Reproducibility of the Respiratory Microbiome in Asthmatic Patients

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Reproducibility of the Respiratory Microbiome in Asthmatic Patients

Thesis

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Candidate Thesis Declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree MD, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work.

Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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Glossary of abbreviations

APC	Antigen presenting cell
AD	Atopic dermatitis
BAL	Bronchoalveolar lavage
BHR	Bronchial hyperresponsiveness/hyperreactivity
BMI	Body mass index
COPD	Chronic obstructive pulmonary disease
C_q	Quantification cycle
CR	Corticosteroid resistant
CS	Corticosteroid sensitive
C_t	Cycle threshold
DLCO	Diffusion capacity for carbon monoxide
$ETCO_2$	End tidal carbon dioxide
EIA	Exercise induced asthma
EIB	Exercise induced bronchoconstriction
EILO	Exercise induced laryngeal obstruction
FEV_1	Forced expiratory volume in 1 second
FENO	Fraction of exhaled nitric oxide
FVC	Forced vital capacity
FRC	Functional residual capacity
ICS	Inhaled corticosteroid
IgE	Immunoglobulin E
INF-γ	Interferon gamma
LABA	Long acting beta agonist
LAMA	Long acting muscarinic antagonist
LPS	Lipopolysaccharide
LTRA	Leukotriene receptor antagonist
NGS	Next generation sequencing
NO	Nitric oxide
OTUs	Operational taxonomic units
PCR	Polymerase chain reaction

PEF	Peak expiratory flow
qPCR	Quantitative or Real-Time polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SABA	Short acting beta agonist
SCFA	Short chain fatty acid
SPT	Skin prick test
T_H	T-helper cell
T_m	Melting temperature
Treg	T regulator cell
VCD	Vocal cord dysfunction

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Summary of presentations and publications

Poster Presentations:

ERS Madrid 2019

1. Antibiotics and the microbiome in asthma
2. The validity of physician-diagnosed asthma

ITS Galway 2019

1. How certain is asthma diagnosis in the absence of spirometry

ATS Philadelphia 2020

1. Airway physiology and the bacterial burden of the lung, oropharynx, skin, and gastrointestinal tract remain stable after exposure to antibiotics in asthmatic patients

Oral Presentations:

ITS Galway 2019

1. Antibiotics and the microbiome in asthma

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1. RCSI travel bursary to support travel to the research lab to complete lab secondment, 2017
2. ITS/GSK ERS bursary to support travel to, and participate in the European Respiratory Society Congress 2019, Madrid

Summary

Contrary to classical teaching, the lungs are not sterile. In fact they play host to thousands of diverse microbes including bacteria, viruses, fungi, and protozoa. The collective term for the genetic material of these microbial components is the microbiome. Since the discovery of the respiratory microbiome some ten years ago, there has been much interest in the study of this topic across a range of respiratory conditions. It has been shown that the respiratory microbiome is significantly altered in asthmatics, though the functional impact of these alterations remains unknown. Alterations in the microbiome of the skin and gut have been implicated in the development of asthma. Studies involving concurrent assessment of all these microbial compartments are rare and it is not known how these communities interact with each other. It is also unknown whether the microbiome interacts with the intrinsic differences in airway physiology which define asthma. Furthermore the response of the asthmatic microbiome to treatments such as antibiotics is largely unknown. We aimed to assess whether airway physiology and the bacterial density of the oropharynx, lung, skin and gut change significantly after an asthmatic patient is exposed to an antibiotic.

Patients were included if they demonstrated either reversible airflow limitation or clinically significant bronchial hyperresponsiveness combined with clinically relevant symptoms of asthma. Skin swabs, throat swabs, faecal samples, and bronchoscopy lung brush samples were obtained in thirteen asthmatic patients. Subjects were asked to take oral levofloxacin for 10 days. Repeated skin swabs, throat swabs, and faecal samples were collected at regular intervals until airway physiology measurement and bronchoscopy were repeated 8 weeks later. Biological samples underwent qPCR assessment of bacterial burden. Data were analysed using a combination of paired T-test and Friedman testing.

There were no significant differences in airway physiology measures including fraction of exhaled nitric oxide, end tidal carbon dioxide, and skin prick allergy testing, post exposure to antibiotic. Bacterial counts in faecal samples (n=37), skin

swabs (n=52), throat swabs (n=49) and lung brushes (n=88) did not change significantly over the study period. When patients were stratified by eosinophil count (elevated >$0.4x10^9$) there were no significant changes in bacterial count, p=0.24. When patients were stratified by disease severity (according to GINA guidelines) there were similarly no significant changes in bacterial count, p=0.53. Streptococcus-specific qPCR was assessed for faecal samples and there were no significant changes in bacterial counts at the various time points, p=0.37.

Airway physiology and the bacterial density of faeces, skin, throat, and lung samples do not change meaningfully after an asthmatic patient is exposed to an antibiotic. Sequence data will be key to further assess for community compositional changes in response to antibiotics.

Acknowledgements

This thesis has been the result of a collaborative effort between Connolly Hospital, Blanchardstown, and Imperial College London and would not have been possible without the help and guidance of a number of key individuals.

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Finally I would like to express my sincere gratitude to the patients who gave so much of their time to participate in this study. This was an intensive study protocol to undergo and we thank them for their selfless and enthusiastic participation.

Dedication

I dedicate this thesis to my family. I am constantly grateful for their unending support, guidance, and patience without which I would not be where I am today. I cannot express the gratitude I feel towards them all.

I would also like to dedicate this thesis to my boyfriend Ronan who has put up with the inevitable frustrations that come with writing a body of work such as this. He has lent a supportive shoulder and an attentive ear any time it was needed and this support has been invaluable.

Chapter 1: Introduction

1.1.1. Asthma Background

The word asthma comes from the Greek verb aazein meaning to exhale with the mouth open or to pant (1). This word has been applied to an increasingly common condition and asthma is now widely known as a disease of variable, reversible airflow obstruction (2). The prevalence of asthma is estimated to be as high as 20% in some countries and seems to be increasing over time (3). It is most commonly diagnosed in paediatric populations and from childhood tends to take one of three possible clinical courses (4). These courses, in decreasing order of frequency, are resolution in teenage years with no recurrence, resolution with recurrence in later life, or persistence. It can also occur de-novo later in life when it tends to be associated with the aspirin triad (4). Asthma, though most commonly mild in its clinical course, has the potential to be a devastating disease with a minority of patients requiring invasive ventilation and intensive care unit admissions. It is estimated that one patient per week in Ireland dies of complications directly attributable to their asthma (5). Asthma poses a significant burden on healthcare systems with the overall cost to the NHS estimated at £1.1 billion in 2010, the majority of which was spent in the primary care setting (6).

Asthma is a disease characterised by symptoms of cough, shortness of breath, and wheeze, which occur intermittently and cause varying levels of functional impairment for patients. The pathophysiology of asthma is complex and involves significant dysregulation of multiple immunological and physiological mechanisms. The hallmark of the disease is reversible airflow obstruction caused by bronchial hyperresponsiveness, bronchospasm, and airway inflammation with mucus production, often in response to certain triggers such as upper respiratory tract infections and inhaled respiratory allergens (7).

The immune system is intrinsically involved in the pathogenesis of asthma, particularly with regard to allergic asthma. Antigen presenting cells (APCs) present inhaled allergens to T-helper cells (T_H) which activate the humoural immune system via cascades of interleukins (IL). Two subclasses of T_H cells are implicated in airway

inflammation, T_H1 and T_H2 (8). T_H1 cells produce mainly IL-2 and interferon gamma and are crucial in orchestrating many cellular defence mechanisms against infection, often mediated by neutrophilic inflammation (8). T_H2 cells mediate allergic inflammation via generation of a host of cytokines including IL-4, IL-5, IL-9, and IL-13 which contribute to Immunoglobulin E (IgE) production, mast cell degranulation, eosinophil recruitment, and release of inflammatory mediators such as histamine and leukotrienes (9). An imbalance between T_H1 and T_H2 pathways has been implicated in the pathophysiology of the disease with the disease further dichotomised according to whether T_H2 levels are high or low (10).

This inflammatory cascade is responsible for the gross alterations in lung function and structure associated with the disease as it progresses over time (11). This process leads to a multitude of changes including acute bronchoconstriction, airway oedema, mucus formation, mucus gland hypertrophy, and eventually airway remodelling (12). It is generally held that the degree of airway inflammation correlates well with clinical asthma severity (13).

Asthma is a heterogeneous disease with both the clinical presentation and the severity of symptoms varying widely between individual patients. Attempts have been made recently to classify asthmatics into distinct phenotypic clusters according to BMI, gender, age, lung function, atopic status, and even healthcare utilization (14). For example, older patients with high BMI tend to present with asthma that is more severe (14). It remains unclear as to whether this widespread variety is due to specific underlying molecular mechanisms, environmental exposures, or additive effects of comorbid disease. This heterogeneity lends significant difficulty to both the diagnosis and treatment of asthmatic patients in the clinical setting.

1.1.2 Diagnosis of asthma

Asthma has classically been a physician-diagnosed entity based on clinical suspicion of the disease. Shortness of breath, cough, chest tightness, and wheeze tend to be the cardinal symptoms, particularly when occurring together. As mentioned, asthma is a disease of reversible airway obstruction and so, by definition, these symptoms tend to be intermittent with partial or complete resolution between episodes. Symptoms tend to be worse at night or in the early morning and can often be triggered by a range of stressors such as viral infection, allergen exposure, or inhalation of certain irritants. Clinical suspicion is heightened by presence of certain co-morbidities such as eczema, rhinitis, and sinusitis giving rise to the so-called atopic nature of these patients. It cannot be overstated that the clinical history is a key tool in the diagnosis of this condition.

Presence of the aforementioned factors make a diagnosis of asthma extremely likely, and in turn asthma diagnosis is less likely where there is a history of productive cough, dizziness, or chest pain, with little resolution of symptoms between exacerbations. In practice, there is considerable variation in asthma symptoms over time and this leads to uncertain diagnoses at the primary care level as often patients are very well between exacerbations. In fact, a recent JAMA-published article (15) found that 33.1% of patients recruited with physician diagnosed asthma did not fulfil diagnostic criteria for the disease when assessed for reversible airflow obstruction and bronchial hyperresponsiveness. This rate has been reproduced throughout the literature (16) . These findings call into question the reliability of the diagnostic process involved for our asthmatic patients and this is often a question replicated in other chronic respiratory conditions which can be similarly difficult to diagnose (17).

Perhaps the most crucial reason for this diagnostic discrepancy in asthma is lack of access to precise diagnostic tools such as spirometry. Often a diagnosis is established based on clinical suspicion followed by a trial of bronchodilator therapy, response to which is used as a surrogate marker for a reduction in bronchial

hyperresponsiveness and airflow limitation. This is unfortunately an imprecise and subjective approach which can lead to over-diagnosis. This leaves many patients with a disease label and has huge ramifications for the individual both in terms of the financial burden of long-term medications and the psychological burden of chronic disease. Irish adults are found to miss 12 days of work per year on average as a direct result of their illness (5). Asthma is also extremely relevant in the realm of competitive sports where therapeutic use exemptions for inhaled medications are proving to be so controversial at present. These factors combined have led to a renewed interest in the employment of precise physiological methods to aid the diagnosis of this complex disease, increasing both the sensitivity and specificity of our diagnoses (18).

Diagnostic criteria help greatly in the streamlining of asthma diagnosis and the two most widely cited resources are the guidelines of the British Thoracic Society (BTS) and those of the Global Initiative for Asthma (GINA). In both resources huge emphasis is placed on the clinical recognition of the constellation of symptoms as mentioned above which alert suspicion to the diagnosis. After careful history and examination, focus then shifts to documenting variable expiratory airflow limitation. Specific criteria for documenting this limitation are outlined in table 1.1. These form the diagnostic criteria favoured by the GINA guidelines (19).

Table 1.1: Criteria suggested for the diagnosis of asthma, adapted from the Global Initiative for Asthma (GINA) guidelines (20).

Confirmed variable expiratory airflow limitation	
Diagnostic Feature	**Criteria for making the diagnosis of asthma**
Documented excessive variability in lung function (one or more of the tests below) AND documented airflow limitation	The greater the variations, or the more occasions excess variation is seen, the more confident the diagnosis At least once during diagnostic process when FEV_1 is low, confirm that FEV_1/FVC is reduced (normally >0.75–0.80 in adults, >0.90 in children)
Positive bronchodilator (BD) reversibility test (more likely to be positive if BD medication is withheld before test: SABA ≥4 hours, LABA ≥15 hours)	Adults: increase in FEV_1 of >12% and >200 mL from baseline, 10–15 minutes after 200–400 mcg albuterol or equivalent (greater confidence if increase is >15% and >400 mL). Children: increase in FEV_1 of >12% predicted
Excessive variability in twice-daily PEF over 2 weeks	Adults: average daily diurnal PEF variability >10% Children: average daily diurnal PEF variability >13%
Significant increase in lung function after 4 weeks of anti-inflammatory treatment	Adults: increase in FEV_1 by >12% and >200 mL (or PEF by >20%) from baseline after 4 weeks of treatment, outside respiratory infections
Positive bronchial challenge test (usually only performed in adults)	Fall in FEV_1 from baseline of ≥20% with standard doses of methacholine or histamine, or ≥15% with standardized hyperventilation, hypertonic saline or mannitol challenge

Diagnostic Feature	Criteria for making the diagnosis of asthma
Positive exercise challenge test	Adults: fall in FEV_1 of >10% and >200 mL from baseline Children: fall in FEV_1 of >12% predicted, or PEF >15%
Excessive variation in lung function between visits (less reliable)	Adults: variation in FEV_1 of >12% and >200 mL between visits, outside of respiratory infections Children: variation in FEV_1 of >12% in FEV_1 or >15% in PEF between visits (may include respiratory infections)

There is a notable caveat in these published guidelines: if clinical suspicion is high enough, treatment should commence without delay for patients. This dramatically reduces the reliability of various markers of airflow limitation (20) and means it may never be possible to conclusively demonstrate reversible limitation for our patients. This is echoed in the BTS guideline diagnostic algorithm (21) as illustrated in figure 1.1.

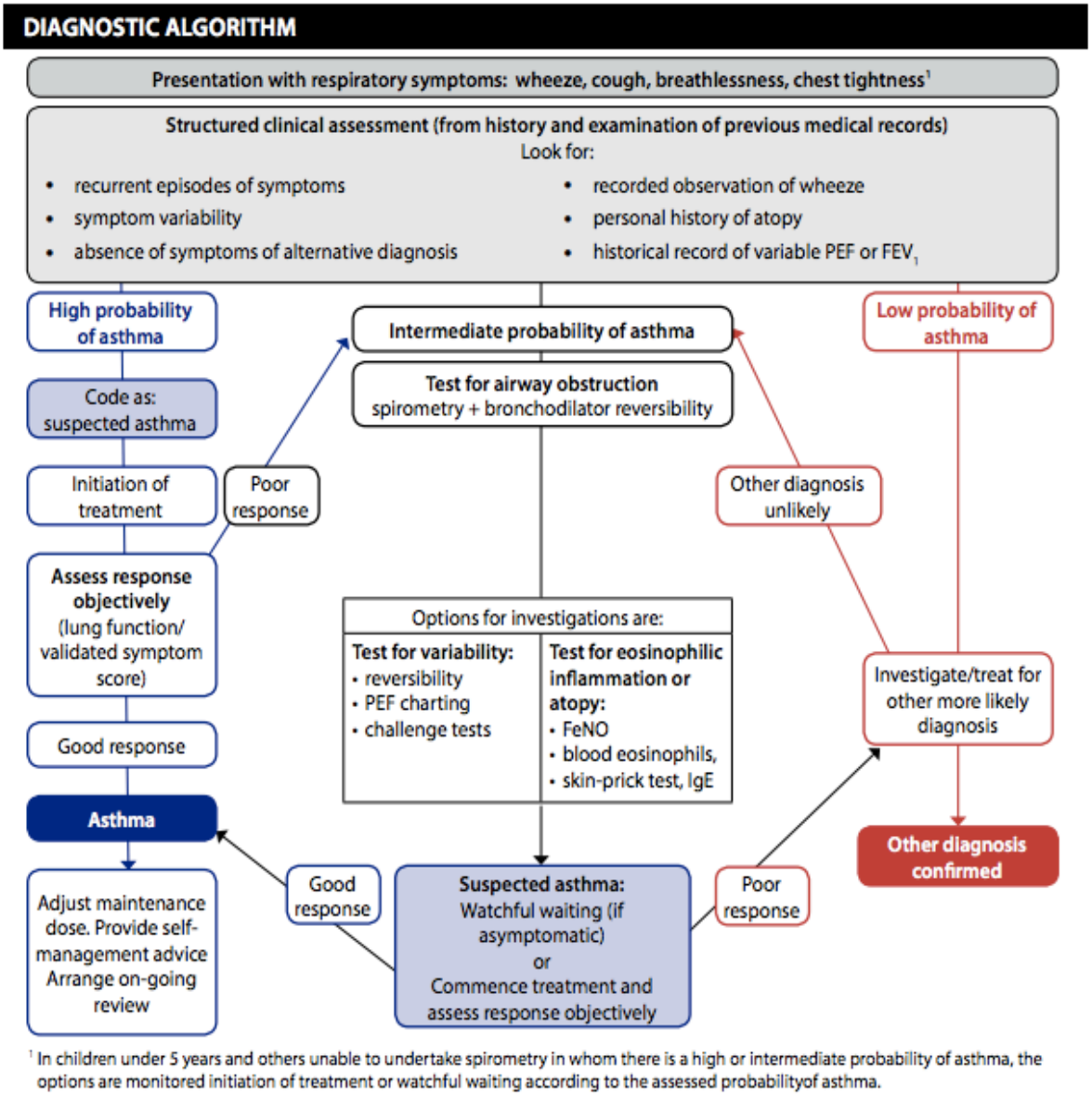


Figure 1.1: Diagnostic algorithm as set out by the British Thoracic Society. Reproduced from (22).

