769
ACQ	0.061	0.6568	-0.162	0.2363
ICS dose	-0.067	0.6253	0.208	0.1283

Table 4.21: Spearman's rank correlation coefficients of clinical measures with microbiota diversity (Shannon's, Simpson's and richness) and composition (composition measure based on non-multidimensional scaling plot axis 1)

4.4 Discussion

The results of this study indicate that there is no significant difference in bacterial community composition or total bacterial load of the airway microbiota between BTS Step 2 and Step 4 asthma groups or the BTS Step 4 subgroups taking budesonide or fluticasone. However, significant differences in the abundance of many bacterial species (OTUs) between the groups were noted.

4.4.1 Sputum microbiota diversity/composition and quantitation between the BTS Step 2 and BTS Step 4 groups

The first point of interest from the study is the average sputum bacterial load of the cohort (~$1x10^7$ cfu/mL) which is in between that observed in healthy controls (~$1x10^4$ cfu/mL) and in individuals with CF (~$1x10^9$ cfu/mL). Even after accounting for oral/upper airway contamination and assuming that a significant number of these bacteria are viable this represents a substantial bacterial load which is likely to be biochemically and immunologically active and exert various effects upon the airways. It is possible that the increased bacterial load observed in this and other cohorts of subjects with airways disease could be used as a biomarker to distinguish patients with airways disease from those without although this requires further study.

The lack of differences observed in the total bacterial load or the community composition of the microbiota between the BTS Step 2 and BTS Step 4 groups imply that the use of higher ICS doses in asthma does not lead to fundamental changes in the microbiota. The observation that several different species are found in higher abundance in subjects with less severe asthma is an interesting finding that requires further investigation.

There are relatively few published studies in this area although two previous studies have compared the airway microbiota in asthmatic subjects with different severities of disease.

Zhang *et al.* (821) compared the airway microbiota from of “severe” and “non-severe” asthmatics and found significant differences in bacterial community structure between the two groups. The severe group in this study consisted of subjects requiring “either continuous or near-continuous oral corticosteroids, high-dose inhaled corticosteroids, or both” whilst the non-severe asthmatics were defined as those with no symptoms and minimal use of rescue medication using ≤2000 µg BDP. It is clear from these definitions that the severe group from Zhang *et al.* had more severe asthma requiring higher steroid doses (equivalent to BTS Step 5) than the BTS Step 4 group from the present study. In fact, the non-severe group from the Zhang *et al.* also had a higher average ICS dose than the BTS Step 4 group of the present study (mean 1453 µg; SD 563 µg vs median 1000 µg; IQR 200 µg). A significantly increased abundance of *Proteobacteria* (including *Haemophilus*) and reduced numbers of *Firmicutes* were detected in the sputum of non-severe asthmatics when compared to severe asthmatics.

These findings would seem to be in contrast with those of the current study. However, the significant differences noted by Zhang et al were determined by comparing the relative abundance of bacteria grouped as phyla. The validity of comparing the relative abundances of whole phyla of bacteria is unclear due to the massive variation of organisms classified within the same phyla. Although such comparisons may allow the detection of a broad difference in microbiota community structure between two selected groups they are not biologically informative. This is because they allow no further assessment of the functionality of the organisms detected within these phyla and hence are unable to advance further understanding regarding the roles of particular species in the progression of disease. The current study did not make comparisons at phylum level for these reasons, instead comparing abundances at an OTU level, i.e. a much higher resolution level of sequencing, in order to try and identify specific species that could plausibly be linked to any observed differences between the two groups.

Huang *et al.* (819) compared the microbial content of bronchial brushings from 30 "severe" and 41 "mild to moderate" asthmatic subjects. Severe asthmatics were defined as having a FEV_1 of 40-80% predicted, an ACQ of >1.5 and a daily dose of ≥1000 µg beclomethasone. Subjects in the mild to moderate group with "sub-optimal" asthma control were defined by an ACQ >1.25 after 4 weeks of standardised treatment with 88 µg of fluticasone twice daily. These two groups were taking similar doses of ICS to the BTS Step 2 and 4 groups in the current study. However, both of these groups had sub-optimal asthma control whilst the groups in the present study had a lower ACQ score/better asthma control (BTS Step 2 0.9 (0.6), BTS Step 4 1.2 (0.8)). The microbiota from the severe asthmatic group was found to be enriched in 53 genera compared to the mild to moderate subjects, the majority of which were *Actinobacteria* (with the remaining 5 all classifying to *Gammaproteobacteria*). Forty-two genera were more abundant in the mild to moderate group with 19 of these belonging to the taxa *Proteobacteria*. These results contrast with those of the current study in which only 8 genera were enriched in the more severe (BTS 4) group and 31 were enriched in the less severe (BTS 2) group.

At the species level there were significant differences in the abundance of a number of organisms between Step 2 and Step 4 groups. Upon review of the possible sources of these organisms they can be divided into several different groups (Table 4.22).

Phylum	**OUT.ID**	**BTS 2**	**BTS 4**
Environmental contaminants/uncertain significance			
Actinobacteria	*Microbacteriaceae1*		↑
Bacteroidetes	*Weeksellaceae*	↑	
Frequent members of the oral microbiota (839)			
Actinobacteria	*Actinomyces.spp. (840)*	↑	
Proteobacteria	*Aggregatibacter.segnis*	↑	
Actinobacteria	*Atopobium.spp.*	↑	
Bacteroidetes	*Capnocytophaga.spp.*	↑	
Proteobacteria	*Cardiobacterium.spp.*	↑	
Firmicutes	*Catonella.spp.*	↑	

Actinobacteria	*Corynebacterium.durum*	↑	
Firmicutes	*Dialister.spp.*	↑	
Firmicutes	*Granulicatella.spp.*	↑	
Proteobacteria	*Lautropia.spp.*	↑	
Fusobacteria	*Leptotrichia.spp.*	↑	
Firmicutes	*Megasphaera.spp.*	↑	
Firmicutes	*Oribacterium.spp.*	↑	
Firmicutes	*Parvimonas.spp.*		↑
Actinobacteria	*Rothia.aeria*	↑	
Actinobacteria	*Rothia.dentocariosa*	↑	
Actinobacteria	*Rothia.mucilaginosa*	↑	
Firmicutes	*Selenomonas.noxia*	↑	
Firmicutes	*Selenomonas.spp.*	↑	
Firmicutes	*Streptococcus.anginosus (841)*	↑	
	TM7 (842)	↑	
	TM_7.Rs_045 (842)	↑	
Frequent members of the GI microbiota			
Proteobacteria	*Acidocella.spp. (843)*	↑	
Firmicutes	*Bacillaceae1 (844)*		↑
Firmicutes	*Bacillaceae2 (844)*		↑
Proteobacteria	*Campylobacter.spp. (844)*	↑	
Verrucomicrobia	*Cerasicoccaceae (845)*		↑
Bacteroidetes	*Dysgonomonas.spp.*		↑
Proteobacteria	*Enterobacteriaceae1*		↑
Firmicutes	*Moryella.spp. (846)*	↑	
Organisms previously noted in lung microbiota			
Proteobacteria	*Neisseriaceae*	↑	
Bacteroidetes	*Prevotella.melaninogenica*	↑	
Bacteroidetes	*Prevotella.nigrescens*	↑	
Bacteroidetes	*Prevotella.pallens*	↑	
Bacteroidetes	*Prevotella.tannerae*	↑	
Firmicutes	*Streptococcus.spp.*	↑	
Firmicutes	*Veillonella.dispar*	↑	

Table 4.22 The OTUs that demonstrate significantly different abundance between BTS 2 and 4 Groups grouped by most likely microbiota origin

Broadly, the BTS 2 group were relatively enriched in a variety of organisms abundant in the oral microbiota and those previously noted in the lung microbiota whilst the BTS 4 group had a higher abundance of several organisms that are frequent members of the GI microbiota, although these differences are difficult to quantify.

Venkataraman *et al.* (796) have proposed that the lung microbiome in health is largely determined by a constant "neutral distribution" of microbes from the oral cavity via breathing and microaspiration, rather than the selective growth of bacteria within the airways. A model assuming this 'neutral distribution' of microbes from the upper airways in subjects with no lung disease found a strong overlap between OTUs detected in the oral cavity and those in the lung (goodness-of-fit/R^2 of 0.86, where 1 is a perfect fit). By contrast, the poor fit of the model between OTUs in the upper airways and bacterial communities resident in diseased lungs suggest that the lung microbiota in disease states is shaped by processes of active selection. If such a model were to be correct, it may be that the critical determinant of lung microbiota composition "switches" at a certain point from dispersal of upper airway/oral microbes to establishment of a distinct and selective bacterial community during the progression of disease. The point at which this happens would necessarily depend on environmental selection pressures within the lung habitat including temperature, pH, oxygen tension, perfusion and the degree of inflammation and epithelial cell damage (799). The results of the current study would seem to provide some support for this model with the microbiota from the subjects with less severe disease (BTS Step 2) containing comparatively high abundances of species commonly isolated in the oral cavity which could be present in the lungs due to neutral distribution. The higher abundances of several organisms normally associated with the GI microbiota in BTS Step 4 subjects could represent the establishment of a distinct bacterial community that is either a cause or consequence of more severe disease.

An increased abundance of *Prevotella* species was found in the BTS Step 2 group and reduced abundance of *Prevotella* species has previously been reported in subjects with severe asthma (821) and corticosteroid resistant asthma (817) in previous investigations. Hilty *et al.* (777) found controls were more likely than asthmatic subjects to be colonised with multiple species of *Prevotella*, which have previously been shown to directly inhibit the growth of a number of other bacteria. An increased abundance of *Streptococcal* species were identified in the BTS 2 group in comparison to the BTS 4 group which may be an interesting and unexpected finding given the obvious role of *S. pneumoniae* in respiratory disease. Unfortunately however the significance of this result is unclear as these *Streptococcal* OTUs lack the specificity to define distinct species. Cox *et al.* previously reported that 16S rRNA gene sequencing was unable to discriminate between *S. pneumoniae* and *Streptococcus mitis*, the latter being a normal commensal of the oropharynx (847).

4.4.2 Sputum microbiota diversity/composition and quantitation between the BTS Step 4 Fluticasone and BTS Step 4 Budesonide groups

No significant differences were found in the total bacterial load or the community composition of the microbiota between the BTS Step 4 Fluticasone (n=18) and BTS Step 4 Budesonide (n=16) groups. This comparison was a specified outcome of the study as fluticasone use has been demonstrated to increase the relative risk of pneumonia in patients with COPD, with suggestions that this risk may also be increased in asthma and a possible cause of this could be alterations in the microbiota. No other studies have previously investigated this clinical question for comparative purposes.

Although there were no significant differences in the microbiota composition as a whole between these two groups, there were significant differences in the abundances of certain species. Upon review of the likely sources of these organisms several of them appear to be environmental contaminants or of uncertain significance (*Anaerobacillus.spp., Exiguobacterium, Fluviicola.spp., Paracoccus.spp., Rhodobaca.spp., Xanthomonadaceae1*), some are common members of the oral microbiota *(Capnocytophaga.ochracea, Porphyromonas.endodontalis and Veillonellaceae2)* and others of the GI microbiota *(Dysgonomonas.spp., Lactobacillus.reuteri, Peptococcus.spp.).*

One potentially interesting difference between the two groups was the differing levels of *H. parainfluenzae* which was more abundant in Step 4 patients on fluticasone compared to those taking budesonide. This organism is a potential respiratory pathogen which has previously been noted to cause infection in subjects with chronic lung disease (848, 849). Goleva *et al.* (817) previously found an increased abundance of *H. parainfluenzae* in asthmatic subjects "resistant" to a treatment trial of oral prednisolone in comparison to those who were steroid "sensitive". These authors also demonstrated an inhibitory effect of *H. parainfluenzae* on asthmatic airway macrophages *in vitro*.

4.4.3 Sputum microbiota diversity/composition and quantitation between baseline and 24 h samples

No significant differences were found in the total bacterial load or the community composition of the microbiota between baseline sputum samples and those taken at 24 hours (n=20). This is in contrast to a previous study demonstrating significant differences in bacterial composition of sputa samples collected consecutively (850). However, this study was conducted using form of sequencing (T-RFLP) with a lower resolution in individuals with CF where a higher bacterial load and diversity would be expected and hence each individual sample may have been less representative of the overall microbiota.

Another study assessing the day-to-day stability of the sputum microbiota from subjects with CF found no significant variability in the bacterial community structure or overall bacterial load during periods of clinical stability (851).