A wide set of differential diagnoses can lead to symptoms which mimic those of asthma. Commonly, gastro-oesophageal reflux disease (GORD) can cause micro-aspiration resulting in chronic upper airway inflammation. Interestingly, studies have shown that between 36 and 48% of asthmatic patients also have GORD and that their symptoms dramatically improve when given anti-acid treatments (22). In a similar way, chronic rhino-sinusitis can lead to post nasal drip which also results in inflamed upper airways. Eosinophilic bronchitis refers to airway inflammation in the

superficial airways as a consequence of excessive mast cell activation and mimics asthma (23). Vasculitides such as Churg-Strauss and granulomatosis with polyangiitis can contribute to the diverse range of diseases which cause breathlessness and wheeze (24). In young patients there is emerging evidence to show that exercise induced bronchoconstriction (EIB) and exercise induced laryngeal obstruction (EILO - often as a consequence of vocal cord paradoxical movement) are major causes of respiratory symptoms in athletes (25). It is clear that a spectrum of disease processes can cause asthma-type symptoms in a variety of pathological manners and it is vital to objectively diagnose asthma. Of equal import is the need to identify the causative factor in those cases where asthma is not present in order to facilitate effective treatments.

It is clear that the diagnostic process is a difficult one when it comes to asthma, largely owing to the intermittent nature of symptoms, the intermittent nature of airflow obstruction, and lack of access to high-level spirometry at the primary care level. There are many conditions which present in much the same way as asthma and this also contributes to a high level of diagnostic uncertainty in many settings. Guidelines continue to place emphasis on early treatment of symptomatic patients without waiting for confirmatory spirometric results, and response to treatment continues to be held as a diagnostic signpost for many patients. This all contributes to the well-recognised fact that asthma can tend to be over-diagnosed, incurring significant burdens on both patients and the healthcare system. There is also an under-explored potential that the validity of some work in the field of asthma may be attenuated by the uncertain nature of asthma diagnosis in certain subjects involved in clinical trials.

1.1.3 Random measurement error and regression dilution bias:

Establishing the diagnosis of asthma can prove a difficult process, especially when there is a lack of access to precise diagnostic tools such as validated spirometry. Throughout the catalogue of asthma literature, physician-diagnosed asthma has been regarded as an acceptable standard for classification of patients in case control studies and even in randomised control studies. However, recently it has been shown that when the aforementioned diagnostic criteria are strictly applied to cohorts of patients with physician-diagnosed asthma, the numbers of so-called 'true' asthmatics can be substantially lower than expected (15). What remains to be established is whether this potential diagnostic inaccuracy could affect the validity of some established relationships in the asthmatic literature.

Random measurement error is defined as the variability or deviation between a usual value and an observed value and can be a consequence of either technical error or biological variation over a given time period. It occurs when the values of a given variable fluctuate around the true values so that some will be lower and equally some will be higher than the true value (26). The effect which random measurement error has on a relationship depends on whether the error is measured in an exposure variable or an outcome variable in a given study. Measurement error in an exposure variable can lead to attenuation of a relationship, or to missing of an association altogether. It will bias the regression coefficient (the slope of the regression line which represents the rate of change of one variable as a function of changes in the other variable) towards the null. This introduces so-called regression dilution bias or regression attenuation. Measurement error in an outcome variable makes the study underpowered to detect the true effect of an exposure thereby increasing the standard error.

To apply this to our study, we consider the exposure variable as the presence of asthma and the outcome variables as measures of the respiratory microbiome, such as numbers and types of operational taxonomic units in a given sample. Over-measurement of the exposure variable, as happens if those with physician-

diagnosed asthma rather than spirometry-diagnosed asthma were to be included, would introduce regression dilution bias and attenuate the strength of the relationship between the exposure and the outcome variables. Imprecise measurement of the outcome variables will increase standard error of the observed relationship.

Both types of error can be minimised by careful study design, accurate subject selection, and precise measurement methods (26). For example, spirometry could be carried out multiple times on the same subject in order to introduce reproducibility to the measurements taken. Correct profiling of potential subjects as asthmatic or non-asthmatic will introduce diagnostic accuracy and minimise the effects of regression dilution bias. This will in turn increase the strength of relationships demonstrated between the exposure variable and multiple outcome variables.

1.1.4. Role of Pulmonary Function Testing in the diagnosis and physiological profiling of asthma

1.1.4.1. Spirometry

In practice, asthma diagnosis at the primary care level is reliant on observation of peak expiratory flow rates, variation in which leads to a clinical suspicion for the diagnosis. Peak flow variability is usually calculated based on the following formula: ([day's highest reading – day's lowest reading]/mean of day's highest and lowest readings)x100 (27). Patients are instructed to measure their 'best of three' at the same time twice per day, usually for 2 weeks consecutively and variability is calculated from this. A value of >10% for adults is considered to be excessive variation, thus confirmatory of asthma, and this is based on the knowledge that the upper 95% confidence interval limit of twice daily peak flow variability in healthy adults is 9% (28). However, the reliability of this method can vary greatly depending on individual technique and adherence to self-testing and self-reporting (29). There is evidence that newer electronic peak flow meters allow for superior reliability and patient engagement in the outpatient setting (30). These meters show promise as a method of addressing the intrinsic difficulties outlined above and there are calls to include them as part of routine patient care as technology is advancing in the field (31).

In the hospital outpatient setting spirometry, which is much more readily available, is the investigation of choice. A combination of reduced FEV_1/FVC ratio (often associated with a characteristic concave outline to the expiratory limb of the flow volume curve) and bronchodilator reversibility of >12% or 200ml clinches the diagnosis (32). Documentation of variable airflow limitation should be sought as early as possible for new patients due to the fact that as treatment improves lung function, variability tends to reduce . Additionally in patients with high levels of inflammation, the airways can become irreversible or 'fixed' over time (33). Additionally, false negative bronchodilator responses can occur for patients who have current viral infections, or in those who have taken beta-agonists prior to testing (32). Difficulty arises when there is a clinical suspicion of asthma in terms of

history and examination, but spirometry and peak flow are equivocal. Further initial investigation is best served by identifying and treating any potential contributors to symptoms such as GORD, rhino-sinusitis, allergy, and vasculitis as discussed above. In cases where clinical suspicion is high but subjects do not demonstrate bronchodilator reversibility, bronchial provocation testing is the next port of call in line with diagnostic algorithms.

1.1.4.2. Bronchial Provocation

In those patients who have a negative bronchodilator response on spirometry, assessment of bronchial hyperresponsiveness (BHR) is the next step taken in most specialist centres. This is achieved using either direct or indirect bronchial provocation testing. Direct testing is via inhalation of methacholine which acts directly on bronchial smooth muscle or inhalation of histamine which has additional neural effects on muscle (34). After baseline spirometry is assessed, patients begin tidal breathing a nebulised saline solution to which methacholine is added in incremental doses with FEV_1 assessed after each dose adjustment (35). If FEV_1 does not show a significant decrease after a dose increment, the dose is increased on the next inhalation. Sequential FEV_1 measurement is vital in order to avoid serious provocation of our patients which could lead to significant symptomatic exacerbation. This is best carried out by trained, experienced personnel (36). A positive test result for asthma involves a decrease in FEV_1 by at least 20% and must be accompanied by a clinical suspicion for asthma to make the diagnosis. The amount or dose of the stimulus required to provoke a reaction sub-classifies the severity of BHR (36). Indirect provocation testing is carried out with mannitol or hypertonic saline in a similar way to methacholine challenge and these agents induce osmotic stresses on the respiratory system which attract a multitude of cytokines to the respiratory mucosal barrier in order to provoke bronchoconstriction as a response in those with asthma (37). The magnitude of FEV_1 decrease required for a positive test result varies with the inhaled agent used.

The GINA guidelines outline that tests such as bronchial provocation challenge, both direct and indirect, may be utilised to help establish a diagnosis of asthma in

patients with clinical features of the disease but with normal lung function. There is widespread caution about its use as a stand-alone diagnostic clinical test for asthma, as it has been established that though the tests are associated with a high level of sensitivity, they can have limited specificity (38). Airflow limitation caused by other non-asthmatic conditions such as cystic fibrosis, bronchiectasis, and even COPD, can occasionally be associated with positive challenge test results indicating that a level of BHR can occur with other diseases (39). Therefore it is clear that a positive test result does not always automatically equate to a diagnosis of asthma (40). However if interpreted in the context of a significant clinical suspicion for the disease, these tests can be extremely useful. Consequently, a negative test in a patient not already using asthma controlling medications can help to conclusively rule out the disease, even in symptomatic populations (41).

1.1.4.3. Exhaled Nitric Oxide

Nitric oxide (NO) is present in the exhaled breath of all humans (42). It is an important biological mediator in many body systems and in the lungs it plays key roles in inflammation, neurotransmission, vasodilation, and bronchodilation. Its role in airway physiology is complex as it can simultaneously act as a pro-inflammatory mediator to up-regulate levels of bronchial hyperresponsiveness (43, 44), and equally act as a weak mediator of relaxation of airway smooth muscle thereby protecting against BHR (45). Eosinophilic airway inflammation is known to up-regulate levels of inducible nitric oxide synthase in bronchial epithelial cells, in turn producing higher levels of NO. Therefore an elevated fraction of exhaled nitric oxide (FENO) measured in the breath can be regarded as an indirect marker for elevated levels of eosinophilic airway inflammation (46) and correlates well with sputum eosinophil count (47).

FENO is generally not recommended for use as a diagnostic tool in asthma for a few reasons. Firstly, though FENO is certainly higher in eosinophilic asthma, it can also be elevated in other non-asthmatic conditions such as eosinophilic bronchitis, allergic rhinitis, and eczema (48). In contrast there are certain well-recognised asthma phenotypes which will not have elevated levels of FENO such as

neutrophilic asthma (49). Falsely low readings can be observed is asthmatic smokers, those with viral respiratory illness, and it is known to be lower than expected in bronchoconstriction and the early phase of an allergic response (50). An elevated FENO if >50 parts per billion (ppb) is associated with a good response to inhaled corticosteroids (ICS) in steroid naïve patients (51). There is also a case for using FENO as an indirect marker of a patient's compliance with ICS, as an initial elevated FENO would be expected to fall with adherence to ICS therapy (52). Though FENO cannot be used on its own to support a diagnosis of asthma, it can be used as a supporting test to add to the likelihood of asthma diagnosis in patients with otherwise equivocal pulmonary function tests (53).

1.1.4.4. Skin Prick Allergy Testing

A diagnosis of allergic asthma is supported by the presence of atopy and this can be identified using the simple, sensitive, and cost-effective method of skin prick testing (SPT). The test involves introduction of relevant allergens into the skin in patients with a clinical query of atopy. Specific IgE found on the surface receptors of mast cells cross-link to cause mast cell degranulation and histamine release in response to the allergens in question (54). The skin then reacts with a wheal and flare type reaction, the size of which is quantified to assess the strength of the allergic response. Therefore the degree of cutaneous reaction informs us indirectly as to the level of sensitization in target organs (54). This must be interpreted in the context of the clinical history to assess whether certain allergens are actually causing symptoms or if the patient is merely sensitized. Many other asthma phenotypes will not exhibit atopy, and in fact less than one half of asthma cases are thought to be attributable to atopy (55). Similarly there are many conditions in which atopy exists independent of asthma such as eczema, allergic rhinitis, allergic conjunctivitis, and others. Therefore this test much be applied and interpreted in the correct clinical context to be of use in the diagnosis and phenotyping of asthma.

1.1.4.5. End Tidal Carbon Dioxide

Though not included as part of the diagnostic algorithm in asthma guidelines, end tidal carbon dioxide ($ETCO_2$) measurement is a clinically useful, non-invasive test to investigate for the presence of hyperventilation in dyspnoeic patients. Hyperventilation is defined as breathing in excess of metabolic requirements (56). It leads to reduced arterial CO_2 levels, respiratory alkalosis, and symptoms including dizziness and tachycardia. Both acutely and chronically reduced CO_2 levels can lead to contraction of bronchial smooth muscle and increased airflow resistance (57). In this way, hyperventilation may cause dyspnoea and wheeze, exacerbating symptoms or even confounding the diagnosis of asthma.

There is evidence to suggest that hyperventilation syndrome can frequently coexist with asthma (58). It is certainly a common entity, though diagnosis can often be difficult and it can often involve some degree of psychological overlay. Treatment in non-asthmatics requires simple interventions including explanation of the phenomenon combined with simple breathing exercises (59). Its presence in asthmatics can exacerbate symptoms and this simple test can be employed in order to diagnose and address this comorbidity.

1.1.5. Treatment of asthma

The general primary goal of asthma management is to achieve good symptom control while simultaneously reducing exacerbation frequency and risk of future exacerbations. The secondary goals are avoidance of the long term complications of asthma such as fixed airflow limitation and minimisation of the side effects of asthma treatment. The success of asthma treatment is not only dependant on the health care provider, but also on the patient as it is shown that self-management education can reduce asthma related morbidity in adults (60, 61).

Asthma medications have traditionally been based on derivatives of adrenaline and cortisone, initially in oral preparations and now via inhaled routes with inhaler devices continually becoming more varied, potent, user-friendly, and cost-effective. Combinations of inhaled corticosteroids (ICS) and beta-adrenergic agonists (both short acting SABA and long acting LABA) are the cornerstone of treatment and doses are stepped up or down depending on clinical severity of asthma symptoms. With increasing severity other medications are considered such as leukotriene receptor antagonists (LTRA)(62), long-acting muscarinic antagonists (LAMA) such as tiotropium (63), or low-dose theophylline (64).

In cases where patients continue to have symptoms and exacerbations despite adequate inhaler technique and good compliance, specialist intervention is sought and patients may be trialled on add-on therapies. Often oral corticosteroids are required and show good effect (65), however these are associated with substantial systemic side-effects. In recent times, targeted therapies have shown excellent results and have allowed reduction in oral steroid dose and exacerbation rates for severe asthma. Omalizumab is an anti-immunoglobulin E (IgE) treatment which has shown excellent effect in those with high IgE levels (66). Similarly the anti-interleukin 5 inhibitors Mepolizumab (67) and Reslizumab (68) have shown excellent trial data in terms of patient outcomes for those with high eosinophil levels. The benefit of targeted therapies such as these is that they are extremely effective in the patients who are suitable for their use (69). The clear disadvantage

is that in patients who are inappropriate for their use they will have little beneficial effect. Given the expense of these novel agents these considerations further emphasise the need for precise phenotyping. These considerations come under the umbrella of so-called personalised medicine in that for a targeted therapy to have a useful clinical effect, the target itself, such as IL-5 for example, must be present in sufficient quantities to warrant treatment.

With the emergence of targeted therapies for the disease, treatment is moving slowly away from the cornerstones of ICS and LABA and towards a more personalised approach which is designed to target specific causative factors. Outcomes are better for patients who are categorised by their IgE or eosinophil status because targeted treatments exist for these patients. A similarly novel area for asthma research which has emerged as a potential therapeutic target in recent years is the concept of the respiratory microbiome which will now be discussed in detail.

1.1.6. Microbiome introduction

The incidence of asthma is known to be increasing worldwide. A key factor in this observation is thought to be the hygiene hypothesis, namely that reduced exposure to microbes in early life as a result of multiple lifestyle factors predisposes to the development of autoimmune and allergic diseases (70, 71). For example, there is a longstanding observation that children who are raised in traditional farming backgrounds have significantly lower rates of asthma than those raised in other environments. This observation has been reinforced and investigated in more detail recently in the New England Journal of Medicine (72) where asthma prevalence in two distinct farming groups, the Amish of Indiana and the Hutterites of South Dakota, was studied. These two groups originated from Central Europe and remain reproductively isolated since emigration to the United States in the 1700's. They share similarities in lifestyle, have minimal exposure to inhaled pollutants and tobacco smoke, and avoid indoor pets. However their farming practices are entirely opposed, Amish people practice traditional farming while Hutterite people live on large highly industrialised farms. Studies have shown that 5.2% of Amish children have asthma vs 21.3% in Hutterite schoolchildren (73, 74) and this study aimed to find a potential cause for this discrepancy. On blood analysis it was found that the function of the cells of the innate immune system differed significantly between the groups. Peripheral-blood leukocytes from the Hutterite cohort showed increased eosinophils and decreased proportions of neutrophils compared with Amish subjects. Hutterite children had significantly higher levels of circulating cytokines, independent of their asthma status. Investigators concluded that differential microbial exposures between these two groups could account for this. Findings such as these provide potential to unravel the mechanisms involved in the hygiene hypothesis. Strachan, the first to propose the hygiene hypothesis, found that hay fever was less prevalent in children who had older siblings (71) and postulated that this resulted from increased microbial exposure in early life. At the same time, T_H1 and T_H2 $CD4^+$ cells were identified and their differential cytokine profiles were outlined (75), thus providing an immunological basis for the hygiene hypothesis. T_H1 cells stimulate immune response to microbial exposure, acting to supress T_H2

cells via up-regulation of IFN-γ (76). Since the discovery of the microbiome, and in particular the respiratory microbiome, there has been an opportunity for further understanding of the complex relationship between microbial exposure and the immunological basis of asthma.

The human microbiome is the name given to the collective genetic material of the microorganisms which inhabit the human body. The microbiome comprises bacteria, protozoa, fungi, archaea, and viruses and the bacterial component, or bacteriome, is the most widely studied. High-level research into the gastrointestinal microbiome has led over decades to robust observations regarding the existence of core microbes in health and disease, interactions between host and microbe, functional impacts of alterations to the microbiome in disease states, and even the potential of the microbiome as a therapeutic target. In contrast, the airway microbiome is a relatively novel but rapidly evolving area for research in respiratory medicine since its discovery in 2010 . Culture-independent molecular techniques have allowed rapid accurate profiling of whole microbial communities and their genomes which has in turn promoted the recent acceleration of research in the area. These techniques crucially allow for thorough assessment of the interactions between the host and these microbes in both health and disease. It is imperative to understand the basis of these techniques in order to discuss fully the area of microbiome research.

1.1.7. Molecular Genetics

Study of the aforementioned human microbiome has advanced exponentially in recent years due to the advent of highly efficient genetic sequencing methods. This has coincided with the rapid expansion of interest in the area of microbiome research as it pertains to our current concepts of health and disease. These techniques are 'culture-independent' meaning that vast amounts of information can be gleaned from virtually any type of clinical sample in a rapid and accurate fashion, overcoming previous laborious and often time-consuming methods of culture-dependant growth of specific bacteria on specific culture media.

1.1.7.1. Quantitative Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a molecular biology technique which functions to amplify a specific region of DNA, generating thousands to millions of copies of the given DNA sequence of interest. It is a core technique across many research fields and has multiple applications including cloning of DNA for sequencing, diagnosis of hereditary diseases, and pathogen detection in infectious diseases (77).

The components required for a PCR assay include firstly the DNA template containing the target region which is to be amplified. To allow for DNA replication DNA polymerase is required, an enzyme which synthesises new strands of DNA by recruiting nucleotides and binding them to existing single strands of DNA. Next, a primer is required to act as an initiation point for DNA elongation. For a PCR cycle, two primers are required to bind to the three-prime end of the sense and anti-sense strands of the original DNA target. DNA polymerase works from this point to synthesise the new DNA strand, but needs to be heat-stable to do so, therefore a buffer solution is required in order to provide stability to the reaction process.

The starting point of this process is the DNA template which contains the target region to be amplified. DNA extraction products of original samples such as skin swabs or faeces become the DNA template. When heat is applied to the double-stranded DNA target it denatures, the hydrogen bonds between complementary

base pairs break, and this yields two single-stranded DNA molecules. When the temperature of the reaction is lowered as part of the heat cycle, primers can anneal to each of the single-stranded DNA templates. Primers are short pieces of single-stranded DNA that are designed to flank the target region of the DNA template. Stable hydrogen bonds are formed between complementary base pairs when the primer sequence is closely matched to the template or target sequence. DNA polymerase then binds to the primer-template hybrid and commences DNA synthesis. The new DNA strand is elongated by addition of nucleotides present in the reaction mixture, optimally at a rate of a thousand bases per minute.

This entire cycle is repeated multiple times during one PCR 'run' and with each DNA elongation step the original and new strands become templates for the next cycle. This leads to exponential amplification of the original target region of the sample DNA.

Quantitative or Real-time PCR is based on this classic PCR process but allows for measurement of the amplification products of the target DNA throughout the cycle, not just at its end (78). The rate of production of the amplification product in each cycle is calculated in real time and thereby allows for assessment of the abundance of the particular target DNA sequence in our samples. This is achieved by addition of a fluorescent dye to the reaction mixture. This dye binds only to double-stranded DNA, i.e. the amplification products, and is inactive unless bound. It can be detected by measuring fluorescence once the fluorophore of the dye is excited by application of light beam at a specific wavelength. This reaction takes place in a thermal cycler apparatus which provides the wavelength-specific light source and contains sensors to measure the fluorescence of the dye at the end of each cycle through the PCR process. As the number of gene copies increases across cycles, the fluorescence increases in the reaction.

DNA quantification then involves plotting the fluorescence generated in a given reaction against the number of cycles in that reaction on a logarithmic scale., generating an amplification curve. An arbitrary threshold for detection of

fluorescence is set usually a number of times higher than the standard deviation of any signal noise present in the reaction at baseline. The number of cycles it takes for a sample to cross that threshold is called the quantification cycle (C_q) or threshold cycle (C_t).

This technique is open to inaccuracies, usually due to poor primer design or incorrect melting temperatures. In order to ensure a valid result, the efficiency of a qPCR run is tested in one of 2 ways: generation of a melt curve/dissociation curve, or establishment of a standard curve.

The melting temperature (T_m) of DNA is the temperature at which 50% of the double stranded DNA in a sample has denatured into single stranded DNA under heat application. Assessment of this parameter is achieved by measuring the changes in fluorescent signals from the DNA binding dye as DNA denatures. Fluorescence reduces as DNA denatures. The rate of change in fluorescence is plotted against temperature to generate a melt curve. The shape of this curve can infer qualitative information about the amplicons generated in the qPCR run and the curves tend to be generated after the amplification cycles are completed. A single spike in the curve illustrates that the amplification product is pure and uncontaminated. It also provides quantitative information in that the melting temperature is specific to each amplified fragment therefore analysis of T_m can help identify the amplification product.

The efficiency of an amplification process can often vary among templates and primers. This is something which needs to be assessed for each PCR run in order to allow extrapolation of meaningful quantification data. Efficiency can be assessed in a titration experiment. To generate a standard curve, serial dilutions of a known quantity of target DNA are amplified in the same run-process used for sample analysis. C_q will be different for each dilution and this allows us to plot a standard curve. The slope of the linear regression is then used to calculate the efficiency of the amplification, an ideal result lying between 90% and 110%.

qPCR forms the basis of 16S rRNA sequencing techniques which have further progressed the microbiome research field.

1.1.7.2. 16S rRNA sequencing

16S rRNA gene sequencing was the first tool used to explore the human microbiome and since then has been the foundation on which much of the vast emerging research into the field of microbiome has been laid. It permits quantitative and qualitative assessment of exactly which bacteria are present in a given sample, allowing investigation of whole microbial communities. The 16S rRNA gene codes for 16S rRNA which forms the 30S small subunit of a prokaryotic ribosome (79). The gene itself is highly conserved meaning it is present across all prokaryotes and changes very little in structure or function even in distantly related species. In addition to the highly conserved region, the 16S rRNA gene contains nine hypervariable regions, V1 – V9 which are involved in the secondary structure of the small ribosomal subunit. These can be widely variable between species and allow discrimination between microorganisms.

16S rRNA sequencing first involves extraction of total DNA from the given sample, for example bronchoalveolar lavage or sputum. Specific genes are then amplified using PCR as previously outlined. Specific primers can be chosen which allow amplification of only the target gene, in this case 16S rRNA. The amplicons generated by 16S rRNA qPCR are then analysed for identification of their variable regions (V1-V9). This was originally achieved with processes such as denaturing gel electrophoresis or cloning and sequencing of the PCR products. Now however, 16S rRNA PCR is coupled with next-generation sequencing (NGS) and allows for rapid culture-independent analysis of many microbes in a cost-effective manner.

In order to aide analysis of 16S rRNA data, sequences are organised into operational taxonomic units (OTUs) by clustering related sequences at a particular level of identity and counting the representatives of each cluster. The operator defines a level of sequence homology which is felt to be significant and this is usually set at 97% which represents significant enrichment of a cluster. OTUs are

then analysed against specific microbial databases to identify them to the level of species, family, or genus. In this way rapid identification of the microbes in a sample and their relative abundance occurs, thus providing a huge amount of information regarding the richness and evenness of the microbiome in a given system.

There are a few widely recognised key limitations involved with 16S rRNA sequencing. Firstly, the whole process relies on highly specific PCR primers but often these primers can introduce bias to the system as their design may select for or against particular organisms. Secondly, the accuracy of the final analysis is fully dependant on the reference database which is chosen. It is generally recognised that databases (such as SILVA and Greengenes) where alignments are manually optimised and sequences undergo continuous quality assessment are highly reliable (80). Conversely the NIH sequence database GenBank is thought to be prone to error with up to 5% of its 16S rRNA sequences potentially being spurious (81). Thirdly, the process is open to contamination at many levels particularly in relation to bacterial contamination of the reagents used. This requires extensive use of focussed controls at many key phases of the process.

16S rRNA sequencing allows evaluation of the identities of microbes present in a given sample and also permits calculation of parameters such as species richness, diversity, and evenness of the microbiome. This can be linked in the research setting with relevant clinical and environmental elements to gain huge insight into the potential alterations of the resting microbiome in response to disease states. However a limitation of 16S rRNA sequencing is that it cannot infer information about an organisms' function, a vital step in establishing the role of the microbiome in health and disease.

1.1.7.3. Whole genome sequencing

Shotgun sequencing is an advance on 16S rRNA sequencing which can generate massive amounts of qualitative and quantitative information regarding microorganisms in a given sample. DNA strands extracted from samples are

sheared into random fragments and then cloned into a bacterial vector, historically E.coli. Clones are then sequenced to produce reads which are in turn assembled into the original sequence using software programmes. This method is known to be open to errors due to the long length of reads generated and indeed the huge amounts of complex DNA data analysed results in a slow and labour intensive sequencing process.

High throughput sequencing, also known as next-generation sequencing, employs the same principle as shotgun sequencing but generates hundreds of thousands of smaller reads in parallel. The reads generated are anywhere from 25 to 500 base pairs as opposed to the 50 to 200,000 base pairs required for shotgun sequencing. This causes the process to be extremely fast, but in order to guarantee accurate assembly of the cloned reads, the process becomes much more computationally intense. Fortunately due to the high demand for fast, effective DNA sequencing, competition between software providers has ensured that NGS is an accessible and affordable platform in molecular medicine.

Whole genome sequencing through shotgun and next-generation sequencing forms the foundation for understanding a given organism's function, in that the provision of the raw nucleotide sequence of a given organism's DNA can allow inference of vital information regarding the downstream effects of the organism's genetic makeup. Where it is possible, whole genome sequencing has begun to advance understanding of the pathway from non-pathogen to disease-causing pathogenic strains for many organisms.

1.1.7.4. Other techniques

The majority of the existing literature around the topic of the microbiome in various body systems associates the particular organisms sequenced to various clinical measurements. However, as of yet true causality of disease has not been established in these studies. It is thought that by borrowing further techniques from environmental microbial ecology it could be possible to bridge this gap. In particular the techniques of metabolomics, metaproteomics, and

metatranscriptomics show huge promise in measuring activity of the sequenced community so that causal relationships between the microbiota and the human host could potentially be established in a clinically relevant manner. Table 1.2 outlines the main nomenclature involved with regard to study of the microbiome.

Table 1.2: Nomenclature involved in microbiome research

Term	**Description**
Microbiota	The collection of all microorganisms living in association with the human body or a specific system/environment
Microbiome	The combined genetic material of the microorganisms in a particular environment
Taxonomy	Classification system of organisms
Operational taxonomic unit	Categorisation of organisms into species by similarity of DNA sequence data
Metagenome	The collection of all genes obtained from microorganisms in a habitat
Next-generation sequencing	A collective term to describe high-throughput technologies. These allow rapid parallel DNA sequencing, i.e. Illumia (Solexa) sequencing, SOLiD sequencing
16S rRNA ribosomal gene	Gene encoding a 16S subunit of bacterial ribosomes that is highly conserved between different bacterial species
16S rRNA gene sequencing	Determining the DNA sequence of a 16S ribosomal gene
Metatranscriptomics	Analysis of mRNA transcripts associated with an organism
Metaproteomics	Analysis of microbial proteins

Term	Description
Metabolomics	Analysis of the set of metabolit present within an organisms
Richness	The number of unique taxonomic units in a sample
Evenness	The relative number of taxonomic units in a sample
Alpha diversity	A measure of the richness and evenness in a sample
Beta Diversity	A measure of the similarity of the bacterial composition between samples

1.1.8. The Respiratory microbiome in health

Classical teaching dictated that the lungs were fully sterile and that any isolation of bacterial species in culture was representative of active infection at the time of sampling. In fact, few bacterial species are ever isolated via incubation in culture media (82) and so with development of the above culture-independent techniques our understanding of the microbial composition of this presumed sterile environment has progressed greatly. The index study of lung microbiota proved for the first time that the lungs, far from being sterile, play host to a wide number of microbes when assessed using 16S rRNA techniques (83). In addition, this study demonstrated that the microbial community was significantly altered in asthmatic patients and patients with COPD, opening up the possibility that dysregulation of host-microbe interactions may contribute to the development or propagation of airway disease (83).

The lungs have a substantial luminal surface area of between $50m^2$ and $75m^2$ and estimates of the total bacterial load vary depending on the type of sampling employed. Bronchoalveolar lavage generally will give results in the order of 4.5-8 log copies per ml of fluid, while endobronchial tissue samples can reveal 10-100 bacterial cells per 1000 human cells (84). The main component phyla of the airway microbiome in healthy controls are Protebacteria, Firmicutes, Actiniobacteria, Fusobacterium, and Bacteroides (85). There is considerable overlap between the microbiome of the oral cavity and the lower respiratory tract, suggesting some element of migration of species along the contiguous anatomical pathway from the mouth to the lungs via a process of microaspiration (86). There is evidence also to suggest that some species which migrate to the lung are selectively eliminated from healthy lungs, indeed *Prevotella* prevalence is lower in the respiratory tract than in the mouth (86). Therefore it has been proposed that the composition of the respiratory microbiome is determined through a balance of microaspiration leading to bacterial migration and colonisation, and microbial elimination via mechanical mechanisms such as coughing and mucociliary clearance. It is also postulated that various immune methods involving both the adaptive and innate immune

responses can help regulate the composition of the airway microbiome, mainly in terms of eliminating species from the environment (87).