4.4.4 Sputum microbiota diversity/composition and quantitation between baseline and 2 week samples

No significant differences were found in the total bacterial load or the community composition of the microbiota between baseline sputum samples and those taken at 2 weeks (n=20).

This comparison was included in order to assess the longitudinal stability of the microbiota in stable asthma which has not previously been investigated. Longitudinal studies taking multiple sputum samples from individuals with CF over multiple time points have demonstrated an inherent stability in the microbiota even after short scale perturbations caused by exacerbations of disease or courses of antibiotics (800, 851-854).

4.4.5 Sputum microbiota diversity/composition and quantitation vs clinical measures

No significant correlations were found between the total bacterial load or the community composition of the microbiota and the FEV_1, sputum differential cell count, FE_{NO} level, PC_{20}, LCQ score, ACQ score or inhaled steroid dose (BDP).

Previous studies have found significant correlations between bacterial community structure and clinical measures in asthma. Huang *et al.* (814) found that the relative abundance of certain bacterial taxa primarily belonging to the *Proteobacteria* phylum were highly correlated with BHR. The cohort in the present study had an unusually low number of positive PC_{20} tests considering each had a physician diagnosis of asthma, the reasons for which are unclear. A standard protocol for the methacholine challenge was followed using appropriately calibrated equipment and subjects were asked to withhold their inhaled medication prior to the test as per the ATS guidelines on methacholine challenge testing (855). The fact that all subjects were clinically stable, had been using the same ICS dose for the past year and had not had any infections/asthma exacerbations in the last month would be expected to lessen the degree of AHR seen in these subjects, but this is unlikely to account for such a large number of negative tests. It is possible that some systemic error with the equipment or methacholine used for testing occurred, but this again seems unlikely given that the two staff who performed the test were experienced in performing the test and the methacholine used was replaced several times during the course of the study upon expiry.

Huang *et al.* (819) found significant associations between poor asthma control (i.e. between visit differences in the Asthma Control Questionnaire) and increased sputum leucocyte values and a high relative abundance of *Proteobacteria*, whereas high BMI was associated with high relative abundance of *Bacteroidetes/Firmicutes*. However, no association between asthma control and microbiota diversity or composition were found in the present study.

4.4.6 Study limitations

The study results suggest there may be no true difference in the bacterial load or microbiota diversity between the groups compared. However, there are a number of possible confounding factors that could have influenced the lack of overall difference seen in the bacterial abundance and composition seen between the groups in this study. The first of these relates to a lack of specificity in selection of “asthmatic” patients. As described previously, asthma is a disease with a range of different endotypes/phenotypes. Previous investigations have found associations between certain disease characteristics and high relative abundance of certain organisms including subjects with severe neutrophilic asthma and abundant potentially pathogenic micro-organisms (635) and subjects with eosinophilia and high levels of *Streptococcus* (821). The subjects in this study were selected simply on the basis of a previous physician diagnosis of asthma but may contain several different endotypes/phenotypes with distinct differences in their microbiota.

Another potential confounding factor in this study was the effect of age on the microbiota. This cohort was older than those examined in previous asthma microbiota studies with a mean age of 56 compared to a mean age in previous studies ranging between 26 and 48 (777, 814, 815, 819, 821). Although the effect of age on the microbiota in asthmatic subjects

has not previously been investigated, studies from subjects with CF suggest that microbiota diversity decreases with age (856-858) potentially reducing any difference in microbiota composition between two groups of older subjects.

A limitation inherent in all microbiota studies involving sputum is the contamination of sputum samples with upper respiratory tract and oral micro-organisms. Although it is likely that this contamination would be similar for most subjects some significant differences in the relative abundance of organisms commonly found in the oral cavity between BTS 2 and BTS 4 groups and BTS 4 fluticasone vs BTS 4 budesonide groups were noted. It is unclear if these differences are due to varying levels of oral contamination of sputum samples in subjects from different groups or if they represent changes in the lower respiratory tract microbiota. A degree of contamination is inevitable in studies of the respiratory microbiota as even in studies utilising bronchoscopy to collect samples directly with a protected specimen brush there may still be contamination of the bronchoscope when traversing the upper airways. Bronchoscopy studies in patients with asthma carry an inherent degree of risk and as such collecting sputum for microbiota analysis may represent the only suitable sampling method for studies involving significant numbers of subjects with asthma.

Healthy controls were not included in the present study as it has already been quite well established that there are significant differences in the microbiota of those with asthma compared to those without. However, the inclusion of controls might have been useful in determining likely environmental contaminants.

Another limitation in this study is the lack of information regarding other micro-organisms. Whilst 16S rRNA gene sequencing allows detailed profiling of the bacterial content of a sample it does not enable identification of viruses and fungi. For a comprehensive understanding of the airways microbiota to form, the abundance and community composition of these other organisms needs to be quantified.

4.4.7 Further Work

Due to the relative paucity of knowledge in this area a number of questions are still to be answered. Ultimately to answer the question of whether ICS or other inhaled medications affect the microbiota a double blind RCT needs to be performed with microbiota sampling before and after an intervention. This could consist of either starting ICS in a steroid naïve cohort of asthmatic subjects or increasing ICS dose in a cohort of asthmatics already taking ICS. This design would minimise the problem of significant intra-subject baseline variability in microbiota composition that causes difficulty in the interpretation of cross-sectional studies.

Another question that requires answering is the significance of high levels of potentially pathogenic bacteria detected using qPCR or 16S rRNA gene sequencing. Some of the subjects in the study had high levels of potential pathogens such as H. parainfluenzae detected but did not have active features of infection. This individuals could however be at risk of more severe features of disease such as more frequent exacerbations due to airway colonisation and further studies to investigate this possibility and the potential need for antibiotic treatment of these patients should be performed.

The findings of this study suggest that single sputum samples are well representative of the underlying microbiota and that the composition of the microbiota in asthma is largely stable over a short period when subjects are clinically stable. The stability of the microbiota in subjects with asthma over longer periods of time now needs to be established with longitudinal studies sampling the microbiota over a number of time points, ideally including periods of clinical stability and disease.

Owing to the increasing awareness of different phenotypic/endotypic variants of asthma it will be necessary in future to specifically recruit subjects for microbiota studies that have already been phenotyped or incorporate into studies a planned post-hoc analysis of different sub groups or clusters of disease based on measured clinical or biochemical parameters. This is to avoid the potential error of failure of detection of significant differences in the microbiota between different sub-groups that may be specifically linked to certain features of disease.

4.5 Conclusion

In conclusion, there is no significant difference in the airway microbiota of BTS Step 2 and Step 4 asthma groups or BTS Step 4 subgroups taking budesonide or fluticasone in terms of overall bacterial load or microbiota diversity. However, certain species are more abundant in BTS 2 subjects and vice versa and these findings require further evaluation. The microbiota is likely to have an important role in the pathophysiology of airways disease, especially in severe disease.

Chapter 5: Conclusion

Review of the existing literature in the field suggests airways diseases are heterogeneous phenomena which are currently categorised using ill-defined diagnostic labels that artificially separate patients with airways disease into 'distinct' groups. This categorisation is often based on symptoms and measurement of physiological markers such as airflow obstruction which lack specificity for the pathophysiological processes underlying these changes and hence are poor predictors of treatment response. This process has improved the recognition and outcomes of many patients that display the 'classical' features of these diagnostic labels, who probably represent frequently occurring phenotypes or 'clusters' of disease. However, the limitations of this approach for subjects elsewhere on the 'spectrum' of airways disease, who are often excluded from RCTs (859, 860), are becoming apparent. These limitations may account for the lack of further improvement in treatment outcomes of airways disease seen in Westernised nations over the last 10 years despite increasing expenditure.

Evidence is accumulating that a number of different cellular and molecular pathways ('endotypes') underlie the clinical features ('phenotype') of airway disease expressed by an individual. These endotypes may combine and interact to produce an individual phenotype on the "spectrum" of airways disease (861), which may have the physical disease characteristics of any of the classically described conditions, either in isolation or combination. Therefore, rather than attempting to classify a subject as having a "disease" based on the measurement of certain physiological parameters and proceeding to treat them for that 'disease', outcomes may improve if treatment is specifically targeted at the underlying endotype(s) recognised in that individual. Studies demonstrating improved outcomes when treatment of airway disease is based on biomarkers appear to justify such an approach (299, 303). A new paradigm for the management of airways disease has been outlined based on the identification of characteristics (biomarkers or phenotypic characteristics) that are good predictors of treatment response ('treatable traits') in airways disease (160).

The studies presented in this thesis provide further evidence of the potential benefits of such an approach.

The first study "The utility of exhaled nitric oxide in patients with suspected asthma" demonstrated that the measurement of a biomarker of Th2 inflammation (FE_{NO}) has the potential to allow stratification of a cohort of patients presenting with "asthma-like" symptoms into those who are likely to benefit from ICS treatment and those who are not. Such an approach might avoid the problems associated with the current system of 'treatment trials' of ICS which are complicated by issues of 'regression to the mean' of symptoms whilst using ICS and retrospective, often incorrect, diagnoses of "asthma". This approach is currently being tested in a placebo controlled RCT which aims to determine whether a low level of exhaled nitric oxide can identify patients who will not benefit from inhaled steroid treatment. If this proves to be the case an algorithm that targets treatment

based on FE_{NO} level could potentially reduce the substantial costs associated with unnecessary ICS prescription, adverse effect exposure and incorrect diagnoses of asthma.

Similar points regarding the identification of treatable characteristics in subjects with airways disease also emerge from the second study "An open label trial of azithromycin in chronic productive cough". This cohort of patients with the symptom of chronic productive cough of ill-defined cause demonstrated a significant symptomatic improvement to azithromycin. These subjects are not easily categorised by any of the existing diagnostic labels for airways disease and many had experienced delayed recognition, consultation for and treatment of their symptoms, despite in some cases having had symptoms for years. The study suggests that using a biomarker (sputum neutrophil count) and/or biopsy evidence of inflammation may allow even more effective targeting of azithromycin than the CPC symptom alone, although the findings are limited by the lack of a placebo control and the small size of the study. Neutrophilic inflammation in airways disease appears to be a 'treatable trait' which has been demonstrated to respond to macrolide therapy across groups of subjects with different disease labels. Further work in this area should concentrate on attempting to elucidate the pathological basis for this condition. The first step in doing this will be investigation of the bacterial content of the airways of these patients using the microbiota profiling techniques outlined in Chapter 4. Changes in microbiota composition have been noted in bronchiectasis and if this condition is indeed a precursor to bronchiectasis early changes to the microbiota such as increased abundance of potentially pathogenic organisms may be observed. Samples were taken and stored from this study and some initial microbiota analysis on these will be performed. Ultimately now this cohort has been identified and described further longitudinal studies need to be performed in patients from this cohort to determine the natural history of this condition. Such studies should aim to clarify whether these subjects will inexorably progress to develop radiological bronchiectasis and if treatment i.e. an RCT of azithromycin can delay or even potentially stop such progression.

Both of these studies suggest that stratifying subjects with certain symptoms using biomarkers can allow accurate predictions of treatment response to be made. New studies investigating the potential of such an approach are currently underway.

The third study "Microbiota in Asthma" aimed to characterise the airway bacterial microbiota in groups of subjects with diagnoses of asthma using specified dose ranges and types of inhaled steroids. The airway microbiota is a poorly described pathological component of airways disease, and this study aimed to describe in detail the bacterial communities extant within the airways of these different groups to determine whether inhaled steroid dose or type affected airway community composition. Broadly no differences in the abundance or community structure of bacteria in the airways were found between the BTS Step 2 and 4 treatment groups or the BTS Step 4 subgroups taking budesonide or fluticasone. This suggests that varying inhaled steroid dose or type does not fundamentally alter the airway microbiota, although significant differences in the abundance of certain bacterial species between the groups were noted. As the roles of particular bacterial species in the microbiota are further elucidated the importance of these

findings may become clearer. With the need to further characterise airways disease and recognise biomarkers for targeting treatment further studies of the airway microbiota, especially those based on answering clinically relevant questions, are required. Further work in this area should include the investigation of whether overall bacterial load or relative abundance of potentially pathogenic organisms such as *H. influenzae* or *P. aeruginosa* could serve as an early "biomarker" for airways disease that could be used to guide treatment. A future clinical interventional study should be planned in which ICS are introduced to steroid naïve subjects with longitudinal microbiota sampling over a prolonged time course. This study design would be the best way of answering the question of whether or not ICS affect the microbiota as it would allow comparison of intra subject comparison of bacterial airway composition pre and post ICS thus eliminating the considerable difficulties caused by significant inter subject microbiota variability. Systematic examination of whether microbiota composition varies between different airways disease phenotypes and interventional studies examining the effects of other therapeutic agents such as azithromycin on the microbiota should also be performed.

In summary, the studies presented in this thesis provide support for the consideration of airways disease as a spectrum of disease with a number of underlying pathophysiological components, the recognition of which can be used to selectively target treatment potentially resulting in improved patient outcomes. As the biochemical and eventually fundamental genetic abnormalities that predispose to airways disease are further elucidated it is hoped that the continuing use of such an approach will ultimately abolish the need for existing archetypal disease labels and eventually lead to a new era of precision medicine in which specific treatments can be accurately targeted to an individual subject.

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APPENDIX A:

PATIENT INFORMATION SHEET

(Version 2.0, 06/09/2012)

Title: Reducing Costs in Asthma Management – A Pilot Study

Chief Investigator: Dr Tim Harrison

Introduction

You are being invited to take part in a research study. Before you decide, it is important for you to understand why this research study is being conducted and what it will involve. Please take the time to read the following information carefully and discuss it with others if you wish. Please feel free to ask us if there is anything that is not clear or if you require any further information. You may keep this information for future reference.

Purpose of the Study

Your GP has recommended treatment with an inhaled steroid for suspected asthma. Although asthma is very common and you have symptoms which could be caused by asthma, we now know that a large number of patients treated for asthma have no firm evidence that they either have asthma or need inhaled steroid treatment. This is mainly because we have no really good tests to confirm if somebody does or does not have asthma.

Researchers at the Respiratory Research Unit at Nottingham City Hospital wish to establish whether there are baseline tests which can reliably identify patients who will not benefit from inhaled corticosteroid treatment. If successful this will prevent patients taking a treatment, sometimes for many years, which they do not really require.

Why have I been chosen?

You have been chosen because you have been prescribed an inhaled steroid for suspected asthma.

Do I have to take part?

It is entirely up to you whether or not you decide to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form (you will be given a copy of this as well). If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw, or a decision not to take part, will not affect the standard of care you already receive or your legal rights.

What will happen to me if I take part?

If after reading the information sheet, you would like to take part in this study, please get in touch with a member of the study research team (their contact details are on the bottom page of this information sheet) and we will arrange an appointment for you at Nottingham City Hospital to discuss the study further and if agreeable we will ask you to sign a consent form.

In addition to any tests your GP has already arranged, we would like to perform some additional tests and then review your response to your inhaled steroid at various time points. This will allow us to determine whether there are better ways of deciding who does or does

not need this treatment and will help your GP determine whether you need to continue taking your inhaled steroid.

Visit 1 (Respiratory Research Unit, Nottingham City Hospital)

This visit will ideally take place before you start taking your new inhaled steroid; and your GP will advise you whether this will or will not be possible. During this visit (approximately 2 hours), you will be asked about your past medical history and the following procedures will be carried out:

- **Respiratory Questionnaires:** You will be asked to fill out 2 short questionnaires (Juniper Asthma Control Questionnaire & Asthma Control Test) about your asthma symptoms and how it affects your daily life.
- **Spirometry Test**: This simple blowing test is used to measure your lung function (the size of your lungs and how quickly you can empty them). You will be asked to breathe in and then blow out very fast into a mouth piece.
- **Blood Sample**: We would like to take a small amount of blood (20mls or the equivalent of 4 teaspoons) from your arm for further analysis.
- **Bronchial Challenge**: This test is performed to measure how irritable your airways are. You will be asked to breathe in very small quantities of a drug (Methacholine) that is designed to make you wheezy. After each dose we will measure your lung function as described previously and we will stop when your blowing test falls by 20% or earlier if you feel unwell.
- **Exhaled Nitric Oxide:** This simple test measures the amount of inflammation in the breathing tubes by measuring the concentration of exhaled nitric oxide. It involves breathing into a tube connected to an analyser for a few seconds at various flow rates.
- **Allergy Skin Prick Test:** This test is performed to determine whether you have a specific allergy to something and involves pricking your skin through a solution containing an allergen. This is a standard test, which measures your reaction to 6 allergens; cat, dog, house dust mite, aspergillum, tree pollen & grass pollen.
- **Sputum Induction**: We would like to take a sputum/phlegm sample to be analysed in our laboratories, to look for inflammatory cells and chemicals that may be responsible for causing your chest problem. If you cannot produce a sample spontaneously, we would like to ‘induce sputum’. This procedure involves inhaling mildly salty water for 5 minutes to produce a sputum sample.

Visit 1a (Optional Visit, 24 hours after Visit 1)

If after Visit 1, we believe you may have airflow obstruction, we would like you to attend for an optional visit, up to 24 hours after Visit 1, which will provide further information about your airways. However; please be aware that this visit isn’t an essential part of the study should this be inconvenient.

During this visit (approximately 30 minutes), we will perform a spirometry test as before, but will also repeat this test again after you have taken a drug called salbutamol to look for an improvement in your lung function; this is called reversibility testing.

Please be aware that if you are already taking salbutamol, you must stop taking this medication up to 6 hours before this test is conducted. However, your study doctor will advise you in more detail about this.

Visit 2 (Follow-Up – 1 month)

We will ask you to attend for a follow-up visit at Nottingham City Hospital, 1 month after your initial visit, and whilst you are taking your prescribed inhaled steroid. The following tests will be repeated:-

- Respiratory Questionnaires
- Spirometry
- Exhaled Nitric Oxide
- Bronchial Challenge
- Sputum Induction
- Blood Sample

Visit 3 (Final Visit – 3 months)

We will ask you to attend for a final follow-up visit at Nottingham City Hospital, 3 months after your initial visit, and after you have finished taking your inhaled steroid where the following tests will be repeated:-

- Respiratory Questionnaires
- Spirometry
- Exhaled Nitric Oxide Measurements
- Bronchial Challenge
- Sputum Induction
- Blood Sample

At this visit we will also be able to provide you and your GP with an in-depth review of your response to the inhaled steroid you will have been taking for 3 months, allowing your GP to make a clear decision on whether or not they feel you should continue with this treatment.

What do I have to do?

You should continue to carry on with your normal daily activities and take your usual medication. We also ask that you attend the scheduled study visits (although there is some flexibility in terms of the days and times when these occur) and complete the study paperwork.

What are the possible benefits of taking part?

It is hoped that the results of this study will lead to a greater understanding of asthma and which patients will and will not benefit from inhaled corticosteroid treatment. By taking part in this study you and your doctor will also have very detailed information about whether or not you have benefitted from the inhaled steroid you were prescribed and therefore whether or not it should be continued.

What are the possible disadvantages/risks of taking part?

As with all tests/procedures some people experience side effects, some of which are detailed below:-

Sputum Induction: Occasionally the inhalation of salt solution in order to produce a sputum sample can make you wheezy. However, we will monitor you closely, and if necessary, this can quickly be reversed by using a Salbutomol inhaler (Ventolin) which will be present

throughout this procedure. Please be aware that this test will be performed by an experienced research nurse/officer.

Bronchial Challenge: This is a simple and safe test widely used in the assessment of asthma. Nevertheless you may experience chest tightness, wheeze and a cough; however these symptoms are usually mild and only last for a few minutes. They are easily reversed by inhaling a drug for treating asthma (salbutamol).

Allergy Skin Prick Test: This is a very safe test but can commonly cause itching around the site where the allergen has been introduced into the skin. The itching will last for about an hour, and can be reduced by taking an antihistamine if necessary.

Blood Tests: Occasionally, some people feel faint during a blood test. If this occurs, please tell the person doing the test, as you should immediately lie down to prevent fainting. Sometimes after donating blood, a bruise develops where the needle was inserted.

If you experience any unwanted side effects during the study you should inform a member of the research team.

What will happen if I don't want to carry on with the study?

You are free to withdraw from this study at any time and without giving a reason. A decision to withdraw, will not affect the standard of care you already receive. However, please be aware, that should you wish to withdraw, the information collected so far cannot be erased and may still be used in the final project analysis. Any stored tissue samples that can still be identified as yours will be destroyed if you wish.

Will my taking part in the study be kept confidential?

We will follow ethical and legal practice and all information about you will be handled in confidence.

If you join the study, some parts of your medical records and the data collected for the study will be looked at by authorised persons from the University of Nottingham who are organising the research. They may also be looked at by authorised people to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty.

All information which is collected about you during the course of the research will be kept strictly confidential, stored in a secure and locked office, and on a password protected database. Any information about you which leaves the hospital will have your name and address removed (anonymised) and a unique code will be used so that you cannot be recognised from it.

Your personal data (address, telephone number) will be kept for up to 12 months after the end of the study so that we are able to contact you about the findings of the study. All other data (research data) will be kept securely for 7 years. After this time your data will be disposed of securely. During this time all precautions will be taken by all those involved to maintain your confidentiality, only members of the research team will have access to your personal data.

Information on the storage and use of tissue samples for research

Any tissue sample you donate will be stored in a secure research facility at the University of Nottingham (Respiratory Research Unit, Clinical Sciences Building, Nottingham City Hospital), for as long as is required for the purposes of this study. The study researchers wish to measure the small particles (molecules/cells) found in your blood and sputum/phlegm in order to better understand asthma.

Your sample will have your code which is unique to yourself, a barcode and date of study. By using these numbers, we can trace which sample belongs to you. The analysis of samples will take place within the Respiratory Research Unit at Nottingham City Hospital. Please note; your sample will not be sold for profit or used in any animal research.

With your permission we would like to retain any remaining tissue/blood in a link-anonymised form for future laboratory research into respiratory disease (as yet unspecified). If you agree, the remaining tissue/blood will be stored on University premises under our Human Tissue Authority License. Finally, we often work together with scientists at other universities, as well as with commercial companies, and this often involves sharing research samples with them. With your consent we may wish to send some of your sample to third parties; including EU and Non-EU countries and commercial companies. Please be aware that your personal details would be removed in order that you cannot be identified by these third parties. If you agree to this, please indicate on the consent form as this is **optional**.

Will any genetic tests be carried out?

No

Will travel expenses be reimbursed?

Participants will not be paid an inconvenience allowance to participate in the study. However, we will cover the cost of travelling to the hospital (maximum £20 allowance per visit).

Involvement of the General Practitioner/Family Doctor (GP)

With your permission we will write to your GP to notify them that you are going to take part in this study and provide him/her with an in-depth review of your response to the inhaled steroid you will have been taking for 3 months; allowing your GP to make a clear decision on whether or not they feel you should continue with this treatment.

Who is organising and funding this study?

The research has been organised by the University of Nottingham and funded by the National Institute for Health Research (NIHR). Please be aware that the research team involved in this study are not being paid for including you in this study.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people called a Research Ethics Committee, to protect your interests. This study has been reviewed and given a favourable ethical opinion for conduct in the NHS by the Derby 1 Research Ethics Committee and will be subject to the Data Protection Act.

What will happen to the results of this study?

We intend to publish the results of this study in a medical respiratory journal. A summary of these results will also be made available on the Nottingham Respiratory Research Unit's website (www.nrbru.org.uk). Furthermore, a copy of any published material regarding the study will be made freely available to you. Please be aware that you will not be identified in any publications – all data used in the publications will be anonymous.

What if there is a problem?

If you wish to complain or have any concerns about the way in which you have been treated, please get in touch with the research team (see below), who will do their best to answer any problems you might have. In addition, the normal NHS complaints procedures are also available to you (e.g. Patient Advice and Liaison Service – PALS); please telephone 0115 92449924 ext 65412 for Nottingham University Hospitals NHS Trust.

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation but you may have to pay your legal costs.

Contact for Further Information

If after reading this information sheet, you would like to take part in this study, please get in touch with a member of the research team (see contact details below, or please complete the reply slip and return it to us in the pre-paid envelope) and we will arrange an appointment for you at Nottingham City Hospital to discuss the study further.

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Appendix B: Juniper Asthma Control Questionnaire (ACQ)

Please complete questions 1-6. Circle the number of the response that best describes how you have been during the past week.