In health, the homeostatic balance of the microbiome is achieved mostly through the aforementioned processes of inhalation and elimination. In disease however this balance is altered (83). Multiple local factors tend to affect the preferential growth or elimination of certain bacterial species. Factors such as pH, oxygen tension, temperature, nutrient availability, and the abundance and activity of immune defences tend to be somewhat uniform in health however these factors change dramatically in disease states and this can give rise to areas in the lung which allow enriched growth and reproduction of some species (85). It is well recognised that bacterial colonisation is higher in chronic disease states and this reflects the permissive environment caused by altered local factors. In a sense, the normal inhalation-elimination relationship as outlined above becomes overwhelmed by the reproductive advantage of the diseased lung. This gives rise to altered microbial communities in many various respiratory disease states.

1.1.9. The respiratory microbiome in asthma

The index study of the respiratory microbiome conducted by Hilty et al (83), investigated the composition of the airway microbial community in healthy controls, patients with chronic obstructive pulmonary disease (COPD), and patients with asthma. There were distinct differences in the asthmatic cohort, namely increased frequency of Proteobacteriae, particularly *Haemophilus, Moraxella,* and *Neisseria* species, and decreased frequency of Bacteroidetes.

This finding has been reproduced in the literature. Huang et al (88) found that both bacterial diversity and abundance are increased in asthmatic patients when compared with controls. Once again, Proteobacteria such as *Neisseria* and *Haemophilus* were found to be significantly increased in asthmatics compared to controls. Their study was designed to test relationships between physiological parameters in asthma and microbial composition of asthmatic airways. They found that the composition of airway microbiota is significantly associated with bronchial hyperresponsiveness (BHR), as measured via methacholine challenge, in patients with poor asthma control. They assessed the response of BHR to administration of clarithromycin in their subjects and found that BHR improved greatly in those patients with increased microbial diversity prior to the administration antibiotics. Finally they assessed whether their subjects were corticosteroid responsive or resistant in terms of improvement in BHR, and found that microbial community composition was significantly different when this stratification was applied to the cohort. This was the first study to link the microbiome with concrete physiological measures of disease activity and has paved the way for further studies to assess whether altered microbial composition of the lungs could potentially contribute to disease pathogenesis.

Exacerbation is another important consideration when it comes to chronic lung diseases such as asthma. Exacerbations are common entities in asthmatics and cause a vast majority of the morbidity, mortality, and financial burden experienced by patients (89). Exacerbations tend to be precipitated by a range of insults including allergens, pollutant inhalation, and exercise, though they are most

commonly associated with viral infections. It is thought that up to 75% of exacerbations in asthmatics are associated with various respiratory viruses with the most commonly detected viral species being rhinovirus (90). Less is known about the role of bacterial species in exacerbation as studies have concentrated on culture-dependant sputum analysis, bacteria rarely being isolated from samples in exacerbating asthmatics. In recent studies, *C. pneumoniae* and *M. pneumoniae* have been found to be frequently isolated via PCR-based serology approaches in exacerbating asthmatics (91), indicating a potential role for bacterial involvement. Though there have been no dedicated studies assessing the changes, if any, in the respiratory microbiome during an asthma exacerbation, the fact that the microbiome demonstrates distinct differences in asthmatics at baseline would suggest a potential relationship between microbiota, inflammation, and susceptibility to exacerbation.

The treatment of a chronic disease such as asthma must also logically impact on microbiome composition over time and equally it is feasible that the response to treatments may be affected by alterations in the microbiome. We have already seen that the level to which BHR improves after administration of a macrolide antibiotic is associated with increased microbial diversity (88). Huang's trial also suggested that there is a relationship between microbial community diversity and corticosteroid responsiveness and this has been further described in a study conducted by Goleva et al (92). This study categorised asthmatics as corticosteroid sensitive (CS) or corticosteroid resistant (CR) based on their FEV_1% response to administration of a course of oral prednisolone after baseline bronchoscopy with bronchoalveolar lavage (BAL). They found that some specific bacteria were expanded in the airways of CR asthmatics and these were mostly gram negative bacterial species known to produce lipopolysaccharides (LPS) with documented endotoxic activity. The patients who had this bacterial expansion had elevated levels of IL-8 expression in BAL macrophages and increased levels of LPS in their BAL fluid suggesting microbe-driven activation of airway macrophages. Activated pulmonary macrophages are more difficult to supress with treatments such as corticosteroids and persistence of active macrophages in the airway lumen leads to

production of multiple pro-inflammatory mediators involved in host defences and tissue remodelling. In essence, failure of response of pulmonary macrophages to treatments such as corticosteroids can lead to persistent airway inflammation in CR asthmatic patients (93).

Recent work has aimed to elucidate any differences in airway microbiome composition when patients are grouped phenotypically. The majority of studies in the area have included asthmatics of all phenotypes using a wide variety of treatment strategies. As such it is not clear whether the alterations seen in microbial composition were related to the effects of treatments such as ICS, or indeed if they related to phenotypic elements such as atopic status. Atopy itself is known to alter mucosal immune function (94) and a recent study conducted by Durack et al aimed to assess microbial differences in asthma versus atopy (95). This study found that there was a similar expansion of certain proteobacteria such as *Neisseria* and *Haemophilus* species as previously documented in the literature, and importantly that this enrichment was also present in steroid-naïve asthmatics, a finding which had not previously been established. Other taxa found in asthmatics included *Fusobacterium* and *Prophyromonas* species, known for their ability to interact with and augment the pathogenic actions of certain respiratory pathogens including *Pseudomonas*. The abundance of *Fusobacterium* species was strongly correlated with sputum eosinophil level in addition. A similarly novel finding was that atopic non-asthmatic patients (ANA) had specific alterations of their bronchial microbiome including enrichment in *Aggregatibacter* and *Corynebacterium*. This supports previous findings (14) that suggest an interaction between airway colonisation and altered mucosal immune functions in atopic patients. Though there was significant taxonomic overlap between the asthmatics and non-asthmatic atopics, specific bacterial expansions were characteristic of each group and tended to be associated with biomarkers of atopic disease such as serum IgE and eosinophil levels.

Certainly strong correlations exist between microbiome alterations and specific parameters such as bronchial hyperresponsiveness, response to steroid treatment, and markers of atopy including sputum eosinophilia and serum IgE levels. It is

widely believed that rather than individual species having marked effects on asthma pathogenesis, the functional outcome of community interactions is the more likely culprit. The Durack study (95) attempted to assess this hypothesis by employing PIRCUSt, an algorithm that uses 16S rRNA sequences to generate predicted bacterial metagenomes (96). The most significant predicted function was increased metabolism of short chain fatty acids (SCFA) which are intrinsically involved at baseline in maintaining correct epithelial barrier function in the lungs. Increased metabolism could lead to reduced SCFA bioavailability and increase epithelial permeability to aeroallergens and pathogens but this is largely a hypothetical downstream effect.

There is clearly a distinct microbiome associated with asthmatic airways and this finding has been replicated throughout the literature. However, as the concept of the lung microbiome in health and disease is a relatively new one, there is somewhat a dearth of reproducible information as to what role that microbiome plays in both disease pathogenesis and clinical progression for asthmatic patients. Studies such as the aforementioned help to build the foundations for the understanding of multiple complex interactions between the microbiome and core physiological parameters, but the field remains an emerging one.

1.1.10. Reproducibility of the lung microbiome

A reproducible core set of microbiota inhabit the lungs in both health and disease states and are referred to as 'keystone species'. However, relatively little is known about the reproducibility of the respiratory microbiome on a longitudinal basis, particularly after common interventions such as antibiotic administration or even treatment with ICS. There are many reasons for this. Some of the most robust data from investigative trials into the airway microbiome has been gained from bronchoscopic evaluation, most commonly employing endobronchial brush samples as the primary sampling method. Bronchoscopy is an invasive test for patients to undergo even once and so the concept of repeat bronchoscopy to investigate microbial composition of the airways is a difficult one. Nevertheless, Durack et al randomised 42 atopic asthmatic patients to receive either ICS or placebo and assess the respiratory microbiome via bronchial brush samples at baseline and 6 weeks after intervention (95). 16S rRNA sequence levels were insufficient for analysis of paired samples in all participants. Eight patients from each of the placebo and ICS responder groups had sufficient 16S rRNA for analysis. ICS administration was shown to confer lasting changes to the endobronchial microbiome after the six week study period. Those patients who demonstrated clinical response to ICS showed enrichment in *Neisseria* and *Moraxella* species and depletion of a specific *Fusobacterium* post ICS treatment. Very interestingly, there was also a significant compositional shift observed in the placebo group which had been inhaling a lactose-containing particles, indicating that multiple inhalants have the potential to confer alterations on the microbiome over time, not limited to treatments.

With regard to the effects of antibiotics on the airway microbial communities, little is known. Huang et al conducted a microbiome investigation (88) in a subset of patients enrolled in a larger trial studying the effect of clarithromycin on sub-optimally controlled asthmatics (97), endobronchial brush samples being obtained prior to randomisation to either antibiotic or placebo. Due to the fact that repeat

bronchoscopy was not performed in the parent study, this study was limited in its investigation of possible post-treatment microbial shifts. Instead the authors opted to test relationships between pre-treatment airway composition and a certain number of clinical outcomes post antibiotic treatment. Increased bronchial bacterial diversity pre-treatment was shown to correlate well with a reduction in bronchial hyperresponsiveness and this was not observed placebo group. One study in the literature examined paired bronchial samples using bronchoalveolar lavage before and after a 6 week treatment course of 250mg azithromycin (98). They found that bacterial richness tended to decrease post treatment however this relationship was not significant. The study was limited by the small number of participants (n=5).

Knowledge of the reproducibility of the lung microbiome remains elusive and its assessment is limited by the invasive nature of bronchoscopic sampling, particularly when undertaken in a longitudinal fashion.

1.1.11. The gastrointestinal microbiome and asthma

The gut microbiome is generally held as being the most diverse of the human environments studied to date. The huge interest in this field has contributed vastly to our understanding of host-microbe interactions and continues to direct the majority of advancing research in this area. Microbes resident in the gut contribute to many protective mechanisms along the gut barrier, and dysbiosis in the system has been associated with multiple disorders including inflammatory bowel diseases (99), cancer (100), obesity (101), and even neurological disorders (102). The most commonly occurring phyla in the healthy gastrointestinal tract include Bacteroidetes and Firmicutes, with Actinobacteria, Proteobacteria, and Verrucomicrobia contributing also but with much less frequency (103). Commensal species are responsible for nutrient extraction in the gut, for example there is an association between a single locus on a particular species of *Bacteroides* and microbial digestion of dietary xyloglucans found in vegetables (104). Complex carbohydrates are metabolised to short chain fatty acids in a microbe-dependant fashion, SCFAs in turn being an essential source of energy for intestinal epithelial cells. SCFA production can acidify the intestinal barrier environment making local pathogenic overgrowth by species such as *Salmonella* and *E.coli* more difficult (105). The gut microbiome has also been implicated in influencing host immune responses, particularly responses of the adaptive immune system. T-regulatory (Treg) cells can be induced by certain members of the *Bacteroides* family and specific *Clostridium* species, these cells playing essential roles in immune regulation including immune tolerance and protection against allergic and autoimmune disease types (106).

Multiple observations have led, over time, to the hypothesis that allergic disease may develop in response to shifts in the microbial communities of the gastrointestinal tract. Many of these observations relate to early life factors which are known to be associated with allergic type disease. For instance, early antimicrobial exposure, formula feeding, and caesarean birth are all associated with

a risk of developing allergic disease in later life (107-109). It is generally agreed that the development of the gut microbiome begins in utero, this hypothesis being strengthened by the description of a placenta-related microbiome (110). Multiple factors influence the development of the intestinal microbiome from this time onwards and it is thought that the first 24 months of life are critical in development of both the gut microbiome and the relationship between it and the developing immune system (111). Birth mode can even influence the microbiome and it has been established that caesarean section is associated with significantly lower diversity of Bacteroidetes and Actinobacteria in early life (112).

Loss of microbial diversity in early life could potentially reduce the gut's aforementioned ability to induce T-regulator cells via commensal bacteria, hypothetically leading to T-helper cell imbalance and promoting the development of allergic phenotypes. Other mechanisms which could potentiate susceptibility to immunologic diseases include accumulation of invariant natural killer T cells when biodiversity is lower. Germ-free mice tend to accumulate these cells in their colonic mucosa and this increases morbidity of allergic diseases (113). Conversely, mouse models trials have shown many bacteria to be specifically protective against allergic disease. For example, Mice treated with *Lactobacillus johnsonii* which is a species found commonly in the human vaginal tract just before birth shows significant suppression of T_H2 pathways via downregulation of IL-4, IL-5, and IL-13 (114).

There is emerging evidence for cross-talk between the gut and lung microbiomes. As we have discussed, there is a near-certain role for the gut microbiome in the protection against allergic diseases such as asthma via the methods already described. Another important relationship involves short chain fatty acids (SCFAs) and their ability to induce Treg cells and IL-10 production. A recent study of infants with atopy and wheeze found microbial dysbiosis in stool samples at 3 months old and found these samples to be low in levels of the SCFA acetate (115). There was a large decrease in the frequency of *Lachnospira, Veillonella, Faecalibacterium,* and *Rothia* and when investigators inoculated germ free mice with these four species, asthma incidence was lower in the progeny of the treated mice. This indicated a protective role of these strains. When dietary fermentable fibre is fed to mice,

there is a shift in the ratio of Firmicutes to Bacteroidetes in both gut and lung microbiota (116). This alteration caused an increase in circulating propionate, another SCFA, and this was associated with protection against inflammatory responses normally involved in the allergic response via an increase in $CD25^+$, $CD4^+$ and Treg cells.

There is emerging evidence, as discussed, that the GI microbiome likely plays some role in the development of allergic type diseases but causality has yet to be established. Antibiotic use and reduced exposure to environmental microbes in youth are known to be associated with both an increased risk of development of atopic diseases and also with significant gastrointestinal dysbiosis. Whether these observations are truly related in a causative manner remains to be established, though work is ongoing. There are no concurrent assessments of the lung and gut microbiome compartments in the literature and this type of study could potentially begin to unravel these complex relationships.

1.1.12. The skin microbiome and asthma

The skin functions as a barrier against the external environment and in doing so is exposed constantly to innumerable microbes, many of which are adapted as commensal components of the skin's microbiome. It is the largest organ of the body and as such demonstrates great topographical variance as many areas differ in anatomical composition and function. This topographical variance confers vast compositional differences in the microbiome of the skin. Thermophilic bacteria such as *Staphylococcus aureus* and various gram negative bacilli preferentially dominate the microbiome in areas of higher humidity and temperature such as the axillae and groin (117). Lipophilic bacteria such as *Malassezia spp* dominate areas where sebaceous glands are plentiful such as the back and chest (117). This topographical variance has been well established via culture-dependent research over many decades. Newer culture-independent techniques have enabled a deeper understanding of the skin microbiome in health and disease.

The microbiome of the skin in health is dominated Actinobacteriae, Firmicutes, Bacteroidetes, and Proteobacteria (118). The relative abundance of community members varies according to topographical location and community composition seems to be related to various functional roles of the skin. For instance, the malodour of human sweat is a consequence of the processing of apocrine secretions by bacteria such as *Staphylococcus spp,* and *Corynebacterium spp* (119).

Dysbiosis of the skin is implicated in some dermatological disease processes and is primarily driven by factors such as ageing, hydration, nutrition, and lifestyle factors such as environmental exposures to sunlight, cosmetics, and certain medications (120, 121). While there is little known regarding the relationship, if any, between the skin microbiome and asthma, there is a wealth of information regarding the role of the skin microbiome in the development of atopic dermatitis (AD), a condition closely associated with asthma. Overpopulation of *Staphylococcus aureus* characterises this disease, crowding out other components of the microbiome community and reducing diversity of the system in affected areas (122). This

discovery has led to targeted therapeutic interventions which have shown promising results in treating the condition. Topical supplementation of *Vitreoscilla filliaformis* is shown to reduce *S. aureus* overgrowth, restore diversity to the microbiome, and objectively improve AD symptoms according to validated assessment tools (123).

AD is closely associated with asthma and is also known to be associated with gastrointestinal dysbiosis along to 'Skin-Gut-Axis' in a similar manner to the 'Gut-Lung Axis'. There are no clear links between the skin dysbiosis which characterises AD and the lung dysbiosis particular to asthma, though this may be due to a paucity of research in the area.

1.2. Summary and hypotheses

Classically, asthma diagnosis tended to rely heavily on physician-led assessment of the clinical signs and symptoms involved in the disease process. Often this clinical diagnosis was strengthened in cases where patients demonstrated partial or complete clinical resolution in response to trial of treatment with standard inhaled bronchodilators and inhaled corticosteroids. However there has recently been a definite move towards the incorporation of more precise and objective physiological criteria in the various diagnostic algorithms designed to aid our navigation of this complex disease.

Random measurement error is defined as the fluctuation of a measured value around the true value of a given variable. This error, if introduced to a study, can affect both the exposure and outcome variables. Consider, for example, a diagnosis of asthma to be the exposure variable and the microbial composition of the airways to be the outcome variable. If we introduce error into the measurement of the exposure variable (asthma) we will bias the regression coefficient towards the null, the regression coefficient being the slope of the regression line representing the rate of change of one variable as a function of changes in the other variables involved in a given relationship. Measurement error in an exposure variable can lead to attenuation of a relationship, or to missing of an association altogether. Asthma needs to be defined in an objective and precise manner in order to avoid the phenomenon of regression dilution bias, or relationship attenuation, in studies involving the disease. Combining the clinical suspicion for the disease with solid physiological observations measured in a reproducible and standardised manner can help to reduce random measurement error and in turn attenuate regression dilution bias when testing statistical relationships involving asthmatic patients.

The microbiome is a newly evolving target for research in the realm of respiratory medicine. In terms of the literature, the discovery of a distinct microbiome in the lungs is a relatively novel finding and there is certainly a need to explore this area further across many respiratory diseases, not least asthma. While recent studies

have concentrated greatly on improving our understanding of the microbial composition of the lungs in both health and disease, there is relatively little information known regarding the relationships between the microbiome and certain reproducible measurements of airway physiology. Furthermore, the effects of routine clinical treatments is poorly understood in this context.

The gastrointestinal microbiome is a better understood entity in both composition and function, thanks to the vastly wider expanse of studies on the topic. Certain keystone species in the gut have effects on the immune system's function and this can cause so-called 'cross-talk' between the gut and the lungs. The skin is known to have a distinct microbiome of its own and less is known about its ability to interact with other microbial systems such as that of the skin.

Little is known regarding the effects on the respiratory microbiome of the various treatment strategies involved in the management of asthma.

The central hypothesis of this thesis is that airway physiology, and the bacterial burden of the lungs, skin, and gastrointestinal tract, will maintain relative stability over time despite the addition of an antibiotic in carefully selected asthmatic patients. It is not clear whether different microbiome compartments operate independently of each other and are shaped by local factors such as disease, or whether they are related with dysbiosis occurring as a consequence of and contributing to overall immune dysregulation. We postulate that these microbiome compartments are related and potentially communicate via their immunomodulatory functions and that concurrent assessment of these systems is a vital yet underexplored topic of research in this area. To our knowledge, concurrent assessment of these microbiome systems has not been undertaken to date.

1.3. Aims and Objectives:

The aims of this thesis were:

To establish full physiological profiles in a cohort of asthmatic adults and to assess the reproducibility of these physiological parameters after a standard course of antibiotic treatment

To assess the microbiome of asthmatic lungs using qPCR techniques and to assess the reproducibility of the microbiome after a standard course of antibiotic treatment

To assess the relationship between the microbiome of the lungs, the skin, and the gastrointestinal system of asthmatic adults before and after antibiotic treatment

1.4. Summary Project Overview

This project aimed to comprehensively profile the microbiome of patients with physiologically documented asthmatic airways. The parent study of this MD study will be the largest investigation of the endobronchial microbiome in the literature to date and it is being conducted in three international asthma centres in London, Swansea, and Dublin. Asthmatic patients and non-asthmatic control subjects were included in this analysis. This research project was funded by a Wellcome grant which allowed for limited related smaller projects to occur. We conducted an investigation in a subset of the asthmatic patients in the Dublin centre, the methods of which are outlined below. This sub-study aimed to profile the microbiome of asthmatic airways, gut, and skin, and to assess changes in the microbial composition of these communities, as well as the airway physiology of these asthmatics, after a course of standard quinolone antibiotic.

Initially the aim of this collaborative research project was to analyse a large bank of biological samples obtained from asthmatics, and from controls in the parent study, for bacterial burden using qPCR and for community composition using 16S rRNA sequencing. The laboratory secondment placement which took place in the Asmarley Genomics Laboratory of Imperial College London occurred in May 2017, a time during which uncertainty regarding the real-world outcomes of the affirmative Brexit referendum was high. As a consequence of this widespread uncertainty, there was a pause placed on the Wellcome grant funding of this and other projects which included international collaborators. It was therefore not possible to continue the laboratory analysis of these samples through to 16S rRNA sequencing as initially planned.

These samples are stored in the laboratory in London and it is hoped that when funding allows, these will be analysed as intended. However for the purposes of this MD it was decided after much consideration to limit the scope of the thesis to analysis of the bacterial burden of these samples using qPCR methods. This type of assessment, particularly as is was undertaken in a longitudinal fashion, is novel and

helps to advance the understanding of the complex relationships intrinsic to any analysis of the microbiome.

Chapter 2: Methods

2.1. Study design

2.1.1. Recruitment

Patients who attended the asthma clinic in the Respiratory Department of Connolly Hospital were invited to participate. All patients had been referred to the clinic complaining of a combination of cough, shortness of breath, and wheeze. The majority of patients included were newly referred to the clinic for diagnosis of potential asthma, however some suitable patients who were already under review in the respiratory outpatients department were also invited to participate.

Patients were required to be at least eighteen years old and to have a clinical suspicion of asthma. Patients were admitted to the study regardless of atopic status. Exclusion criteria were more extensive and are outlined fully in Appendix 1. Exclusion criteria included smoking, both active or previous with a greater than five pack-year history, elevated body mass index (BMI) above 30, significant occupational exposure to an inhaled irritant as interpreted by the investigators, and use of disease-modifying medications. Patients on newer monoclonal antibody therapies for eosinophilic asthma were also excluded. Patients could be later excluded from participation if they developed asthmatic exacerbations or lower/upper respiratory tract infections during the study period. Exacerbations were defined as any increase in symptoms of cough, shortness of breath, or wheeze which necessitated the use of either oral antibiotics, steroid courses, or both.

2.1.2. Asthma diagnosis

Patients who met the inclusion and exclusion criteria were invited to participate. The first step of the investigation included strict pulmonary function testing to accurately diagnose asthma. Patients were considered to be asthmatic if they demonstrated either of two results in combination with convincing clinical signs and symptoms: reversible airflow obstruction during spirometry as shown by post bronchodilator improvement in forced expiratory volume (FEV_1) of 12% and 200ml, or a positive bronchial challenge result as shown by a drop in FEV_1 of greater than 20% on incremental dosing of methacholine. Patients who were non-asthmatic by

the above criteria were returned to usual clinical follow up in order to assess for the cause of their presenting symptoms. Patients in whom asthma was confirmed were invited to proceed further with the study protocol.

2.1.3. Study protocol overview

This study involved a high level of patient interaction. After recruitment in the respiratory outpatient clinic, patients were first asked to attend for full lung function testing. This involved not only spirometry with bronchodilator reversibility and bronchial challenge testing, but also fraction of exhaled nitric oxide measurement, end tidal carbon dioxide measurement, and skin prick allergy test analysis in order to generate robust data on the patient's airway physiology. Patients then attended for their first bronchoscopy at which time they signed their informed consent to participate in the trial. The patient information sheet and consent forms are outlined in Appendix 2. Skin swabs, faecal samples, and oropharyngeal swab samples were also collected at this time. After their first bronchoscopy patients were given a prescription for 10 days of oral Levofloxacin 500mg once per day. They were asked to record the start and end dates for this medication as well as any adverse reactions. Patients were instructed of the major side effects of this medication and were asked to stop the antibiotic if they developed any allergic-type symptoms or heel pain (risk of Achilles tendonitis).

Patients returned to the outpatient department for visits approximately 3 weeks and 6 weeks after their bronchoscopy. This was to facilitate collection of skin swab samples, faecal samples, and oropharyngeal swab samples. Patients were given the number of a dedicated phone which they could contact in the event of any adverse reactions, intercurrent illnesses, or changes to medication such as prescriptions of antibiotic or steroid by their primary care physicians.

8 weeks, on average, after their first bronchoscopy, patients returned for their second bronchoscopy. Skin swabs, faecal samples, and oropharyngeal throat swabs were again collected at this point. Once they had completed the trial patients were

allocated outpatient appointments for regular follow up of their asthma. Appendix 3 outlines the flowchart of patient visits.

2.2. Lung function testing

2.2.1. Spirometry

Patients were asked to withhold inhalers sequentially prior to testing. Long acting muscarinic antagonists (LAMA) required a one week washout period, long acting beta agonists (LABA) and inhaled corticosteroids (ICS) required 48 hours washout, and short acting beta agonists (SABA) were withheld the morning of the test. Patients were also asked to refrain from eating a heavy meal or exercising 2 hours prior to the test. Height and weight were measured using standardised scales. Patients were then positioned in a comfortable upright seated position with both feet on the ground. Pulse oximetry was recorded. Patients were asked to breathe at tidal volume firstly for an amount of time specified by the testing physiologist. At the point of functional residual capacity (FRC, i.e. the end of tidal breathing), the patient was instructed by the respiratory scientist to inhale maximally and rapidly and then to exhale maximally. A minimum of three acceptable FVC manoeuvres were performed and results were considered reproducible when the difference between the largest and next largest FVC and FEV_1 measurements was less than or equal to 150ml. If results were found to not be reproducible then further efforts are attempted but no more than 8 trials should were completed. To assess for bronchodilator reversibility, an albuterol nebuliser (400mcg) was administered and the spirometry was repeated 15 minutes later. Reversibility was established if the FEV_1 increased post bronchodilator inhalation by 12% and 200ml.

2.2.2. Diffusion Capacity testing

Single-breath determination of Diffusion Capacity (DLCO) involves measuring the uptake of carbon monoxide by the lungs over a period of breath-holding. Patients were asked to withhold their inhalers on the day of the test. Patients were positioned in a comfortable upright seated position with their back straight and legs uncrossed. Subjects first had vital capacity measured and this must have exceeded 1.5l on spirometry to allow for testing to continue. Once the patient was sitting comfortably with the mouthpiece and nose peg in place, they were asked to tidal breathe. The test commenced when the patient was asked to expire in an unforced

manner to residual volume (RV) and then inspire to total lung capacity (TLC). The test gas was given to this patient at the point of inspiration and they were then asked to hold their breath for 10 +/- 2 seconds for an accurate test. Inspiration must have been rapid and must not have exceeded 4 seconds. The patient then expired into the mouthpiece so that analysis of the alveolar gas could take place. The test gas was composed of carbon monoxide (CO), a tracer gas and oxygen. The tracer gas Methane (CH4) was used to estimate alveolar volume because its gaseous diffusivity is close to CO therefore it does not interfere with measurement of CO concentration and is not present in alveolar gas. In order for a valid test result, 2 or more measurements were performed with a recommended 4 minute interval between trials. Both trials were required to agree to within 10% of each other in order to establish an accurate result. DLCO was measured in ml/min/mmHg and a level of less than 60% predicted indicated a low diffusion capacity.

2.2.3. Lung Volume Measurement

Lung volumes were measured for this cohort using body plethysmography. This method for lung volume assessment is based on Boyles law whereby the absolute pressure exerted by a given mass of a gas is inversely proportional to the volume it occupies if the temperature and amount of gas remain constant in a closed system. The plethysmograph ('body-box') provides the closed system and the pressure and volume changes generated by breathing manoeuvres can be used to calculate functional residual capacity (FRC), residual volume (RV), and total lung capacity (TLC). Patients were asked not to use their inhaled medications on the day of the test. The patient sat as comfortably as possible in the body plethysmograph and the door of the box was shut. Measurement occurred via a computer programme and measurements were extrapolated in this way also. The patient was instructed to begin tidal breathing via the mouthpiece with a disposable filter attached, and a nose-clip was applied. Once breathing was felt to be stable and consistent with end-tidal FRC, the mouthpiece was occluded and the patient was instructed to tidal breathe against the closed shutter at FRC. The patient then exhaled without force to RV, inhaled maximally to TLC and exhaled maximally to RV. This set of

manoeuvres were repeated twice more. The readings of FRC were compared to ensure a valid result, and the values were required to agree to within 5% in order to be considered an accurate and reproducible attempt.

2.2.4. Bronchial Provocation Testing with Methacholine

Prior to the test day, patients were asked to withhold medications as follows:

- Short-acting beta agonists 6 hours
- Oral Theophyllines 12 to 24 hours
- Long acting beta agonists 36 to 48 hours
- Long acting antimuscarinics 1 week

They were also asked to abstain from alcohol during the 4 hours directly preceding the test. The test was undertaken using a Jaeger spirometer as above with baseline spirometry measured first in accordance with the same protocol as outlined above. The best of three FEV_1 measurements was taken as the baseline. The test was explained to the patient, including the risk of developing respiratory symptoms and some mild discomfort. An Aerosol Provocation System (APS) nebuliser was employed which is a flow-controlled system that produces an exact dose of the given aerosol, in this case methacholine. Firstly the patient was challenged with nebulised saline through the APS. They were asked to tidal breathe through the nebuliser mouth-piece with a nose-clip in place for exactly 2 minutes after which FEV_1 was measured at 30 and 90 seconds. An FEV_1 drop of 10% of more from baseline was considered a positive test result at this point and the test was terminated after administration of salbutamol/ipratropium via nebuliser to counteract bronchoconstriction. If FEV_1% did not decrease by 10% then the test was continued. Serial dilutions of nebulised methacholine were administered at each inhalation through the APS apparatus, again requiring the patient to tidal breathe with a nose-clip in place for 2 minutes. The lowest FEV_1% obtained post inhalation was chosen as this reflected maximum bronchoconstriction after each dose. This procedure was repeated until the FEV_1 either fell by 20% or more from baseline, or the final cumulated dose of methacholine of 1.44mg was reached. The dose at which FEV_1 dropped by 20% or more was called the PD20 and was

measured in μg of methacholine. The results of this measure were then interpreted as follows:

- >1440μg Normal bronchial responsiveness
- 360-1440μg Borderline bronchial hyperresponsiveness
- 90-360μg Mild bronchial hyperresponsiveness
- <90μg Moderate to severe bronchial hyperresponsiveness

The patient was reviewed throughout for signs and symptoms of respiratory distress. Patients were instructed to discontinue inhalation if symptoms become troublesome at any point. Whether the test was positive or negative, 2.5mg/2.5ml of salbutamol was administered via a small nebuliser at the termination of the test protocol. Spirometry was then repeated a few minutes after the salbutamol to ensure that the patient had returned to their baseline. If FEV_1 remained less than 90% of baseline then salbutamol was repeated or a physician was asked to review the patient.