1. On average, during the past week, how often were you woken by your asthma during the night?

0 Never
1 Hardly ever
2 A few minutes
3 Several times
4 Many times
5 A great many times
6 Unable to sleep because of asthma

2. On average, during the past week, how bad were your asthma symptoms when you woke up in the morning?

0 No symptoms
1 Very mild symptoms
2 Mild symptoms
3 Moderate symptoms
4 Quite severe symptoms
5 Severe symptoms
6 Very severe symptoms

3. In general, during the past week, how limited were you in your activities because of your asthma?

0 Not limited at all
1 Very slightly limited
2 Slightly limited
3 Moderately limited
4 Very limited
5 Extremely limited
6 Totally limited

4. In general, during the past week, how much shortness of breath did you experience because of your asthma?

0 None
1 A very little
2 A little
3 A moderate amount
4 Quite a lot
5 A great deal
6 A very great deal

5. In general, during the past week, how much of the time did you wheeze?

0 Not at all
1 Hardly any of the time
2 A little of the time
3 A moderate amount of the time
4 A lot of the time
5 Most of the time
6 All the time

6. On average, during the past week, how many puffs of short-acting bronchodilator (e.g. Ventolin) have you used each day?

0 None
1 1±2 puffs most days
2 3±4 puffs most days
3 5±8 puffs most days
4 9±12 puffs most days
5 13±16 puffs most days
6 More than 16 puffs most days

To be completed by a member of the clinic staff:

7. FEV_1 pre-bronchodilator: .....
FEV_1 predicted: .....
FEV_1 % predicted: .....
(Record actual values on the dotted lines and score the FEV_1 % predicted in the next column)

0 >95% predicted
1 95-90%
2 89-80%
3 79-70%
4 69-60%
5 59-50%
6 <50% predicted

Appendix C: Asthma Control Test

1) During the past 4 weeks, how often did your asthma prevent you from getting as much done at work, school or home?

1 All of the time
2 Most of the time
3 Some of the time
4 A little of the time
5 None of the time

2) During the past 4 weeks, how often have you had shortness of breath?

1 More than once a day
2 Once a day
3 3-6 times a week
4 1-2 times a week
5 Not at all

3) During the past 4 weeks, how often did your asthma symptoms (wheezing, coughing, chest tightness, shortness of breath) wake you up at night or earlier than usual in the morning?

1 4 or more times a week
2 2-3 nights a week
3 Once a week
4 Once or twice
5 Not at all

4) During the past 4 weeks, how often have you used your reliever inhaler (usually blue)?

1 3 or more times a day
2 1-2 times a day
3 2-3 times a week
4 Once a week or less
5 Not at all

5) How would you rate your asthma control during the past 4 weeks?

1 Not controlled
2 Poorly controlled
3 Somewhat controlled
4 Well controlled
5 Completely controlled

APPENDIX D:

Participant Information Sheet
Final version 1.0

Title of Study: **An open label trial of azithromycin in chronic productive cough**

Name of Researcher(s): Dr Tim Harrison, Dr Matthew Martin, Dr Dominick Shaw, Dr Helen Roberts

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. Talk to others about the study if you wish. Ask us if there is anything that is not clear.

What is the purpose of the study?

We have noticed a group of patients presenting with a longstanding wet cough which has often been treated as asthma. The cough is productive of phlegm which frequently contains bacteria, and it does not get better with standard antibiotic treatment.

A very similar cough is seen in people who smoke, have exposure to airbourne dusts or chemicals or have a condition known as bronchiectasis in which there is scarring of the airways in the lung leading to coughing up lots of phlegm. In our research study these problems have already been ruled out.

We have found that prolonged treatment of people with longstanding wet cough with an antibiotic called azithromycin is very effective at improving the cough. However, using azithromycin in this way has not yet been studied in detail to work out how effective it is.

Our research will try to work out what the cause for your cough is and if it is actually caused by a new condition which does not yet have a name. We will also try to determine how effective azithromycin is at improving the cough.

Why have I been invited?

You are being invited to take part because you have a longstanding wet cough of unknown cause. We are inviting 50 participants like you to take part.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This would not affect your legal rights.

What will happen to me if I take part?

Investigations

Participants in this study will first have a series of additional investigations aimed at trying to discover the cause of their wet cough. These include:

- **Sputum microbiology and differential cell counts:** Taking a sputum sample to look for inflammatory cells, bacteria and chemicals that may be responsible for causing your chest problem. We will take a further sputum sample with your agreement for storage and future analysis by a research team. This future analysis may involve looking for other inflammatory cells, chemicals or for DNA of any bacteria that may be present in the sputum.
- **Exhaled nitric oxide measurement:** Measuring the amount of exhaled nitric oxide in your breath to measure the amount of inflammation in the breathing tubes of your lungs.
- **Exhaled carbon monoxide measurement:** Measuring the amount of carbon monoxide in your breath to assess any exposure you may have had to cigarette smoke.
- **Leicester Cough Questionnaire:** Completing a questionnaire assessing how your cough affects your life.
- **Blood samples:** Blood samples will be taken at a certain point to ensure the azithromycin treatment does not affect your liver, which is one of its recognised side effects.

All of these investigations will take place in our research clinic at the Nottingham City Hospital. Further information on these investigations can be found on our website: (http://www.nrru.org/NRRU_Patient_Information.html). If you do not have access to the internet, printed information leaflets describing these tests in further detail are available upon contacting the Nottingham Respiratory Research Unit on the telephone number given below.

Bronchoscopy

We also intend to perform a procedure called a bronchoscopy on each of the participants in the study. Bronchoscopy is a routine diagnostic examination which allows us to directly examine the large air passages in the lung and retrieve cells from the lining of the airways. Bronchoscopy is a safe procedure and carries little risk.

The test takes about 15 minutes but you will need to be at the hospital for about half a day. It involves the following:

You will be asked not to eat or drink anything for at least 6 hours prior to the bronchoscopy. You can take your medication including inhalers as normal.

You will be given oxygen to breathe throughout the procedure and the amount of oxygen in your blood will be measured throughout the test with a monitor around a finger (oximeter). A small drip (cannula) will be placed in a vein in your arm and we will monitor your heart rate, and measure your blood pressure.

A local (topical) anaesthetic called lignocaine, or an alternative, is then sprayed on to the back of the mouth and into the nose. This anaesthetic numbs the nerves so that the bronchoscope can be easily inserted into the wind passage without discomfort. Lignocaine is the same as the local anaesthetic used by dentists (you should let us know if you are allergic to any local anaesthetic agents). Midazolam, or an alternative sedative, injected through the drip, is also given at this point to make you more relaxed and drowsy. You may be given an injection of alfentanyl or fentanyl which are medicines which will also calm you and will prevent you from coughing too much.

The bronchoscope, which is a thin flexible instrument, is then passed usually through the nose and down the back of the throat. If it is not easy to pass the bronchoscope through the nose, it will be passed through the mouth instead. More local anaesthetic is then placed on the vocal cords and the bronchoscope passed through the voice box and into the lungs. The following samples will be collected:

1) Wash – a small amount of fluid is injected and sucked out. In all, we do this four times in succession.
2) Biopsies – Small tissue samples (maximum of 10) will be taken from the airway wall.

The samples obtained will be used to evaluate the structure of the airway, the types of inflammatory cells found in the airway wall and to measure the presence of proteins which could be involved in inflammation. With your consent some of the samples will be stored for future analysis which may include detecting the DNA of any micro-organisms present in the lung.

After your examination, the lining of your mouth and throat will remain numb just in the same way as your mouth would after a dental procedure. You will experience a sore-throat and a cough. These discomforts will wear off within the next 2 hours or so. You should not eat or drink for at least 2 - 3 hours. This precaution is necessary to keep food or liquids from accidentally entering the windpipe or lungs.

As you will be given midazolam you will not be able to drive or operate a machine for 24 hours after administration.

If clinically relevant information is obtained at the time of the bronchoscopy this will be shared with you and the relevant doctors involved with your care. The samples obtained will be anonymised and the results will not be put in your medical records

Following bronchoscopy, we will invite you to start part 2 of the study, which involves treatment with the azithromycin antibiotic for a period of 12 weeks.

Azithromycin (Other names: Zithromax)
In this study, one tablet of azithromycin (dose 250mg) must be taken by mouth 3 times per week, on Monday, Wednesday and Friday.

Timing of study
Overall, participation in this study will require five visits to the Nottingham City Hospital over a period of 17-18 weeks, which is summarised here:

PART 1 OF STUDY

Clinic appointment visit

You will be identified potential candidate for the study in a respiratory clinic appointment by one of the study team or one of our colleagues. You should have already had a number of investigations for your cough that have not fully explained the cause. You will be asked if you would like to take part in the study and any questions you may have will be answered. You will be asked to give written informed consent in order to take part in the study.

Duration: 10-15 minutes

Hospital visit 1

Face to face visit with our research team in the hospital. This visit will take place in the Nottingham City Respiratory Research Unit. You will be seen by a doctor who will ask some questions relating to your condition to make sure you are eligible for the study. If so, we will measure the exhaled gases (nitric oxide and carbon monoxide) in your breath (explained above) and take some sputum samples with your permission for future analysis. We will perform a set of breathing tests (spirometry), and ask you to collect the sputum you produce over a day in a container, also making a note of the colour of this sputum.

You will be asked to complete a questionnaire assessing how your cough affects your life.

Finally, we will also take some blood tests and perform a simple electronic tracing of your heart activity (ECG) to ensure azithromycin will be safe for you to take.

After these investigations we will provide you with the full 3 month course of azithromycin, to begin taking following bronchoscopy.

Duration: 1.5 -2 hours

Bronchoscopy visit (week 2)

You will attend the endoscopy unit in the hospital for a bronchoscopy (as described above). We will ask you to start taking the azithromycin after your bronchoscopy.

Duration: 4 hours

PART 2 OF STUDY

Hospital visit 2 (4 weeks after starting treatment)

Face to face visit with our research team at the Nottingham City Respiratory Research Unit. We will ask you some questions to see if you have experienced any problems with treatment and repeat some blood tests.

Duration: 0.5 hours

Hospital visit 3 (after 12 weeks of treatment)

Face to face visit with our research team at the Nottingham City Respiratory Research Unit. You will be seen by a doctor who will repeat the investigations you had in visit 2, including the questionnaire, spirometry, sputum collection and colour, blood tests and ECG to assess the effects of the treatment.

Duration: 1 – 1.5 hours

Hospital visit 4 (follow up visit)

Face to face visit with our research team at the Nottingham City Respiratory Research Unit. You will be seen by a doctor who will assess your symptoms after treatment. We will again ask you to complete a questionnaire assessing how your cough affects your life.

We will also take some final samples of your sputum including, with your permission, samples for future analysis.

Duration: 0.5 – 1 hours

Expenses and payments

Participants will be paid an inconvenience allowance to participate in the study of up to £150 including time and travel expenses.

What are the possible disadvantages and risks of taking part?

Investigations

Providing sputum and blood samples and having the levels of carbon monoxide and nitric oxide in your breath measured do not pose any risks and are usually well tolerated.

Bronchoscopy

Bronchoscopy with lung biopsy is usually a very safe procedure but there are some very small risks associated with this.

The medication for the bronchoscopy may make you feel lightheaded or dizzy. The initial medication, or placement of an IV drip, may cause local pain, bleeding and swelling. There is a very small risk (less than 1 in 100) of infection at the IV site.

Likely side effects of the bronchoscopy and the related lavage and biopsy (occurring in 25 out of 100 procedures) include discomfort (coughing and occasionally gagging) and nosebleed (if the bronchoscope was passed through your nose). You may cough up small flecks of blood for 24 hours after the procedure.

More serious complications from the bronchoscopy, lavage, and biopsy include major bleeding, collapse of the lung, vocal cord and windpipe spasms, pneumonia or bronchitis and irregular heartbeats. These have been reported but are extremely rare (occurring in less than 1 out of 1000 procedures). One death has been reported after research bronchoscopy in the USA, but not in the UK. Many thousands of research bronchoscopies have been performed, so the risk of death is extremely remote.

Azithromycin

Azithromycin is a very safe medication which is commonly used to treat infection, and is often used over long courses of 3 months or longer. As with all medicines, it has side effects. Common side effects include stomach upsets and diarrhoea (which may occur in around 1 in 10 people) and headaches and dizziness, tiredness or skin rashes (which may occur in around 1 in 100 people), but these are usually temporary and not serious. In rare cases azithromycin may cause more serious side effects relating to the heart or liver (less than 1 in 10,000) which will be carefully monitored for as part of the study. If you are allergic to any medications including antibiotics please let us know.

It is important to note that in this study, azithromycin is being used "off licence" which means that the manufacturers of this drug do not currently have enough information to recommend or not recommend using the drug in this way. However, the drug has been licensed for use in very similar conditions as it has proven to be of benefit, and is already used off licence in our respiratory unit for people with your symptoms.

What are the possible benefits of taking part?

You may benefit from the 3 months of azithromycin treatment although we cannot promise that this study/trial will cure your cough. The information we get from this study may help explain what is causing it and help us treat patients in the future.