2.2.5. Fraction of Exhaled Nitric Oxide Testing

This measurement was achieved using the Aerocrine NIOX VERO® nitric oxide analyser which is a high sensitivity detector based on a gas phase chemoluminesent reaction between nitric oxide and ozone. NO measurements are given in parts per billion. Patients were asked to withhold all respiratory medications as follows:

- Short acting bronchodilators 6-8 hours
- Long acting bronchodilators 48 hours
- Inhaled or oral corticosteroids 72 hours
- Antihistamines 4 days
- Long acting antimuscarinics 1 week

Patients were advised not to smoke or eat in the hour before measurement and also to avoid caffeine and chocolate on the day of the test. NO measurement was generally carried out prior to spirometry as spirometric manoeuvres have been shown to transiently reduce exhaled NO levels and this may affect the validity of the test. The patient was seated in a comfortable upright seated position and a single use bacterial filter was applied to the mouthpiece of the device (NIOX VERO

filters, single use). The patient was instructed to expel air from the lungs entirely and then inspire deeply. They exhaled smoothly into the mouthpiece and produced a pressure of around 16cmH2O at a flow rate of 0.05L/s and held this rate until test criteria were met as evaluated by the physiologist. A minimum of two valid measurements were required. If the largest measurement was <50ppb the results should be within 10ppb of each other to qualify as a valid result. If the largest measurement was >50ppb, then there should be less than 20% differential to qualify as a valid result. If valid, results were then interpreted as follows:

- <5ppb: low Eosinophilic inflammation unlikely
- 5-25ppb: normal Eosinophilic inflammation unlikely
- 35-50: intermediate Mild eosinophilic inflammation likely
- >50ppb: high Significant eosinophilic inflammation likely

Patients were then instructed to resume all medications as prescribed.

2.2.6. End Tidal Carbon Dioxide Testing

This measurement was achieved using the BCI 9004-000 Capnocheck® Plus Capnometer and its associated calibration adapter, flow-meter, and tubing. Patients were positioned in a comfortable upright seated position and a disposable oral/nasal CO2 sample line (Salter Labs 4001-7-25) was placed in the nares of the patient. This was connected to the moisture trap apparatus of the capnometer. The patient was asked to tidal breathe for a period of 5 minutes prior to recording values to allow their breathing rate to normalise after connection to the apparatus. The resulting End Tidal CO2 measurement as per the capnogram was recorded every two minutes for a total of 10 minutes. The average $ETCO_2$ and respiratory rate are calculated from these measurements. Normal values lie between 35mmHg and 45mmHg.

2.2.7. Skin Prick Allergy Testing

Allergen solutions were first removed from the refrigerator and allowed to approach room temperature before use, usually over a thirty minute time period. The subject was positioned in a comfortable upright seated position with their bare

forearm in a resting position on a level surface. Patients had been requested to discontinue antihistamine medications at least four days prior to their test and this was confirmed with the patient before commencing the test protocol. The forearm was cleaned with an alcohol wipe and positions for each allergen were marked on the skin with a site marker. Sites should be at least 2cm apart to avoid overlapping reactions. Allergen solutions were then dropped on to the skin from dropper bottles. Sterile lancets were used to prick the skin where the allergen had landed with a separate lancet used for each allergen to avoid contamination. Excess allergen solution was wiped from the skin 1 minute after the sites had been pricked. Allergen reaction was read 15 minutes after application of the lancets. The diameter of the resulting wheal was measured in millimetres, or where there was an irregular wheal, the long and short axis were evaluated and divided by 2. A negative and positive control solution was also applied to two separate sites. In order for the test to be considered valid, the patient must have reacted to the positive solution which contains a dilution of histamine. If they also react to the negative solution, a saline solution, then the size of this reaction is subtracted from all other reactions measured. The sensitivity of the reaction was classified as follows:

- Negative <3mm
- Mild 3-10mm
- Moderate 10-15mm
- Severe >15mm

The patients arm was then cleared fully of any residual allergen and an antihistamine cream was applied to the area.

2.3. Biological sampling

2.3.1. Skin swab sampling

Skin swab samples were obtained from each patient at various times throughout the study period. The index swab set was taken on the day of first bronchoscopy. Sterile cotton-tipped swabs were pre-moistened in a sterile container which contained 10ml of sterile water. One control swab was pre-moistened but not applied to skin for each set. A sample was then taken from each of the right and left antecubital fossae and the right and left axillae. Firm pressure was applied to the skin and the swab was moved in a circular motion for at least thirty seconds to obtain the sample. Samples were labelled and batched into a specimen bag, then frozen at -80^{o}C within 20 minutes. This procedure was repeated twice in the interval between first and second bronchoscopy and also repeated on the day of the second bronchoscopy, meaning four sample sets were obtained during the research period.

2.3.2. Faecal sampling

Faecal samples were obtained from each patient at various times throughout the study period. Patients were asked to provide faecal samples on the same day as skin samples were being obtained, i.e. four samples were obtained throughout the study period. For comfort reasons, patients were requested to obtain their samples at home and then transport them to the hospital for freezing. Patients were given a sampling kit which included a disposable tray, latex gloves, sealed specimen bag, and a pre-labelled sterile faecal sample collection pot which had a sterile spatula attached to the lid. Patients were instructed to collect a specimen in the tray and then to take a sample of the specimen with the sterile spatula attached to the collection pot (Sarstedt faeces tube 76x20mm 80.734.001). The pot was sealed and put into the pathology specimen bag which was in turn sealed and dated. If patients could transport their samples directly to the hospital within twenty minutes, then samples could be directly placed in the -80^{o}C freezer. Otherwise patients were instructed to place the sealed samples in their own home freezers and transport them to the hospital within five days from being placed in the freezer.

2.3.3. Bronchoscopy

Bronchoscopy was carried out under sterile conditions in the endoscopy unit of Connolly Hospital, Blanchardstown. Each patient presented fasting from midnight the night before, was checked in by an endoscopy staff nurse and given nebulised salbutamol 3mg/ipratropium500μg (Combivent® UDVs 2.5ml) prior to the procedure. Informed consent was obtained from the patient by a member of the research team and were given an additional patient information leaflet detailing the procedure, sampling, and sample destination. They signed a copy for the hospital and a copy which was to accompany samples to the laboratory in London. Please see Appendix 4 for copies of the additional consent and information leaflet.

Patients were connected to a cardiac monitoring system which measured pulse oximetry and blood pressure. A sterile throat swab sample was obtained by a member of the research team and the throat was then anaesthetised with local topical lidocaine (Xylocaine 10mg Spray) via the nasal route. 50 ml of sterile water was washed through the bronchoscope prior to the procedure into a sterile container in order to identify and confounding microbial DNA present on or in the scope. An intravenous cannula was sited and Propofol was administered by an anaesthetist who then remained in the endoscopy suite for the entirely of the procedure and recovery.

Patients were intubated with the bronchoscope via the nasal route. The vocal cords were further anaesthetised using a dilution of lidocaine in normal saline and this was administered through the scope. The scope was then passed through the vocal cords to commence the procedure. 2 sheathed endobronchial brush samples were obtained from the left upper lobe and two from the left lower lobe. 6 endobronchial biopsy samples were obtained from the right middle lobe. Once an inspection of the airways was complete the scope was removed and the patient returned to the endoscopy recovery area and observed for a period of time not less than 2 hours after the procedure.

2.3.4. Endobronchial sample preparation

2.3.4.1. Brushes

After sampling, the sheathed brushes were removed from the bronchoscope and fully extended or unsheathed before cutting the brush off with sterile single-use scissors. Brush tips were cut into sterile containers and placed in the -80^{o}C freezer within three hours of sampling.

2.3.4.2. Biopsies

Two of the samples were taken for histological analysis and placed immediately into tubes containing 4% formalin. These were then transported immediately to the local histology laboratory to be embedded in paraffin, so-called paraffin block fixation. The paraffin blocks were then transported to the laboratory in Imperial College for analysis.

Two of the samples were taken for RNA extraction and were placed in Quiagen Tissue Protect tubes at sampling. These were firstly incubated at 2-8^{o}C for a maximum of 72 hours after which they were transferred to the -80^{o}C freezer for storage until they were transported to the laboratory in Imperial College London for extraction.

Two of the samples were taken for DNA extraction. These samples were placed into empty sterile tubes and placed in the -80^{o}C freezer immediately or within three hours.

2.4. Laboratory methods

2.4.1. Laboratory Work Overview

Once samples had been couriered on dry ice to the Asmarley Genomic Medicine Laboratory of Imperial College London, they were first entered into a tissue tracking database in line with the UK's ethical standards. This tracked each sample to one specific individual bag and mapped the exact location in which they were stored against unique identifiers for each sample.

Once tracked, DNA extraction was the first step in the process and this is outlined in detail below. This lab analyses respiratory samples regularly however had not carried out skin swab extraction previously. Because of this, it was decided to run a sample extraction using samples obtained from lab staff in order to ascertain whether the bacterial load on the swab would be sufficient for downstream analysis. These samples (right and left antecubital area, right and left axilla, and control) underwent DNA extraction, nanodrop assay, and qPCR analysis (all detailed below). The results of this initial test investigation showed that only the axillary samples carried enough DNA to meet the minimal quantitative threshold necessary for sequence analysis.

In contrast, faecal samples were found on nanodrop assay to have extremely high DNA content and so multiple initial test runs were carried out in order to identify the correct dilution volume needed to allow for accurate qPCR processing.

Oropharyngeal swabs and lung brush samples are routinely processed in this laboratory using the methods as outlined below.

2.4.2. Microbial DNA extraction

Microbial DNA extraction was carried out under supervision at the Asmarley Laboratory of Genomic Medicine. The process outlined here was applied to extraction of lung brushes, skin swabs and oropharyngeal swabs. A double extraction method was used which increases DNA yield through the process.

Swab tips were cut using individual sterile autoclaved scissors and placed into Lysing Matrix E tubes (LME tubes, MPBio, 116914500). Each tube contains 1.4mm ceramic spheres, 0.1mm silica spheres, and one 4mm glass bead, and are designed to facilitate cell lysis when agitated. The tubes had been prepared by addition of 50μl of 0.2M aluminium ammonium sulfate (AINH4(SO4)2.12H0, Sigma 402816), designed to precipitate out any heavy metal particles during the extraction process. 500μl of CTAB (hexadecyltrimethylammonium bromide) extraction buffer was then added and samples were left to incubate for 15 minutes. CTAB acts as a surfactant in DNA extraction processes and serves to remove membrane lipids and promote lysis of cells, binding to polysaccharides when the salt concentration is high.

After incubation, 500μl of Phenol:Chloroform:Isoamyl alcohol (25:24:1 pH 8.0, Sigma, P2069-400ml) was added using sterile pipette tips to each LME tube.

In order to facilitate cell lysis, the LME tubes were then placed in a 'bead-beater' apparatus and subjected to a protocol designed specifically for the CTAB extraction buffer. This applied a speed of 5.5m/s for a 60 second cycle. Tubes were then removed from the apparatus and centrifuged at 16000 x g for 5 minutes. The supernatant was transferred via individual sterile pipette tips to 2ml phase lock tubes (VWR Phase lock: PLG Heavy (733-2478)). These contain pre-aliquoted high density gel which migrates under centrifugal force to form a seal between the aqueous phase of the solution containing the nucleic acid, and the organic phase. These were centrifuged and put aside.

The LME tubes which still contained the original swab material then underwent the second extraction phase, identical to that outlined above. The supernatant was placed into a second set of labelled phase lock tubes and centrifuged.

The broken-down swab material was transferred from the LME tubes to sterile tubes containing 'spin basket' filters so that they could be centrifuged. Spin baskets act as filters, allowing liquid to pass through but retaining any organic material from

the swab itself. The baskets were removed and any excess liquid was then added to the first set of phase lock tubes already collected in order to maximise yield of genetic material.

A Chloroform solution (Chloroform:Isoamyl alcohol 24:1, Sigma, 25666-500ml) was added to all the phase lock tubes prior to a final centrifuge cycle at 16000 x g for 5 minutes at 4^{o}C.

Faecal samples also underwent double extraction however there were subtle differences in the process. Samples were first prepared by weighing out 200mg of faecal matter and aliquoting this amount into the bead beating tubes. The reagents were then added in identical quantities as outlined above. The bead beating cycles were standard for all samples and supernatant from the first cycle was pipetted into the first set of phase lock tubes as described above. Supernatant from the second bead beating cycle was added to the second phase lock tube. Spin baskets were not utilised in the faecal DNA extraction protocols. Phase lock tubes were centrifuged in the same manner as the swab samples with addition of the chloroform solution. Further downstream analysis continued in an identical manner for all samples regardless of nature.

2.4.3. Microbial DNA Precipitation

The aqueous phase of the solution in the phase lock tubes was added to 2ml Eppendorf® Safe-Lock micro centrifuge tubes (T2795-100EA) which had been prepared with 1µl of LPA (Linear Polyacrylamide, GenElute-LPA Sigma 56575-1ml) and stored in a 4^{o}C refrigerator. Polyethylene glycol solution (30% w/v polyethylene glycol 6000 in 1.6M NaCl) was then added in a 2:1 ratio to the Eppendorf tubes. This precipitated DNA into a cloudy solution. Samples were mixed well and placed again into the refrigerator overnight.

Having been left to precipitate overnight, the tubes were centrifuged at 16000 x g for 20-30 minutes at 4^{o}C. This left pellets of DNA material suspended at the base of the tubes in the PEG NaCl solution. The solution was pipetted off to leave only

genetic material in the tube. The next stage was to 'wash' the DNA pellets with ice-cold 70% ethanol. This was repeated three times, first with 500μl of the 70% ethanol solution and then twice with 200μl.

The pellets were then left to air dry for 5 minutes in their tubes. Once dry, they were re-suspended in 30μl of TE buffer (low EDTA TE: 10mM Tris pH 8.0 and 0.1mM EDTA, Invitrogen, 12090-015). Buffers such as TE function to stop DNA degradation by chelating divalent metal ions such as Mg^{++}, essential components for breakdown of DNA and RNA. After remaining in the refrigerator for a few hours, the 2 pellets per original sample (now re-suspended) were combined together in labelled tubes. Therefore in this way the DNA extracted and precipitated through the above processes can be stored at -20^{o}C or -80^{o}C and downstream analysis can then take place.

2.4.4. Nanodrop Spectrophotometry

Once purified DNA was extracted, small quantities were assessed for DNA concentration and purity using spectrophotometry. 1μl of extracted DNA was assessed per sample and this was repeated to ensure agreement between two estimates. The nanodrop platform was used which allowed rapid spectrophotometry of a small volume without the need for other reagents. Concentration of DNA was assessed and recorded as ng/μl. Purity was estimated by assessing for protein contamination at a wavelength ratio of 260/280 and salt contamination at a wavelength of 260/230. The optimal 260/280 ratio of protein contamination for DNA samples is 1.8 or above and 260/230 ratio of salt contamination is 2 or above. If these values were met it indicated that the sample was likely to be pure and free from significant contamination which could affect downstream analysis.

To operate this equipment the pedestal, the platform on which the DNA is placed and read, was cleaned with sterile water. The system was then primed or 'blanked' with TE buffer, the same solution used in the prior extraction step to suspend the

DNA in solution. If cleaning had been successful, this blank run did not return any detectable DNA. Samples were then mixed and checked for air bubbles and pipetted on to the pedestal. Measurements were taken in duplicate to ensure agreement and recorded. If measurements were discordant, the sample was measured once more and the outlier was discarded.

2.4.5. 16S rRNA qPCR: General principles

All biological samples in this study underwent qPCR using the ViiA7 Real-Time PCR system. The selection of this technique was based upon the knowledge that our central hypothesis required quantification of bacterial load. Amplification of target DNA using SYBRGreen fluorescence to quantify the amplification products gives fast and accurate estimates of 16s rRNA burden which can then be back-calculated to identify the bacterial count in the original sample. Biomass is known to be variable depending on which sample is assessed therefore it is often necessary to dilute the extracted DNA product in order to generate clear amplification curves in order for these calculations to take place. In this experiment multiple dilutions of each sample were run in order to find the optimal dilution for each reaction.

2.4.6 16s rRNA SYBRGreen qPCR Reaction Preparation

qPCR was undertaken using the ViiA7 Real-Time PCR system. A 96 well plate (MicroAMP Fast 96-well Reaction Plate 0.1mL) was set up for each qPCR run. This contained 10μl of 'mastermix' and 5μl of either sample, standard, or non-template control, giving a final reaction volume of 15μl in each well of the plate. All components were added to the plate in triplicate.