What happens when the research study stops?

You will be referred back to your usual respiratory physician or GP and have further follow up if necessary in the normal respiratory clinic.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. The researchers contact details are given at the end of this information sheet. If you remain unhappy and wish to discuss the matter further, you can do this by getting in touch with the Nottingham Hospitals Patient Advice and Liaison Service (PALS) in person, by telephone or e-mail who can try and resolve the situation. Details are provided below. If you still wish to make a formal complaint you can do this through the NHS formal complaints procedure (further details of which can be provided by the PALS service).

Nottingham City Campus PALS service
By person: PALS is on the South Corridor at Junction S6. Opening times 9:30 – 4:30 pm Monday - Friday
Tel: 0800 052 1195 (free from a landline) or 0115 969 1169 ext 59671
E-mail: pals@nuh.nhs.uk
Post: NUH NHS Trust, c/o PALS, Freepost, NEA 14614, Nottingham NG7 1BR

Will my taking part in the study be kept confidential?

We will follow ethical and legal practice and all information about you will be handled in confidence.

If you join the study, some parts of your medical records and the data collected for the study will be looked at by authorised persons from the University of Nottingham who are organising the research. They may also be looked at by authorised people to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty.

All information which is collected about you during the course of the research will be kept **strictly confidential**, stored in a secure and locked office, and on a password protected database. Any information about you which leaves the hospital will have your name and address removed (anonymised) and a unique code will be used so that you cannot be recognised from it.

Your personal data (address, telephone number) will be kept for 12 months after the end of the study so that we are able to contact you about the findings of the study *and possible follow-up studies* (unless you advise us that you do not wish to be contacted). All other data (research data) will be kept securely for 7 years. After this time your data will be disposed of securely. During this time all precautions will be taken by all those involved to maintain your confidentiality, only members of the research team will have access to your personal data.

What will happen if I don't want to carry on with the study?

Your participation is voluntary and you are free to withdraw at any time, without giving any reason, and without your legal rights being affected. If you withdraw then the information collected so far cannot be erased and this information may still be used in the project analysis.

Involvement of the General Practitioner/Family doctor (GP)

We will inform your GP about your participation in the trial and send him/her a copy of this information sheet.

What will happen to any samples I give?

We would also like to seek your consent so that any remaining samples may be stored and used in possible future research – this is optional (please indicate you agree to this on the consent form). The samples will be stored with a code unique to you and securely at the University of Nottingham under the University's Human Tissue Research Licence (no 12265).

Some of these future studies may be carried out by researchers other than current team of Dr Harrison including researchers working for commercial companies. Any samples or data used will be anonymised, and you will not be identified in any way. If you do not agree to this any remaining samples will be disposed of in accordance with the Human Tissue Authority's codes of practice.

Will any genetic tests be done?

No tests will be performed on any of your samples to determine any of your genetic information. In future studies, tests may be performed to detect the DNA of any micro-organisms (including bacteria) that may be present in the samples

What will happen to the results of the research study

We will publish the results of the trial in a high-profile respiratory medicine journal, present the results at various scientific conferences, and this work will form part of a thesis for a higher degree. You will not be identified in any report/publication. We will send you a newsletter with a summary of the results.

Who is organising and funding the research?

This research is being organised by the University of Nottingham and is being funded by the Nottingham Respiratory Medicine Department.

Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by Yorkshire & The Humber – Leeds West Research Ethics Committee.

Further information and contact details

Further information can be obtained from our website:
http://www.nrru.org/

Or by telephoning us on:

Matthew Martin
(Study Doctor)
Tel: 0115 8231935

Denise Barber
(NRRU Secretary)
Tel: 0115 86231317
Fax: 0115 8231946

OR

Tina Wilkinson
(CTU Receptionist)
Tel: 0115 8404844
Fax:0115 84026217

Alternatively, you can write to us at the following address:

Nottingham Respiratory Research Unit
Room B28
Clinical Sciences Building
Nottingham City Hospital
Hucknall Road
Nottingham
NG5 1PB

Appendix E: Leicester Cough Questionnaire

This questionnaire is designed to assess the impact of cough on various aspects of your life. Read each question carefully and answer by CIRCLING the response that best applies to you. Please answer ALL questions, as honestly as you can.

1. In the last 2 weeks, have you had chest or stomach pains as a result of your cough?

1 All of the time	2 Most of the time	3 A good bit of the time	4 Some of the time	5 A little of the time	6 Hardly any of the time	7 None of the time

2. In the last 2 weeks, have you been bothered by sputum (phlegm) production when you cough?

1 Every time	2 Most times	3 Several times	4 Some times	5 Occasionally	6 Rarely	7 Never

3. In the last 2 weeks, have you been tired because of your cough?

1 All of the time	2 Most of the time	3 A good bit of the time	4 Some of the time	5 A little of the time	6 Hardly any of the time	7 None of the time

4. In the last 2 weeks, have you felt in control of your cough?

1 None of the time	2 Hardly any of the time	3 A little of the time	4 Some of the time	5 A good bit of the time	6 Most of the time	7 All of the time

5. How often during the last 2 weeks have you felt embarrassed by your coughing?

1 All of the time	2 Most of the time	3 A good bit of the time	4 Some of the time	5 A little of the time	6 Hardly any of the time	7 None of the time

6. In the last 2 weeks, my cough has made me feel anxious

1 All of the time	2 Most of the time	3 A good bit of the time	4 Some of the time	5 A little of the time	6 Hardly any of the time	7 None of the time

7. In the last 2 weeks, my cough has interfered with my job, or other daily tasks

1 All of the time	2 Most of the time	3 A good bit of the time	4 Some of the time	5 A little of the time	6 Hardly any of the time	7 None of the time

8. In the last 2 weeks, I felt that my cough interfered with the overall enjoyment of my life

1 All of the time	2 Most of the time	3 A good bit of the time	4 Some of the time	5 A little of the time	6 Hardly any of the time	7 None of the time

9. In the last 2 weeks, exposure to paints or fumes has made me cough

1 All of the time	2 Most of the time	3 A good bit of the time	4 Some of the time	5 A little of the time	6 Hardly any of the time	7 None of the time

10. In the last 2 weeks, has your cough disturbed your sleep?

1 All of the time	2 Most of the time	3 A good bit of the time	4 Some of the time	5 A little of the time	6 Hardly any of the time	7 None of the time

11. In the last 2 weeks, how many times have you had coughing bouts?

1 All the time (continuously)	2 Most times during the day	3 Several times during the day	4 Some times during the day	5 Occasionally throughout the day	6 Rarely	7 None

12. In the last 2 weeks, my cough has made me feel frustrated

1	2	3	4	5	6	7
All of the time	**Most of the time**	**A good bit of the time**	**Some of the time**	**A little of the time**	**Hardly any of the time**	**None of the time**

13. In the last 2 weeks, my cough has made me feel fed up

1	2	3	4	5	6	7
All of the time	**Most of the time**	**A good bit of the time**	**Some of the time**	**A little of the time**	**Hardly any of the time**	**None of the time**

14. In the last 2 weeks, have you suffered from a hoarse voice as a result of your cough?

1	2	3	4	5	6	7
All of the time	**Most of the time**	**A good bit of the time**	**Some of the time**	**A little of the time**	**Hardly any of the time**	**None of the time**

15. In the last 2 weeks, have you had a lot of energy?

1	2	3	4	5	6	7
None of the time	**Hardly any of the time**	**A little of the time**	**Some of the time**	**A good bit of the time**	**Most of the time**	**All of the time**

16. In the last 2 weeks, have you worried that your cough may indicate a serious illness?

1	2	3	4	5	6	7
All of the time	**Most of the time**	**A good bit of the time**	**Some of the time**	**A little of the time**	**Hardly any of the time**	**None of the time**

17. In the last 2 weeks, have you been concerned that other people think something is wrong with you, because of your cough?

1	2	3	4	5	6	7
All of the time	**Most of the time**	**A good bit of the time**	**Some of the time**	**A little of the time**	**Hardly any of the time**	**None of the time**

18. In the last 2 weeks, my cough has interrupted conversation or telephone calls

1 All of the time	2 Most of the time	3 A good bit of the time	4 Some of the time	5 A little of the time	6 Hardly any of the time	7 None of the time

19. In the last 2 weeks, I feel that my cough has annoyed my partner, family or friends

1 Every time I cough	2 Most times when I cough	3 Several times when I cough	4 Some times when I cough	5 Occasionally when I cough	6 Rarely	7 Never

LCQ score: ☐

Appendix F: AZCC Bronchoscopy Sample Collection

Order of collecting samples*:

Order can be changed if patient not tolerating procedure/frequently coughing as washing first may stimulate increased cough. However, this order is preferable to minimise contamination of wash samples with blood

[1] RUL bronchial wash (15ml total – split into 3) – collected into **universal sample containers**

- **1 x 5ml sample labelled with patient details/NHS no and sent to microbiology labelled** 'CLINICAL TRIAL 13RM015'
- **2 x 5ml samples labelled with patient study number only and sent to CSB lab**

[2] 4-8 bronchial biopsies from R bronchus intermedius:

- **2-4 into formalin** containing histopathology sample container (Labelled with patient details/NHS no) and **sent to histopathology** labelled 'Azithromycin in Chronic Cough Study FAO Dr Soomro'

- **2-4 into universal sample containers containing PBS** (labelled with patient study number only) **sent to CSB lab**

OPTIONAL – depending on specific patient consent and tolerance of procedure

[3] 2 bronchial brushings of sub-carina/right bronchus intermedius

- into **1x 3ml Falcon tube containing BEGM + 1%PSF** (labelled with patient study number only) **sent to CSB lab**

AZCC Bronchoscopy Sample Processing

Bronchial Washes (x2)

1x 5ml sample for cell count/viability/supernatant frozen for cytokine analysis

- Centrifuge for 10 min at 400g
    - **Pellet – For cell count/differential**
    - **Supernatant – divide into aliquots and freeze at -70^{0}C** (for later cytokine analysis)

1x 5ml sample for storage for microbiota work

- Centrifuge at 1000 rpm for 5 mins
- Supernatant transferred to a fresh tube and centrifuged at top speed (4180xg) for 15 mins.
- Pellet from the first spin labelled "**pellet 1**" along with the donor information/date
- Second pellet labelled as "**pellet 2**" again with donor information
- Supernatant stored (backup for cytokine analysis)

Both cell pellets are stored at -80^{0}C for bacterial PCR at a later date

Bronchial Biopsies (x4)

2 sent to histopathology (FAO Dr Soomro)

Processed, paraffin blocks made

Initial report made. Samples saved for later reporting once responders/non responders identified

2 for Biobank

Bronchial Brushes (x2)

For Biobank

Appendix G: Radiology Scoring Sheet for CTs

Characteristic				**Areas affected**
Bronchial wall thickening	0 None	1 Some	2 Prominent	
Airway dilatation	0 None	1 Minor dilatation	2 Minor bronchiectasis	
Mosaic perfusion	0 None	1 Some	2 Prominent	

Atelectasis	0 None	1 <3 areas <3cm bands	2 >3 areas	3 Large bands	

Characteristic			**Areas affected**
Lymphadenopathy	Yes	No	
Pleural thickening	Yes	No	
Patulous oesophagus	Yes	No	
Collapsible airways	Yes	No	
Endobronchial mucus retention	Yes	No	
Ground glass change	Yes	No	
Tree in bud changes	Yes	No	
AP diameter			
Other changes/ Relevant clinical info			

Obesity	0 Underweight	1 Expected	2 Overweight	3 Obese

APPENDIX H: AZITHROMYCIN STUDY CT FEATURES DATA

Study No	**Bronchial wall thickening**	BWT Areas affected	**Airway Dilatation**	AD Areas affected	
AZCC01	0		0		
AZCC02	0		0		
AZCC03	1	3,4	1	3,4	
AZCC04	0		1	4	
AZCC05	1	perihilar	2	3,4	
AZCC06	0		1	2,3	
AZCC07	0		1	3,4	
AZCC08	1	perihilar	1	4	
AZCC09	0		0		
AZCC10	0		2	3,4	
AZCC11	2	2,3,4	2	3,4	
AZCC12	1	perihilar	1	2,3,4	
AZCC13	1	perihilar	1	4	
AZCC14	0		1	2	
AZCC15	0		1	2,3,4	
AZCC16	0		1	4	
AZCC17	0		2	3,4	
AZCC18	2	perihilar	0	0	
AZCC19	0		0		
AZCC20	1	2,3	1	2,3	
AZCC21	0		1	4	
AZCC22					
AZCC23	0		1	2	
AZCC24	1	perihilar	1	3	
AZCC25	1	3	2	3,4	
AZCC26	0		1	4	
AZCC27	0		0		
AZCC28	1	perihilar	0		
AZCC29	1	perihilar	0		
AZCC30	1	3,4	1	3,4	

Study No	Atelectasis	Atel Areas affected	Mosaic Perfusion	MP Areas affected	Lymphadenopathy
AZCC01	0		0		0
AZCC02	1	2	0		0
AZCC03	0		0		0
AZCC04	0		0		0
AZCC05	2	4	1		0
AZCC06	2	4	0		0
AZCC07	0		0		0
AZCC08	2		0		0
AZCC09	1	4	0		0
AZCC10	1	4	0		0
AZCC11	2	3,4	1		0
AZCC12	2	4	1		0
AZCC13	2	4	1		0
AZCC14	0		0		0
AZCC15	0		0		0
AZCC16	1	4	0		0
AZCC17	2	3,4	0		0
AZCC18	1	3	0		0
AZCC19	1	4	0		0
AZCC20	1	3,4	1		0
AZCC21	3	4	0		1
AZCC22					
AZCC23	1	4	1		0
AZCC24	2	3,4	1		1
AZCC25	1	4	1		0
AZCC26	0	0	0		0
AZCC27	0		0		0
AZCC28	1	2	0		0
AZCC29	0		0		1
AZCC30	1	4	1	2,3	0

Study No	Pleural changes	Patulous oesophagus	Collapsible airways	Endobronchial mucus
AZCC01	0	0	0	0
AZCC02	0	0	0	0
AZCC03	0	0	0	0
AZCC04	0	0	0	0
AZCC05	0	1	0	0
AZCC06	0	1	0	0
AZCC07	0	0	0	0
AZCC08	0	0	0	0
AZCC09	0	0	0	0
AZCC10	0	0	0	1
AZCC11	0	0	1	1
AZCC12	0	0	0	1
AZCC13	0	0	0	1
AZCC14	0	0	0	0
AZCC15	0	0	0	0
AZCC16	0	0	0	0
AZCC17	1	0	0	0
AZCC18	0	0	0	0
AZCC19	0	0	0	0
AZCC20	0	0	0	0
AZCC21	1	1	0	1
AZCC22				
AZCC23	0	0	0	0
AZCC24	0	0	0	0
AZCC25	0	0	0	0
AZCC26	0	0	0	0
AZCC27	0	0	1	0
AZCC28	0	0	0	0
AZCC29	0	0	0	0
AZCC30	0	0	0	0

Study No	Ground glass change	Tree in bud	AP Diameter	Obesity	Other changes
AZCC01	0	0		1	Poor quality scan
AZCC02	1	0		1	Ground glass changes (4)
AZCC03	0	0		2	
AZCC04	1	0		1	Ground glass changes patchy, small
AZCC05	0	1		3	breathing artefact on scan
AZCC06	0	0	29.8/16.0	1	
AZCC07	0	0		3	Poor quality breath-hold
AZCC08	0	0		1	Longstanding elevated R hemidiaphragm
AZCC09	0	0		1	Enlarged thyroid. Pulmonary nodule RLL
AZCC10	0	0	24.3/9.1	1	Poor quality breath-hold
AZCC11	0	0	21.6/14.0	2	Slight collapse of trachea
AZCC12	1	0	30.6/16.4	1	ground glass change peribronchial
AZCC13	0	1	21.2/13.4	1	patchy tree in bud
AZCC14	0	0	24.4/11.3	1	thymic density ant mediastinum
AZCC15	0	0		2	
AZCC16	0	0	23.1/11.5	2	movement artefact
AZCC17	0	0		1	few small areas pleural thickening (3)
AZCC18	0	0	23.5/21.1	3	
AZCC19	0	0	28.0/20.0	3	motion artefact
AZCC20	1	0		2	ground glass change lingula
AZCC21	1	0		2	hilar +mediastinal LN
AZCC22					
AZCC23	0	0		2	poor breath hold
AZCC24	0	1		2	mediastinal LN >12mm
AZCC25	0	0		2	
AZCC26	0	0		1	
AZCC27	0	0		2	L+R main bronchi narrowed
AZCC28	0	0		1	thyroid enlargement
AZCC29	0	0		1	small volume mediastinal LN
AZCC30	0	0		1	

Appendix I: Microbiota in CF Literature Review

YEAR	NAME	NO OF PTS	SAMPLING TYPE	SEQUENCING METHOD	DESCRIPTION/RESULTS
2008	Bittar *et al.*	16 children 9 adults	Spontaneously expectorated sputum (SES)	Sanger sequencing	1) One of the first studies to compare standard microbiological culture and DNA sequencing for bacterial detection. 2) ~58% of isolated bacteria were detected only after cloning and sequencing. The pathogenic species were only detected after amplification and cloning (7 cases). 3) New or emerging bacteria not or rarely reported in CF patients were detected including *Dolosigranulum pigrum*, *Dialister pneumosintes*, and *Inquilinus limosus*.
2010	Cox *et al.*	51 patients 19 children 32 adults	SES (from adults) Deep throat swab (from paediatric patients)	Phylochip	1) Older CF patients with worse pulmonary function have a less diverse lung microbiota consisting of a 'core' of phylogenetically related colonising pathogenic species in comparison to younger patients. 2) Using longitudinal samples collected from a subset of patients the initially diverse bacterial community observed in younger patients becomes less rich and diverse over time.
2011	Tunney *et al.*	23 patients, before and after IV ABx treatment for CF exacerbation	SES	T-RFLP qPCR	1) One of the first studies examining the respiratory microbiota in CF patients before and after antibiotic treatment of CF exacerbations. 2) Demonstrated significant inter-patient variability in microbiota composition but little intra-patient variability (i.e. stability) in composition of the bacterial community despite treatment with IV antibiotics. 3) There was a decrease in bacterial abundance following treatment, and this effect was more evident for aerobes including *Pseudomonas spp.* than for anaerobes. This may well be because antibiotic treatment was targeted at *Pseudomonas spp*.

2011	Van der Gast *et al.*	14 adult CF patients	SES	Sanger sequencing	1) Study attempted to partition bacterial community into core and satellite taxa. 2) The 'Core' taxa consisted of 15 taxa from 7 genera including *Pseudomonas* (1 taxon), *Streptococcus* (2), *Neisseria* (2), *Catonella* (1), *Porphyromonas* (1), *Prevotella* (5) and *Veillonella*. *Pseudomonas* was by far the most dominant organism. 3) The 'Satellite' taxa consisted of 67 bacterial taxa from 33 genera. 4) The CFTR genotype and antibiotic treatment were significantly correlated with the composition of all taxa and the core group members. 5) A significant correlation was found between FEV_1 and taxa richness (number of different taxa), with a significant positive linear relationship between these two variables.
2011	Sibley *et al.*				1) Comparison of DNA based sequencing of microbiota with standard culture techniques. 2) Standard culture detected a fraction (65.1%) of the organisms in sputum detected using T-RFLP. However, by using extended culture techniques to enhance the growth of organisms which are not classic CF pathogens (particularly anaerobes) this proportion was increased to 84%. 3) Organisms detectable with 10^3 and 10^4 16S rRNA gene sequences recovered by culture in 100% and 86.8% of instances respectively.
2012	Zhao *et al.*	6 adult male CF patients (3 stable disease, 3 'progressive' disease). Multiple samples over 8-9 year period totalling 126	SES	Pyrosequencing	1) Demonstrated significant decrease in bacterial community diversity (measured by Shannon index) over time in patients with typically progressive lung disease but remained relatively stable in patients with a mild lung disease phenotype. 2) Antibiotic treatment was associated with pronounced shifts in community structure, but communities showed both short and long term resilience after antibiotic perturbation. 3) Antibiotic use, rather than patient age or lung function, was the

					primary driver of decreasing diversity. Inter-patient variability in community structure exceeded intra-patient variability in serial samples. 4) Despite decreasing community diversity in patients with progressive disease, total bacterial density remained relatively stable over time.
2012	Stressman *et al.*	14 adult patients, samples collected every month for 12 months	SES	T-RFLP	1) Subjects' bacterial communities were found to be stable over the course of a year, changing little during this time despite intervening respiratory exacerbation periods. 2) Some changes were observed during antibiotic treatment but these did not persist and returned to approximate pre-treatment structures within a month. 3) Concluded that in the CF lung, community richness is inversely correlated with lung disease severity. 4) *P. aeruginosa* was associated with lower community richness and lower lung function.
2012	Delhaes *et al*.	4 adult CF patients, 2 samples each	SES	Pyrosequencing (16S rDNA and ITS2 locus for fungi)	1) First study in CF patients to sequence both bacterial and fungal lung communities. 2) Discovered diverse and complex bacterial and fungal communities, in which more than 60% of the species or genera were not detected by standard cultures. 3) The diversity and species richness of fungal and bacterial communities was significantly lower in patients with decreased lung function and poor clinical status.
2012	Fodor *et al.*	23 adult CF patients. Samples collected before and after antibiotic treatment for exacerbation.	SES Mouthwash samples	Pyrosequencing	1) Antibiotic treatment was associated with a small decrease in species richness but minimal change in overall microbial community structure. 2) Microbial community composition was highly similar in patients during an exacerbation and when clinically stable, suggesting that exacerbations may represent intrapulmonary spread of infection

		Total of 26 matched pairs.			rather than a change in microbial community composition. 3) Mouthwash samples, obtained from a subset of patients, showed a nearly identical distribution of taxa to expectorated sputum, indicating that aspiration may contribute to colonization of the lower airways. 4) Strong correlation between low species richness and poor lung function.
2012	Goddard *et al.*	10 CF subjects undergoing lung transplantation – lung, sputum and throat samples taken 5 non-transplant subjects with FEV_1<30% predicted provided throat and sputum samples. 3 non-transplant subjects provided multiple day sputum samples while clinically stable.	Lung samples, SES and throat samples	Pyrosequencing	1) The microbiota of lung explants from patients with advanced CF was found to be almost entirely comprised of typical CF pathogens (~98%) with *Pseudomonas spp.* by far the most dominant. 2) Throat and sputum samples obtained from the same patients immediately before surgery gave different results. 3) The throat specimens were highly discordant with lung samples, containing a wide range of non-typical organisms not found in the lung explants. 4) The sputum samples identified the dominant lung pathogen. However, in ~1/2 of the cases, sputum contained diverse mixtures of non-typical organisms (comprising ~25% of microbiota) that were either not found or were at very low abundance in the lungs of subjects. 5) Sputum specimens showed day-to-day variation in the abundance of non-typical organisms in the absence of clinical changes. 6) These findings suggest that oropharyngeal contamination may confound DNA based measurements on upper airway samples.
2013	Zemanick *et al.*	21 CF subjects 37 sputum samples collected	SES samples	Pyrosequencing	1) Comparison of microbial content of sputum taken during early treatment (days 0-3) and late treatment (>7 days) of pulmonary exacerbation, with concurrent measurement of inflammatory markers.

					2) At early treatment, lower diversity was associated with high relative abundance of *Pseudomonas spp.* (r =-0.67, p<0.001), decreased FEV_1% predicted (r = 0.49, p = 0.03) and increased CRP (r =-0.58, p = 0.01). 3) Obligate and facultative anaerobes were associated with less inflammation and higher FEV_1. 4) *P. aeruginosa* abundance decreased with treatment (by qPCR), while anaerobic genera showed a variable response. 5) Change in the relative abundance of *Prevotella* was associated with more variability in FEV_1 response to treatment than changes in *Pseudomonas* or *Staphylococcus* abundance.
2013	Carmody *et al.*	28 patients 68 paired baseline/exacerbation sputa	SES	Pyrosequencing	1) There was no significant difference in bacterial community diversity and bacterial density between baseline and exacerbation samples. 2) However, in a subset of patients considerable changes in community structures were observed. In these patients, the initial level of community diversity and dominant taxa were found to significantly predict the magnitude of community structure changes at exacerbation. 3) The diversity of *Pseudomonas* dominant communities increased at exacerbation compared with communities with other or no dominant species. 4) The relative abundance of *Gemella* increased in 24 (83%) of the 29 exacerbation samples and this was the genus found to have the best discriminatory value between baseline and exacerbation samples.