Mastermix is a solution containing the fluorescent dye kit, primers, and sterile water. The dye used was KAPA BioSystems SYRB Fast qPCR Kit Master Mix Universal. This kit contains DNA polymerase, a reaction buffer, nucleotides, SYBRGreen dye, and $MgCL_2$. The universal 16s rRNA primers used were 520F (5'-AYT GGG YDT AAA GNG -3') and 802R (5'-TAC NVG GGT ATC TAA TCC -3') and these were obtained from Eurofins MGW Operon. Sterile molecular grade water was also added (MO BIO PCR Clean water). The below quantities of each component were

added to a 1.5ml microcentrifuge tube in a hood that had been exposed to UV light in order to denature any contaminating DNA. The quantities added to each well of the plate are outlined in column 1 below, however the solution was made up to enough volume for all 96 wells. 10μl of the final mix was added to each well of the 96 well plate using a multichannel pipette.

Table 2.1: Components and quantities of the reaction mixture

Component	**Volume in each well**	**Overall mixture volume**
SYBR Fast qPCR Kit Master Mix	7.5μl	862.5μl
F primer	0.3μl	34.5μl
R primer	0.3μl	34.5μl
PCR water	1.9μl	218.5μl

In order to establish the standard curve, decreasing known concentrations of a standard were added to the plate. The standard used in this preparation was amplified *Vibrio natregens* which was diluted down to a working concentration of 2 x 10^7. This working concentration was then diluted down sequentially and 5μl of the various concentrations added to the first 15 wells of the 96 well plate (5 reducing concentrations run in triplicate).

A non-template control was added to the next three wells and this was achieved using the sterile molecular grade water. This control allowed assessment of the level of noise signal at baseline and therefore set the threshold for fluorescence detection, usually 3 to 5 times the standard deviation of background noise.

Samples were then added to the remaining wells in triplicate. 5μl of sample was added to the 10μl of mastermix solution already in the wells using a different sterile pipette tip for each.

Table 2.2: Layout of the 96 well plate. Standards, non-template controls, and samples were added in triplicate to the reaction mixture already in each well.

S: Standard preparation in decreasing order of concentration

N: Non-template control

U: Sample

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	S8	S8	S8	S7	S7	S7	S6	S6	S6	S5	S5	S5
B	S4	S4	S4	N	N	N	U1	U1	U1	U2	U2	U2
C	U3	U3	U3	U4	U4	U4	U5	U5	U5	U6	U6	U6
D	U7	U7	U7	U8	U8	U8	U9	U9	U9	U10	U10	U10
E	U11	U11	U11	U12	U12	U12	U13	U13	U13	U14	U14	U14
F	U15	U15	U15	U16	U16	U16	U17	U17	U17	U18	U18	U18
G	U19	U19	U19	U20	U20	U20	U21	U21	U21	U22	U22	U22
H	U23	U23	U23	U24	U24	U24	U25	U25	U25	U26	U26	U26

Once the plate was complete it was sealed using a MicroAMP Optical Adhesive Seal (4311971) and MicroAMP Adhesive Seal Aplicator (4348209) ensuring there were no air bubbles visible. The plate was then briefly centrifuged before placing it in the Viia 7 Real Time qPCR Machine.

Once the plate had been placed in the machine a template run application was commenced. This took approximately 90 minutes and involved cycling conditions consisting of 90^{o}C for 3 minutes followed by 40 cycles of 95^{o}C for 20 seconds, 50^{o}C for 30 seconds, and 72^{o}C for 30 seconds. Results were generated by the Viia 7 Real Time PCR software programme into excel sheets and melt curves, standard curves, and efficiency calculations were also established. 16s rRNA copies per well of the 96 well plate were then back-calculated to give bacterial concentrations expressed in both copy count/μl of extracted DNA preparation and copy count/g (faeces) or swab or brush.

2.4.7. Streptococcus-specific SYBRGreen qPCR preparation

Streptococcus-specific qPCR was analysed for any samples deemed to have adequate biomass for detection of streptococcus species. The overall standard operating procedure was similar to the above outline of 16s rRNA qPCR however with some alterations in terms of reaction volumes and primer use.

Based on previous work, the methionine aminopeptidase (*map*) gene was considered to be the gene akin to 16s to allow identification of only streptococcal species in a given sample of DNA. This is similarly highly conserved in streptococcus species. Primers were designed accordingly and obtained from Eurofins, Germany. The forward prime sequence was $^{5'}$GCWGACTCWTGTTGGGCWTATGC$^{'3}$ and the reverse sequence read as $^{5'}$TTARTAAGTTCYTTCTTCDCCTTG$^{'3}$. Forward length was 23 base pairs and reverse length was 24 base pairs and these resulted in the generation of amplification fragments of 348 base pairs respectively. Primers were added in concentrations of 10μM which was found to be the optimal concentration for amplification.

To establish the standard curve, Streptococcus mitis was used in the same serial dilution method as outlined above. Reaction volumes were again made up using a 96 well plate (MicroAMP Fast 96-well Reaction Plate 0.1ml) using 10μl of mastermix and 5μl of sample. Mastermix consisted of SYBRGreen Fast qPCR Kit Master Mix, primers, and PCR water in the following volumes, table 2.3.

Table 2.3: Components and quantities of the reaction mixture

Component	Volume in each well	Overall mixture volume
SYBR Fast qPCR Kit Master Mix	7.5μl	855μl
10μM F primer	0.5μl	57μl
10μM R primer	0.5μl	57μl
PCR water	1.5μl	171μl

The plate was sealed and centrifuged as outlined above and then loaded to the ViiA 7 Real Time qPCR machine. Cycle conditions were set to 90^{o}C for an initial denaturing step, then 40 cycles of 20 seconds at 95^{o}C for further denaturing, 30 seconds at 55^{o}C (assessed to be the optimal temperature for annealing) and 30 seconds at 72^{0}C for elongation. Results were generated by the Viia 7 Real Time PCR software programme into excel sheets and melt curves, standard curves, and efficiency calculations were also established. Streptococcus copies per well of the 96 well plate were then back-calculated to give strep concentrations expressed in both copy count/μl of extracted DNA preparation and copy count/g (faeces) or per skin/throat swab or lung brush.

2.5. Statistical Analysis

Data were analysed using Microsoft excel with data extension software package or GraphPad PRISM 8.0 (San Diego, USA). Students one-tailed paired t-Test was the test of choice in comparing airway physiological parameters where the data were normally distributed. Where data were not normally distributed, the Mann-Whitney U test was employed. The Friedman test was employed to test for differences in bacterial burden along the protocol timeline, as biological data were found to be non-parametric. Post-hoc analysis was used via Dunn's multiple comparisons test to assess at which time point the statistically significant difference, if any, occurred. Mixed effects modelling was used in the case of incomplete data sets. Statistical significance was determined as a two tailed p value <0.05. Results were significant when $p<0.05$.

Chapter 3: Airway Physiology and Biological Sampling

3.1 Introduction

Asthma is a disease characterised and defined by its intermittent nature. It is also a disease which imposes significant burdens on patients and healthcare systems alike in terms of morbidity and mortality, as well as lost labour days and extensive resource utilisation. There is a diagnostic difficulty when it comes to assigning this particular disease label to our symptomatic patients and this is due to the aforementioned intermittent nature of the condition. In recent times there has been a move away from classical physician-diagnosed asthma towards incorporation of objective pulmonary function testing into diagnostic criteria. Multiple trials are pointing towards a potential to over-diagnose the disease in the community. Thorough lung function testing and stringent adherence to criteria can improve our diagnostic accuracy. This can in turn mitigate the psychological and financial impact of an unnecessary or inaccurate disease label and can allow for correct identification of other etiological conditions causing similar symptoms. Additionally in terms of large scale studies, strict inclusion and exclusion criteria based on objective lung function testing can avoid the downstream effect of regression dilution bias which will attenuate the strength of any observed relationships. We aimed to recruit a cohort of patients with strong pre-test probability of an asthma diagnosis and to assess their baseline characteristics.

There is emerging evidence that the clinical phenotypes assigned to asthmatic patients can predict certain inflammatory pathways and vice versa therefore additional information about subjects was gathered in our questionnaire. This involved assessing for the presence of contributing comorbidities such as rhinosinusitis and gastroesophageal reflux. Past medical history and medication lists were documented to assess for other comorbidities. Blood eosinophils were measured in the local laboratory to assess for eosinophilic asthma.

Whole-body microbiome assessment involved sampling of the endobronchial environment using lung brushes, oropharyngeal environment using throat swabs, skin microbiome using skin swabs, and faecal samples as a proxy for gut

microbiome as outlined in the methods section above. The role of lung brush and throat swab sampling has long been defined in various assessments of the respiratory microbiome. The optimal method for assessment of skin microbiome remains a topic for debate. Sampling with a pre-moistened skin swab is an accepted and non-invasive method across the literature but it is noted that swabs result in lower biomass during downstream analysis than other methods such as skin scrapings and punch biopsy (124). In this study multiple sites were swabbed (right and left axilla and right and left antecubital fossa) as skin microbiome composition is known to be site-specific (124). With regard to the gastrointestinal microbiome the choice of optimal sampling technique is even more controversial. It has been shown that microbial composition varies between the lumen and the mucosa of the GI tract (125). It is assumed that faecal sampling represents the luminal microbiome of the distal colon but does not fully capture the complexity of the mucosal microbiome. What is unknown is whether the difference between these two communities infers a functional difference in the microbiome of the gut. Faecal sampling has the advantage of being non-invasive for our research subjects and so was favoured here.

3.2 Hypothesis, Aims, and Objectives

Asthma is a condition which is difficult to diagnose owing in no small part to the intermittent nature of the signs and symptoms which patients experience. Classically it has been diagnosed when patients whose symptom spectrum raises a strong clinical suspicion of the disorder and in whom trial of treatment has a therapeutic benefit. There is evidence to suggest that when there criteria are applied, asthma will be over diagnosed (15). This has many potential ramifications both for the patient, healthcare systems, and the integrity of medical research. We aimed to select a group of patients who demonstrated objective evidence of reversible airflow limitation or bronchial hyperresponsiveness in conjunction with a strong clinical suspicion of asthma to partake in this study of the asthmatic microbiome.

The microbiome is a relatively new topic for research in respect to the respiratory system. There is clear evidence that the microbiome of other systems, particularly that of the gastrointestinal tract, can have effects on the respiratory microbiome. However there is a dearth of studies which investigate multiple microbiome systems in an objective, integrative manner. We aimed to conduct a thorough assessment of the microbiome of the lung, oropharynx, gastrointestinal tract, and skin in a cohort of carefully selected asthmatic patients.

To fulfil these aims, the specific objectives of this chapter were:

Screen a group of physician-diagnosed asthmatics for evidence of objective reversible airflow obstruction or bronchial hyperresponsiveness.
Assess baseline characteristics, eosinophil status, disease severity, and medication use for this cohort of asthmatic patients.
Gather multiple samples from this cohort of asthmatic patients which would allow analysis of the microbiome of lung, oropharynx, gastrointestinal tract, and skin.

3.3 Results

3.3.1 Baseline characteristics

All adult patients attending the asthma clinic in James Connolly Memorial Hospital, Blanchardstown, were invited to participate, including both new and long-term patients. The study protocol was outlined to them and they were given a contact phone number to call once they had decided whether to proceed. They kept this contact number throughout the study period in order to report and adverse effects of intercurrent illness. All patients who wished to participate were offered an appointment to attend on a separate day for extensive lung function testing as outlined in the methods section. Those who had confirmed bronchodilator reversibility >12% and 200ml, or a positive bronchial challenge test result were enrolled in the study.

71 patients were invited to participate having been screened with the exclusion criteria as outlined in Appendix 1. These patients were asked to undergo the aforementioned lung function protocols in order to assess their asthmatic status. 27 (38.1%) were deemed to be non-asthmatic controls based on their results at lung function testing. These patients were informed of their results in the outpatient department by the study team. Clinical history was revisited and alternative diagnoses were assigned in all cases. 10 patients were diagnosed with gastroesophageal reflux disease, 13 with rhinosinusitis, 3 with hyperventilation syndrome, and 1 with vocal cord paradox. Treatment was instigated when necessary and patients were then entered into routine clinical follow-up schedules. No patients required reinstatement of asthmatic medications and none require repeated lung function testing over one year of routine follow up.

Of the 44 patients who fulfilled diagnostic criteria for asthma, all were offered inclusion in either the parent study or in this assessment of reproducibility of the microbiome. The parent study involved one set of lung function tests, one set of blood tests, and one bronchoscopy, rather than the repeated measures and additional biological sampling involved in this assessment. The majority, 31, opted

for this option and were enrolled in the parent study. 13 patients were enrolled in this study having satisfied all inclusion and exclusion criteria and having been diagnosed as asthmatic due to positive bronchodilator response on spirometry or positive methacholine challenge test results. 6 subjects (46%) were diagnosed due to bronchodilator response. Mean % reversibility was 17.33% and mean absolute reversibility was 346.66ml. 7 patients (54%) were diagnosed due to positive bronchial challenge test results with a mean PD20 of 0.5mg methacholine.
Table 3.1 outlines the baseline characteristics of the study cohort.

Table 3.1: Baseline characteristics of the study cohort

Parameter	**n = 13**
Male	7 (53.8%)
Female	6 (46.2%)
Age	46 (18 – 59)
BMI	24 (19 – 30)
Non-Smoker	13 (100%)
SABA	13 (100%)
LABA	9 (69.2%)
LAMA	3 (23.1%)
ICS	10 (76.9%)
LTRA	3 (23.1%)
Other related medications (nasal sprays, antihistamines)	4 (30.7%)
Other unrelated medications (antihypertensives, thyroid replacement medications, aspirin)	4 (30.7%)

All subjects required assessment with chest radiograph to out-rule any confounding conditions which may have contributed to their symptoms. 6 patients had had normal chest radiographs within one calendar month of enrolment in the study and so did not require these to be repeated. 7 patients required assessment with chest radiograph prior to the index bronchoscopy date, these also returned as normal studies.

Once enrolled patients were given a date for their first bronchoscopy test. There was at least a 10 day interval between lung function assessment and bronchoscopy

in order to allow for a time period during which participants could withdraw from the study without giving samples. On the morning of the bronchoscopy patients were asked to sign their consent and baseline biographical details were collected. They were also asked to answer a set of questions designed to assess their functional capacity in terms of illness burden. This information was used to stratify patients as having current mild, moderate, or severe symptoms. Medications were also recorded and allowed for stratification of the patient group in line with GINA treatment groups. The questionnaire is included in full in Appendix 5.

The information gathered in the patient questionnaires and assessment of the medication burden of each patient allowed us to stratify subjects according to their GINA treatment group. This is a method of stratifying patients according to the level of treatment they require to maintain adequate control of their symptoms and can be considered as a surrogate marker for disease severity. Mild asthma can be controlled with GINA step 1 or 2 of treatment, namely with low dose ICS and as-required SABA. Severe asthma requires treatment step 4 or 5 involving high dose ICS in combination with LABA and other pharmacological therapies such as oral theophylline and newer anti-IgE and anti-IL-5 therapy. According to these guidelines asthma is considered to be controlled if patients need little or no reliever (SABA) medication, can carry out their normal activities of daily living, and avoid exacerbations.

Table 3.2: Study participants stratified by GINA treatment group

GINA step	**n = 13**
GINA 1	3 (23.1%)
GINA 2	1 (7.7%)
GINA 3	4 (30.7%)
GINA 4	5 (38.5%)
GINA 5	0 (0%)

The presence of comorbidity was assessed through the questionnaire which was conducted on the day of bronchoscopy prior to the procedure. Results are summarised in table 3.3.

Table 3.3: Prevalence of comorbid conditions in the study cohort

Condition	**n = 13**
Rhinosinusitis	10 (76.9%)
Gastroesophageal reflux disease	2 (15.7%)
Eczema	4 (30.7%)
Other medical conditions	2 (15.7%)

No adverse events were reported by any patients in the group. All patients had been advised to discontinue levofloxacin in the event they developed Achilles tendon pain or discomfort but this did not occur for any subject in the study. One patient developed an intercurrent respiratory tract infection which necessitated treatment towards the end of the study period with antibiotics and steroids. She completed all physiological testing but did not undergo second bronchoscopy as it was deemed unsafe due to illness.