Appendix J: Microbiota in COPD Literature Review

YEAR	NAME	NO OF PTS	SAMPLING TYPE	SEQUENCING METHOD	DESCRIPTION/RESULTS
2011	Erb-Downward *et al.*	3 'healthy controls' 7 'healthy smokers' 4 COPD 6 explanted lungs from severe COPD	BAL Dissected lung explants	Pyrosequencing	1) Subjects had distinct pulmonary microbiome – significantly different from oral cavity/nasopharynx 2) Propose "core" lung microbiome including *Pseudomonas*, *Streptococcus*, *Prevotella*, *Fusobacterium*, *Haemophilus*, *Veillonella*, and *Porphyromonas* species 3) No significant quantitative differences in bacterial numbers between groups 4) Diversity of microbiome lower in moderate/severe COPD than other groups and most commonly dominated by *Pseudomonas spp.* 5) Demonstrated significant heterogeneity in bacterial community between microanatomic sites in severe COPD lung
2010	Huang *et al.*	8 mechanically ventilated COPD patients with 'COPD exacerbation'	Endotracheal aspirates	Phylochip	Suggested 'core' pulmonary bacterial community of 75 taxa detected in all patients including pathogenic species
2012	Sze *et al.*	8 'healthy' non-smokers 8 'healthy' smokers 8 severe COPD (GOLD 4) 8 CF	Lung tissue sections	T-RFLP Pyrosequencing	1) Lower bacterial densities from lung tissue samples than BAL/PBB samples 2) No difference in total bacterial number or diversity between non-smokers, smokers and COPD 3) CF lung much higher bacterial density and lower diversity 4) COPD –increased abundance of *Firmicutes* phylum 5) T-RFLP/sequencing demonstrated 3 distinct bacterial community compositions: Non-smoker/smoker, COPD and CF
2012	Pragman *et*	14 Moderate	BAL	Pyrosequencing	1) Main phyla in all samples were *Actinobacteria*, *Firmicutes*, and *Proteobacteria*

	al.	COPD 8 Severe COPD 4 smokers 6 non-smokers			2) Moderate and severe COPD samples showed significantly higher diversity than control samples but not significantly different to each other (when corrected for age) 3) A few COPD patients by contrast had very low diversity scores 4) Patients using ICS or inhaled bronchodilators found to have consistent differences in microbiota composition compared to those who did not using 'principal co-ordinate analysis'
2012	Cabrera-Rubio *et al.*	6 Moderate COPD (3 surgically treated for lung ca and 1 for breast Ca)	Sputum, bronchial aspirate, BAL and bronchial mucosa from each patient	Pyrosequencing	1) Upper respiratory samples, sputum and bronchial aspirate, showed low diversity and the frequent recovery of phyla that are part of the oropharyngeal flora of the healthy subject, such as *Firmicutes* and *Bacteroidetes* 2) Lower bronchial tree samples (BAL and bronchial biopsy specimens) showed a more diverse microbiome with a close community profile in both samples, a minor representation of oropharyngeal flora, and the recovery of genera that included potentially pathogenic micro-organisms
2013	Zakharkina *et al.*	9 Severe COPD (GOLD 3-4) 9 'Healthy' controls	BAL	T-RFLP Sanger sequencing	1) Suggested 'core' microbiome in the lower respiratory tract comprising of *Prevotella*, *Sphingomonas*, *Pseudomonas*, *Acinetobacter*, *Fusobacterium*, *Megasphaera*, *Veillonella*, *Staphylococcus*, and *Streptococcus* species 2) No difference in diversity between COPD/'healthy' subjects 3) Two COPD patients were identified with significantly lower diversity

Appendix K: Methodology/Sample Collection of Lung Microbiota Literature Review

YEAR	NAME	NO OF PTS	SAMPLING TYPE	SEQUENCING METHOD	DESCRIPTION/RESULTS
2006	Rogers *et al.*	19 adult CF patients	SES Oral wash	T-RFLP profiling	1) Comparison of T-RFLP profiles of sputum and oral wash to determine degree of any possible contamination. 2) T-RFLP profiles significantly different in paired SES and oral wash samples. 3) Suggests that sputum expectorated from the lungs of CF patients is not contaminated to a significant degree by bacteria present in the oral cavity.
2010	Rogers *et al.*	10 adult CF patients. SES and induced sputum (IS) samples collected on days 1,3 and 7	SES IS	T-RFLP profiling	1) Found no significant difference in the bacterial composition of SES and IS samples, regardless of the period for which induction was performed. 2) Showed that analysis of multiple samples is required in order to obtain a comprehensive view of the bacteria present in the lower CF airways. 3) Only after analysis of multiple (≥5) samples did the number of new species detected from each further sample decrease. 4) Estimate that one SES sample only contains about 60% of all of the species identified in total from 5 samples.
2011	Charlson *et al.*	6 healthy subjects	Oral wash Oropharyngeal swabs Nasopharyngeal swabs Serial BAL Lower airway protected brush	Pyrosequencing 16S qPCR	1) Compared samples taken from the URT (oral wash and oropharyngeal swabs) with those taken from the lung (BAL and protected airway brushings). 2) Found no significant difference between bacterial communities in lung and upper airway – but bacteria in lung much less abundant (biomass 2 to 4 logs lower). 3) Some lung specific sequences isolated but these were rare. 4) Also noted low level contamination (with 'environmental'

					organisms) of lavage saline and pre-bronchoscopy channel specimens. 5) Nasopharyngeal samples showed distinctly different bacterial community, with the detection of many species associated with the skin microbiota, including *Staphylococcaceae* and *Propionbibacteriaceae*. NP samples also contained some organisms usually found in the oral cavity e.g. *Streptococcaceae* and *Prevotellaceae*.
2012	Charlson *et al.*	6 subjects: 3 lung transplant recipients 1 subject with sarcoidosis 1 subject with adenocarcinoma 1 subject with bronchiolitis obliterans organizing pneumonia (BOOP)	Matched oral wash and BAL samples	Pyrosequencing	1) Compared BAL and oral wash samples in 'healthy subjects'. 2) Found no significant difference in bacterial communities in lung and oral cavity for 3/6 subjects. 3) BAL samples from the other 3/6 subjects showed a number of sequences that were significantly more abundant in BAL compared to OW, suggesting that contamination of a bronchoscope with upper airway bacteria or repeated micro-aspirations may not fully explain the detection of bacterial communities in the lung. 4) Concluded that oral wash appears to be a reasonable sampling method (in conjunction with bronchoscopic sampling) to use to exclude URT contamination of lower airway samples obtained by bronchoscopy.

APPENDIX L:

Participant Information Sheet
Version 2.0

Title of Study: **Microbiota in Asthma**

Name of Researcher(s): Dr Tim Harrison, Dr Matthew Martin, Dr Dominick Shaw

We would like to invite you to take part in our research study. Before you decide we need you to understand why the research is being done and what it will involve. One of our team will go through the information sheet with you and answer any questions you have. Talk to others about the study if you wish. Ask us if there is anything that is not clear.

What is the purpose of the study?

Previous research has shown that there are differences in the communities of bacteria found in the airways of asthmatic patients compared to those found in the airways of healthy people.

It is not yet clear if these bacterial communities are similar in all patients with asthma or if they are different in people with more severe asthma, or those taking different treatment for their asthma.

This is important to know as any differences in the bacteria present between groups may help to explain why people with asthma do not all have the same symptoms or severity of disease.

This research aims to determine if there are any differences in the number and type of bacteria found in the airways of asthmatic patients (1) with different severities of asthma and (2) who use different types of inhaled steroid treatment for asthma. We will do this by detecting the DNA of bacteria present in phlegm (sputum) samples, as well as taking routine measurements of different features of asthma (explained further below) to see if the bacteria are different in people with different types of disease.

As it is not yet known if the bacteria in the airways change over time, we will be taking more than one sample from some patients to see if the bacteria change over time

Why have I been invited?

You are being invited to take part because you have asthma, are taking an inhaled steroid. In order to take part you must not have had any chest infections or antibiotics within the last month. We are inviting 50 participants like you to take part.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This would not affect your clinical care or legal rights.

What will happen to me if I take part?

Investigations

Participants in this study will have a series of investigations to measure certain features of asthma. These include:

- **Sputum microbiology and differential cell counts:** This procedure will involve giving you salty water to breathe in to help loosen any mucus in your lungs so you can cough it up. We will try and detect the DNA of any bacteria that may be present in the sputum. We will also look for any inflammatory cells that may be present due to your asthma. If possible we will take a further sputum sample with your agreement for storage and future analysis by a research team. This future analysis may involve looking for other inflammatory cells, chemicals or for further analysis of bacterial DNA or proteins.
- **Spirometry test:** This is a simple blowing test that is used to measure your lung function (the size of your lungs and how quickly you can empty them). You will be asked to breathe in and then blow out very fast into a mouthpiece.
- **Bronchial challenge:** This test is performed to measure how irritable your airways are. You will be asked to breathe in small

quantities of a drug called Methacholine which may cause slight narrowing of your airways. After each dose we will measure your lung function as described previously and we will stop when your blowing test falls by 20% or earlier if you feel unwell.

- **Exhaled nitric oxide measurement:** Measuring the amount of exhaled nitric oxide in your breath to measure the amount of inflammation in the breathing tubes of your lungs.
- **Leicester Cough Questionnaire:** Completing a questionnaire assessing how your cough affects your life.
- **Asthma Control Questionnaire:** Completing a questionnaire assessing how your asthma affects your life

All of these investigations will take place in our research clinic at the Nottingham City Hospital. Further information on these investigations can be found on our website: (http://www.nrru.org/patients.html). If you do not have access to the internet, printed information leaflets describing these tests in further detail are available upon contacting the Nottingham Respiratory Research Unit on the telephone number given below.

Timing of study

Overall, participation in this study will usually require 1 visit only to the Nottingham City Hospital, although we will ask some patients to come back for 3 visits over a period of 2 weeks, which is summarised here:

PART 1 OF STUDY

Telephone screening/Clinic appointment visit

You will be identified as a potential candidate for the study in a respiratory clinic appointment by one of the study team or from the Respiratory Research Unit Database. You will be asked if you would like to take part in the study and any questions you may have will be answered. You will be asked to give written informed consent in order to take part in the study.

Duration: 10-15 minutes

Hospital visit 1

Face to face visit with our research team in the hospital. This visit will take place in the Nottingham City Respiratory Research Unit. You will be seen by a doctor who will ask some questions relating to your condition to make sure you are eligible for the study. If so, we will perform a set of breathing tests (spirometry) and ask you to complete questionnaires assessing how asthma affects your life. We will then measure an exhaled gas (nitric oxide) in your breath (explained above) and perform a test to determine
how irritable your airways are (methacholine challenge) which is also explained above.

Sputum samples will then be taken for analysis including extra samples for storage and future analysis with your permission. In order to obtain these sputum samples you will be given salty water to breathe in, which will help to loosen any mucus in your lungs so you can cough it up (induced sputum).

Duration: 2 hours

At this point we may ask you to attend an optional further 2 appointments described below, which will be your decision.

Hospital visit 2 (within 24 hours of first appointment)

Face to face visit with our research team at the Nottingham City Respiratory Research Unit. This will take place the day after Visit 1 and will consist of taking 1 further induced sputum sample only

Duration: 15-20 minutes

Hospital visit 3 (2 weeks after visit 1)

Face to face visit with our research team at the Nottingham City Respiratory Research Unit. This will take place 2 weeks after Visit 1 and will consist of taking 1 further induced sputum sample only.

Duration: 15-20 minutes

What are the possible disadvantages and risks of taking part?

Investigations

Exhaled Nitric Oxide: Having the levels of nitric oxide in your breath measured does not pose any risks and is well tolerated.

Sputum Induction: Occasionally, the inhalation of salt solution in order to produce a sputum sample can make you wheezy. However, we will give you salbutamol (Ventolin) before the test and monitor you closely throughout, giving more salbutamol if necessary during the procedure.

Bronchial Challenge: This is a simple and safe test widely used in the assessment of asthma. You may experience chest tightness, wheeze or a cough during the course of the test, but these symptoms are usually mild and are quickly reversed by using a salbutamol inhaler (Ventolin).

What are the possible benefits of taking part?

It is hoped that the results of this study will help us to understand any possible role that the bacteria in the airways have in causing the symptoms of asthma. This may help us to develop better treatment strategies for asthma in the future.

What happens when the research study stops?

You will be referred back to your usual respiratory physician or GP and have further follow up if necessary in the normal respiratory clinic. If any of your phlegm samples show evidence of infection, we will contact your GP with advice regarding the need for antibiotic treatment.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. The researchers contact details are given at the end of this information sheet. If you remain unhappy and wish to discuss the matter further, you can do this by getting in touch with the Nottingham Hospitals Patient Advice and Liaison Service (PALS) in person, by telephone or e-mail who

can try and resolve the situation. Details are provided below. If you still wish to make a formal complaint you can do this through the NHS formal complaints procedure (further details of which can be provided by the PALS service).

Nottingham City Campus PALS service
By person: PALS is on the South Corridor at Junction S6. Opening times 9:30 – 4:30 pm Monday - Friday
Tel: 0800 052 1195 (free from a landline) or 0115 969 1169 ext 59671
E-mail: pals@nuh.nhs.uk
Post: NUH NHS Trust, c/o PALS, Freepost, NEA 14614, Nottingham NG7 1BR

Will my taking part in the study be kept confidential?

We will follow ethical and legal practice and all information about you will be handled in confidence.

If you join the study, some parts of your medical records and the data collected for the study will be looked at by authorised persons from the University of Nottingham who are organising the research. They may also be looked at by authorised people to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty.

All information which is collected about you during the course of the research will be kept **strictly confidential**, stored in a secure and locked office, and on a password protected database. Any information about you which leaves the hospital will have your name and address removed (anonymised) and a unique code will be used so that you cannot be recognised from it.

Your personal data (address, telephone number) will be kept for 12 months after the end of the study so that we are able to contact you about the findings of the study *and possible follow-up studies* (unless you advise us that you do not wish to be contacted). All other data (research data) will be kept securely for 7 years. After this time your data will be disposed of securely. During this time all precautions will be taken by all those involved to maintain your confidentiality, only members of the research team will have access to your personal data.

What will happen if I don't want to carry on with the study?

Your participation is voluntary and you are free to withdraw at any time, without giving any reason, and without your legal rights being affected. If you withdraw then the information collected so far cannot be erased and this information may still be used in the project analysis.

Involvement of the General Practitioner/Family doctor (GP)

We will write to your GP about your involvement in this study and if your phlegm samples show evidence of infection, we will contact your GP with advice regarding the need for antibiotic treatment.

What will happen to any samples I give?

The sputum samples will be sent to a research team at King's College in London for processing and will then be sent on to another facility abroad for detection of any bacterial DNA. All of these samples will have your name and address removed (anonymised) and a unique code will be used so that you cannot be recognised from it.

We would also like to seek your consent so that any remaining samples may be stored and used in possible future research – this is optional (please indicate you agree to this on the consent form). The samples will be stored with a code unique to you and securely at the University of Nottingham under the University's Human Tissue Research Licence (no 12265).

Some of these future studies may be carried out by researchers other than current team of Dr Harrison including researchers working for commercial companies. Any samples or data used will be anonymised, and you will not be identified in any way. If you do not agree to this any remaining samples will be disposed of in accordance with the Human Tissue Authority's codes of practice.

Will any genetic tests be done?

No tests will be performed on any of your samples to determine any of your genetic information. Tests will be performed to detect the DNA of any micro-organisms (including bacteria) that may be present in the samples.

What will happen to the results of the research study

We will publish the results of the trial in a respiratory medicine journal, present the results at various scientific conferences, and this work will form part of a thesis for a higher degree. You will not be identified in any report/publication. We will send you a newsletter with a summary of the results.

Who is organising and funding the research?

This research is being organised by the University of Nottingham and is being funded by Astra Zeneca (UK)

Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by East Midlands (Derby) Research Ethics Committee.

Further information and contact details

Further information can be obtained from our website:
http://www.nrru.org/

Or by telephoning us on:

Matthew Martin
(Study Doctor)
Tel: 0115 8231935

Denise Barber	OR	Tina Wilkinson
(NRRU Secretary)		(CTU Receptionist)
Tel: 0115 86231317		**Tel: 0115 8404844**
Fax: 0115 8231946		**Fax:0115 84026217**

Alternatively, you can write to us at the following address:

Nottingham Respiratory Research Unit
Room B28
Clinical Sciences Building
Nottingham City Hospital
Hucknall Road
Nottingham
NG5 1PB

APPENDIX M: Bacterial loads of individual MIA subjects (CFU/mL of sputum equiv.)

NO.	PATIENT	BTS STEP 2/4	STEROID	VISIT	TOTAL BACTERIA	*H. influenzae*	*S. pneumoniae*
1	MIA001	2	BEC	1	8.95E+06	2.12E+01	0.00E+00
2	MIA001	2	BEC	2	9.94E+05	1.00E+01	0.00E+00
3	MIA001	2	BEC	3	2.80E+05	0.00E+00	0.00E+00
4	MIA002	4	FLUTIC	1	7.65E+06	1.62E+04	0.00E+00
5	MIA003	2	BUD	1	8.47E+06	3.29E+03	0.00E+00
6	MIA005	2	BEC	1	3.47E+08	2.26E+02	0.00E+00
7	MIA005	2	BEC	2	6.68E+05	0.00E+00	0.00E+00
8	MIA005	2	BEC	3	1.25E+07	4.37E+01	0.00E+00
9	MIA007	2	BEC	1	5.61E+08	1.31E+05	0.00E+00
10	MIA009	2	BEC	1	5.18E+07	0.00E+00	0.00E+00
11	MIA009	2	BEC	2	1.80E+08	0.00E+00	0.00E+00
12	MIA009	2	BEC	3	1.70E+08	0.00E+00	0.00E+00
13	MIA010	2	FLUTIC	1	4.69E+07	4.02E+03	0.00E+00
14	MIA013	4	FLUTIC	1	5.10E+07	1.12E+01	0.00E+00
15	MIA014	4	FLUTIC	1	8.86E+06	0.00E+00	0.00E+00
16	MIA015	4	BUD	1	1.28E+07	1.19E+02	0.00E+00
17	MIA017	4	FLUTIC	1	8.68E+06	0.00E+00	0.00E+00
18	MIA019	4	BUD	1	3.24E+06	0.00E+00	0.00E+00
19	MIA020	4	BUD	1	1.45E+07	0.00E+00	0.00E+00
20	MIA020	4	BUD	2	3.16E+06	0.00E+00	0.00E+00
21	MIA020	4	BUD	3	1.08E+07	3.25E+01	0.00E+00
22	MIA022	4	BUD	1	1.93E+06	0.00E+00	0.00E+00
23	MIA022	4	BUD	2	7.88E+05	3.75E+01	0.00E+00
24	MIA022	4	BUD	3	1.56E+07	2.16E+01	0.00E+00
25	MIA024	2	BUD	1	2.98E+05	1.08E+02	0.00E+00
26	MIA025	4	FLUTIC	1	1.09E+08	0.00E+00	0.00E+00
27	MIA025	4	FLUTIC	2	2.60E+06	0.00E+00	0.00E+00
28	MIA025	4	FLUTIC	3	1.32E+08	0.00E+00	0.00E+00
29	MIA026	2	BUD	1	1.06E+08	6.47E+00	0.00E+00

30	MIA027	2	BEC	1	2.62E+07	2.96E+02	0.00E+00
31	MIA029	2	BUD	1	1.53E+07	5.73E+00	0.00E+00
32	MIA029	2	BUD	2	6.49E+07	1.98E+01	3.80E+01
33	MIA029	2	BUD	3	3.01E+07	2.99E+02	7.26E+01
34	MIA031	4	FLUTIC	1	1.01E+08	0.00E+00	8.14E+01
35	MIA031	4	FLUTIC	2	2.77E+07	0.00E+00	0.00E+00
36	MIA031	4	FLUTIC	3	1.79E+07	0.00E+00	0.00E+00
37	MIA032	4	BUD	1	4.76E+07	0.00E+00	0.00E+00
38	MIA034	4	BUD	1	9.23E+07	2.02E+02	0.00E+00
39	MIA035	2	BEC	1	6.01E+08	0.00E+00	0.00E+00
40	MIA035	2	BEC	2	7.29E+07	0.00E+00	0.00E+00
41	MIA035	2	BEC	3	9.40E+05	0.00E+00	0.00E+00
42	MIA036	2	BEC	1	3.47E+06	0.00E+00	0.00E+00
43	MIA036	2	BEC	2	2.19E+06	0.00E+00	0.00E+00
44	MIA036	2	BEC	3	1.63E+09	3.01E+02	0.00E+00
45	MIA037	2	BUD	1	1.48E+07	1.59E+01	2.86E+02
46	MIA038	2	FLUTIC	1	7.62E+08	0.00E+00	0.00E+00
47	MIA039	2	FLUTIC	1	7.17E+06	0.00E+00	0.00E+00
48	MIA040	2	BUD	1	9.97E+06	3.19E+02	3.88E+01
49	MIA041	4	FLUTIC	1	8.23E+06	3.94E+07	0.00E+00
50	MIA042	2	BEC	1	1.21E+07	0.00E+00	0.00E+00
51	MIA042	2	BEC	2	4.37E+06	0.00E+00	0.00E+00
52	MIA042	2	BEC	3	4.38E+06	5.90E+01	0.00E+00
53	MIA043	4	BUD	1	1.07E+07	0.00E+00	0.00E+00
54	MIA044	4	FLUTIC	1	2.39E+06	3.50E+00	0.00E+00
55	MIA044	4	FLUTIC	2	5.35E+06	0.00E+00	0.00E+00
56	MIA044	4	FLUTIC	3	8.17E+05	0.00E+00	0.00E+00
57	MIA045	2	BUD	1	1.43E+08	0.00E+00	0.00E+00
58	MIA045	2	BUD	2	8.30E+08	0.00E+00	0.00E+00
59	MIA045	2	BUD	3	6.51E+08	3.77E+00	0.00E+00
60	MIA046	4	BUD	1	3.29E+07	0.00E+00	0.00E+00
61	MIA049	4	BUD	1	3.76E+06	1.10E+02	4.24E+00
62	MIA049	4	BUD	2	3.34E+06	4.66E+01	0.00E+00

63	MIA049	4	BUD	3	4.05E+06	1.99E+02	0.00E+00
64	MIA050	4	BUD	1	1.08E+07	0.00E+00	0.00E+00
65	MIA051	4	FLUTIC	1	1.52E+06	0.00E+00	0.00E+00
66	MIA052	4	BUD	1	1.50E+08	2.30E+04	0.00E+00
67	MIA053	4	FLUTIC	1	7.75E+08	0.00E+00	5.14E+01
68	MIA053	4	FLUTIC	2	6.18E+06	0.00E+00	0.00E+00
69	MIA053	4	FLUTIC	3	1.65E+07	4.68E+01	0.00E+00
70	MIA054	4	FLUTIC	1	2.34E+06	1.62E+02	0.00E+00
71	MIA054	4	FLUTIC	2	1.79E+06	4.67E+01	4.14E+03
72	MIA054	4	FLUTIC	3	1.23E+06	1.15E+01	8.75E+01
73	MIA055	2	FLUTIC	1	3.55E+05	0.00E+00	1.33E+03
74	MIA056	2	FLUTIC	1	4.00E+06	0.00E+00	0.00E+00
75	MIA057	4	FLUTIC	1	1.99E+06	1.59E+05	0.00E+00
76	MIA057	4	FLUTIC	2	1.89E+07	2.48E+03	0.00E+00
77	MIA057	4	FLUTIC	3	1.92E+07	1.88E+04	0.00E+00
78	MIA058	4	FLUTIC	1	4.37E+07	5.81E+01	6.40E+00
79	MIA058	4	FLUTIC	2	2.36E+07	6.63E+00	0.00E+00
80	MIA058	4	FLUTIC	3	3.66E+07	5.86E+01	0.00E+00
81	MIA059	2	BEC	1	9.10E+06	4.10E+02	2.28E+02
82	MIA061	4	FLUTIC	1	8.98E+06	3.25E+06	0.00E+00
83	MIA062	4	FLUTIC	1	5.66E+06	3.92E+04	5.27E+00
84	MIA063	4	FLUTIC	1	7.88E+06	0.00E+00	0.00E+00
85	MIA064	4	FLUTIC	1	5.86E+06	5.77E+00	0.00E+00
86	MIA065	4	BUD	1	4.85E+06	1.24E+01	0.00E+00
87	MIA066	4	BUD	1	2.99E+07	0.00E+00	0.00E+00
88	MIA067	4	BUD	1	7.69E+06	1.07E+04	0.00E+00
89	MIA067	4	BUD	2	9.44E+06	2.96E+03	0.00E+00
90	MIA067	4	BUD	3	1.32E+07	3.83E+02	0.00E+00
91	MIA068	4	BUD	1	3.78E+06	2.52E+05	8.53E+01
92	MIA069	4	BUD	1	9.63E+06	3.59E+02	2.45E+01
93	MIA070	2	BEC	1	7.31E+05	0.00E+00	0.00E+00