5 of the 13 patients in this cohort were considered to have elevated eosinophils, >0.4x10^9/L. Average eosinophil count for the elevated group was 0.6x10^9/L (range 0.44x10^9/L – 0.96x10^9/L) and 0.28x10^9/L (range 0.14x10^9/L – 0.31x10^9/L) in those with normal eosinophil levels. Eosinophil status was employed as a criteria for subgroup analysis in later chapters.

3.3.2 Bronchoscopy samples

11 of the 13 patients successfully completed both bronchoscopies through the study course. One patient did not complete the investigation cycle due to intercurrent illness and one patient due to difficulty attending on the scheduled day

for her repeat test. All bronchoscopies were deemed to be uncomplicated. All were performed as per study protocol under intravenous anaesthesia with Propofol and no adverse events were documented by the attending anaesthetists. Each initial bronchoscopy generated 12 samples: 1 sterile saline wash of the bronchoscope prior to intubation of the airway, 1 oropharyngeal throat swab, 2 lung brush samples from the left upper lobe, 2 lung brush samples from the left lower lobe, and 6 endobronchial biopsy samples from the right middle lobe. The repeat bronchoscopy generated one less endobronchial biopsy from the right middle lobe but otherwise samples were taken in the same manner. All samples were processed on the day and stored in the manner outlined in the methods section.

3.3.3 Additional samples

In addition to the above samples generated at the time of bronchoscopy, patients underwent skin swab sampling to yield a further 5 biological samples (1 control, 2 axilla, 2 antecubital samples). Subjects were instructed regarding the process of faecal sampling and were asked to return a sample to the hospital at their earliest convenience prior to commencing their antibiotic. Patients then attended the outpatients department for repeat skin and throat swab sampling at regular intervals between their antibiotic course and the second bronchoscopy. They returned a faecal sample at each visit. The summary table of samples is shown below in table 3.4. Outlined is the intended total number of samples for each patient, the intended total, and the final number of samples generated.

Table 3.4: Samples generated during the study period

Sample type	**Intended number per patient**	**Intended total**	**Final number**
Lung brush	8	104	96
Lung biopsy	11	143	133
Oropharyngeal throat swab	4	52	49

Sample type	Intended number per patient	Intended total	Final number
Sterile saline wash	2	26	24
Control skin swab	4	52	50
Left axilla skin swab	4	52	50
Right axilla skin swab	4	52	50
Left antecubital skin swab	4	52	50
Right antecubital skin swab	4	52	50
Faecal sample	4	52	37
	49	**637**	**589**

92.5% of the samples intended for collection were collected. The biopsy samples and sterile wash samples were collected for use in the parent study of this MD project and were not intended for analysis in this project. These biological samples, having been processed at source in the manner outlined in the methods section, were then transported to the Imperial College London for extraction and analysis.

3.3.4 Time intervals

Taking the first bronchoscopy to be day zero of the study period, it was intended that antibiotics would be taken from day 1 to day 10. Second sampling of the throat, skin and faeces would occur at week 4, third sampling at week 6, and fourth sampling to coincide with the repeat bronchoscopy 8 weeks after the first scope. This however was not the case and the below table, 3.5, represents the real-world time intervals of the study period. The columns represent the weeks of the study and each row represents a subject. AS indicates commencement of antibiotic and AF indicates completion. Each X represents a set of biological samples, skin throat and faeces. Three patients were delayed in starting their antibiotic course and this

led to a slight time discrepancy with the larger group, though the time intervals remained near-constant. One of these three did not complete sampling to second bronchoscopy and so did not give the 4th set of samples (subject no. 8).

Table 3.5: Sample time intervals for the study cohort.

X - collection of samples

AS - start of antibiotic course

AF - end of antibiotic course

	Wk 1	**Wk2**	**Wk 3**	**Wk 4**	**Wk 5**	**Wk 6**	**Wk 7**	**Wk 8**	**Wk 9**	**Wk 10**
1	AS	AF		X		X		X		
2	AS	AF		X		X				
3	AS	AF		X		X		X		
4	AS	AF		X		X		X		
5	AS	AF		X		X		X		
6	AS	AF		X		X		X		
7	AS	AF		X		X		X		
8		AS	AF		X		X			
9	AS	AF		X		X		X		
10		AS	AF		X		X		X	
11	AS	AF		X		X			X	
12		AS	AF		X		X		X	
13	AS	AF		X		X		X		

3.4 Discussion

This study enrolled only those patients with demonstrable bronchodilator reversibility or bronchial hyperresponsiveness as asthmatic subjects. This is a more stringent manner of recruitment than seen in many trials in the area of asthma and is a strength of the study. Strict subject selection leads to a more cohesive study cohort and can have the effect of strengthening any observed relationships in terms of downstream data analysis. It also removes the potential for regression dilution bias, the phenomenon that occurs when the exposure variable is incorrectly measured and relationships between the exposure, asthma, and the outcome are attenuated.

This study found that 38.1% of those patients attending a tertiary referral asthma centre with complaints of cough, wheeze, and dyspnoea did not demonstrate reversible airflow limitation or positive bronchial challenge test results. There are many potential explanations for this. Firstly asthma is known to be an intermittent disease and patients may have been screened out of their usual exacerbation cycle. Subjects were often on inhaled medications when referred and there may have been a false negative reading on bronchodilator response due to non-cessation of medications as instructed prior to their lung function testing. Fixed airflow obstruction is a phenomenon which is well known to affect asthmatics, especially if they are older, male, or have had longer duration of their asthma (126). However, the combination of assessment of bronchodilator reversibility and bronchial hyperresponsiveness gives us detailed information which can help us to decide if patients are likely to asthmatic or not. Methacholine challenge testing confers a high negative predictive value on the diagnostic process involved in assigning an asthmatic label to a patient complaining of the cardinal symptoms of the disease. The test is known to be optimally valuable as a diagnostic tool when the pre-test probability of asthma is 30-70% (127), the pre-test probability increasing linearly with symptom burden. Therefore in patients who actively exhibit symptoms of asthma, as this group did, methacholine challenge testing, when negative, effectively out-rules a diagnosis of asthma (128). Additionally the test is less

sensitive to the above conditions of pre-test medications and the intermittent nature of asthma itself.

Indeed there has recently been much interest in this area and particularly the concept of physician-diagnosed asthma versus physiologically diagnosed disease. Classically asthma has been a condition which has relied heavily on clinical history and examination as the diagnostic criteria. This has been reinforced in guideline documents for many years. Only recently have the guidelines available to physicians reflected a change in the approach to this diagnosis, aiming to incorporate objective measures of reversible airflow limitation in addition to the clinical cornerstones of history and examination. Despite evolution of the diagnostic process, physician-diagnosed asthma cases far outnumber those cases which employ confirmatory physiological tests. The majority of asthma in Ireland, a country in which the prevalence of this condition is high and the incidence increasing, is diagnosed at the primary care level. Often, only complex cases or those in whom achieving asthma symptom control proves difficult are ever referred to tertiary respiratory clinics. Due primarily to lack of access to high quality spirometry and other pulmonary function tests which often fall under the remit of specialist respiratory centres, physicians often have to rely on their clinical acumen in order to establish a diagnosis. However these results demonstrate that not all patients who exhibit clinical signs and symptoms of asthma can be considered strictly asthmatic when physiological criteria are applied. This finding has been replicated across the literature with strikingly similar proportions to the results of this study (15, 129).

The non-asthmatic cohort in our study were all re-evaluated clinically and alternative diagnoses were assigned as outlined above. Patients were taken off inhaled medications in all cases and remained well during their standardised clinical follow-up period. None of the cohort required re-evaluation for a diagnosis of asthma once treatment of the underlying condition had been commenced. This would seem to suggest that exclusion of asthma if correctly applied could reduce inhaled treatment burden, reduce the related cost of medications, and allow

correct identification and treatment of underlying conditions. However our sample size is small and though our results have been replicated in larger international studies, further research is warranted prior to drawing meaningful conclusions.

It is clear that asthma is a heterogenous disease which is often intermittent in its nature and these factors confer significant difficulty on the diagnostic process. There are many other conditions which cause the same symptom spectrum in patients and mis-identification of these can lead to overdiagnosis of asthma and more importantly, underdiagnosis of the actual cause of the symptoms. Though overdiagnosis of asthma can have significant ramifications for patients in terms of the psychosocial and financial burdens of chronic disease, underdiagnosis would certainly be more problematic given that asthma is a disease high in morbidity and mortality. Better access to lung function testing could both streamline the diagnostic process and enable phenotyping of this common disease.

This study protocol generated a large bio-bank of samples from a cohort of physiologically-diagnosed asthmatic patients. The study period was intensive for patients as a result. Not all intended samples were obtained from subjects. Skin swab return was high as the study team carried out the sampling directly. In contrast, 37 out of a possible 52 (71%) faecal samples were returned. Faecal sampling was designated as the microbiome sampling method of choice as it is relatively non-invasive and accurately profiles the luminal microbiome of the gut as discussed in the introduction section. However there remain limitations to the sampling method, making it logistically difficult for our patients. Many patients froze their samples at home prior to transport to the hospital for their clinic visits. This was cumbersome and demanding and this is reflected in the lower proportion of sample return compared to other modalities. Nevertheless the sample number reflects a robust longitudinal assessment of the gastrointestinal microbiome in asthmatics after antibiotics.

Longitudinal analyses such as undertaken in this study are strengthened when there is strict adherence to consistent time intervals. Unfortunately our study is

limited as the time intervals at which samples were collected were often irregular. As already mentioned, this study protocol was intensive and demanding on our subjects. They gave their time freely and were not compensated for their participation. Often subjects would attend the hospital in place of work and other commitments and for this we thank them. As a result the time intervals between sampling visits are not consistent throughout the study period. The study remains valuable as a longitudinal analysis after antibiotics, strengthened by the high sample numbers and repeated sample intervals.

3.5 Conclusion

This study enrolled a carefully selected cohort of asthmatics who were diagnosed when clinical suspicion was confirmed with demonstrable physiological parameters. Assessment of bronchodilator reversibility and bronchial hyperresponsiveness are powerful tools in the diagnosis of this complex disease and can be useful together particularly in the case of out-ruling asthma. It seems that asthma can be ruled out, alternative diagnoses can be assigned, inhaled medications can be stopped, and symptom control can be achieved in approximately one third of patients with physician-diagnosed asthma when physiological criteria are applied. Though the cohort is small in this case, the findings compare favourably with the literature and suggest that there is an important role for the re-evaluation of this diagnosis in many patients attending respiratory centres.

Sampling of the microbiome is a difficult and multi-faceted process particularly when undertaken in a longitudinal fashion. This type of assessment confers significant demands on patients and trial subjects. There is ongoing controversy in the field regarding the optimal sampling methods for various microbiome systems. Many of these methods are laborious for both subjects and researchers and it is not agreed upon as to which samples result in superior results downstream. In the following results chapters we will assess the detailed airway physiology and microbiome analyses which were undertaken on the described cohort and sample bank.

Chapter 4: Airway Physiology and Asthmatic Profiling

4.1 Introduction

Documentation of airway physiology is key to understanding the complex nature of asthma as a disease. Not only is assessment of lung function integral to the diagnosis of this disease as we have already discussed, but accurate knowledge of the underlying physiology can help us to direct treatment options for our patients.

Spirometry is a non-invasive and easily performed test which allows accurate assessment of multiple lung function parameters, most importantly FVC and FEV_1 . It is a test that is readily available and though it is not always easy to access at the primary care level, it enables practitioners to diagnose and follow a range of disease processes. Asthmatic airways will result in an obstructive pattern on spirometry and will be accompanied by characteristic reversibility of this obstruction on administration of bronchodilators. Despite its ubiquitous nature, spirometry tends to be under-utilised by practitioners and remains mostly used by respiratory specialists in a hospital setting. This can contribute to over-diagnosis and over-treatment of obstructive airways diseases in primary care and more worryingly, undertreatment of the causative condition. Equally, underutilisation of this user-friendly assessment can lead to under-diagnosis of asthma, particularly in those with intermittent symptoms. Even when used correctly in the context of clinical suspicion for the disease, a certain proportion of asthmatic patients will not show acute bronchodilator responsiveness. Even more confusingly, those with other obstructive airways diseases such as COPD may show significant reversibility and similar symptoms (130).

Thorough and accurate profiling of asthmatic patients with other lung function tests can strengthen diagnoses, direct treatments, and even monitor compliance when used correctly in conjunction with clinical history and examination. Bronchial challenge testing is highly sensitive for asthma and has an associated high negative predictive value (131). In practice it is used as an add-on test where there is doubt over the validity of an asthma diagnosis. While there are situations in which false negative and false positive results may arise, overall when used in the correct

clinical context it is a hugely valuable asset in the diagnosis of asthma. Fraction of exhaled nitric oxide measurement is a proxy measure for endobronchial eosinophilic inflammation. Eosinophils upregulate inducible nitric oxide synthase in bronchial cells to produce higher levels of NO. Though FENO cannot be used on its own to support a diagnosis of asthma, it can be used as a supporting test to add to the likelihood of asthma diagnosis in patients with otherwise equivocal pulmonary function tests (53). Additionally, this test can help physicians to direct treatment strategies as an elevated FENO if >50 parts per billion (ppb) is known to be associated with a good response to inhaled corticosteroids (ICS) in steroid naïve patients (51, 132, 133). There is also a case for using FENO as an indirect marker of a patient's compliance with ICS, as an initial elevated FENO would be expected to fall with adherence to ICS therapy (132).

Much of the background literature with regard to end tidal CO_2 measurement in asthma centres around acute exacerbations of the disease. CO_2 has been documented to be lower (<35mmHg) in asthma exacerbation independent of respiratory rate when compared with a control group (134). The level of $ETCO_2$ does not seem to correlate well with the degree of disease severity in the acute setting (135). Carbon dioxide is known to be a potent bronchodilator (136, 137). Therefore it follows that hypocapnia may be associated with bronchoconstriction and there is some evidence in the literature to support this (138). Hyperventilation is known to complicate asthma both in times of exacerbation and between acute episodes and this is one mechanism proposed to explain hypocapnia in asthmatics. However one interesting study by Osborne et al. (139) concentrated on the role of hyperventilation in chronic asymptomatic asthmatic patients and found that $ETCO_2$ was significantly reduced in these patients independent of hyperventilation and seemed to correlate with airway hyperresponsiveness. The majority of these studies were small observational studies and therefore it is difficult to ascertain the exact relationship between hyperventilation, hypocarbia and bronchoconstriction in asthma but it is certain that there is a role for further investigation.

Inhaled allergens play a pivotal role in asthma, both in terms of pathogenesis and propagation of the disease (140, 141). There is clear evidence that prolonged avoidance of known allergens can result in reductions in bronchial hyperreactivity (142) and sputum eosinophil counts (143) with overall improvements in subjective measures of symptom control. Therefore identification of specific allergens is important for our asthmatic patients. Often avoidance of allergic triggers is infeasible and in these cases there is building evidence for the employment of sub-lingual immunotherapy (SLIT). This involves application of allergen to the sublingual tissue via tablet or drop formulation in order to induce immune tolerance to the particular causative allergen. It has been shown to be safe in mild asthma and though there is debate regarding its efficacy, it is suggested in the GINA guidelines to consider the therapy as an adjunct to inhaled treatments. Therefore accurate profiling of the allergic tendencies of an asthmatic may be beneficial with regard to full exploration of treatment options.

Asthma guidelines endorse the use of these adjunctive tests, when applied in the correct clinical context, to support a diagnosis of asthma (19). Regarding bronchial provocation testing, the GINA guideline states "a negative test in a patient not taking ICS can help to exclude asthma, but a positive test does not always mean that a patient has asthma – the patterns of symptoms and other clinical features should be taken into account." Guidelines support the use of skin prick allergy testing as the presence of atopy, as identified by skin prick testing or measurement of specific immunoglobulin E, increases the probability that a patient with respiratory symptoms has allergic asthma. It can also inform on the role for adjunctive therapies such as sub-lingual immunotherapy (SLIT) which is suggested as an add-on therapy in mild allergic asthma with persistent respiratory symptoms. There is level B evidence that this therapy is useful in those asthmatics with allergic rhinitis sensitised to house dust-mite who have troublesome symptoms but an FEV_1>70% predicted (144, 145). There is more reticence regarding FENO assessment as a potential diagnostic adjunct. However there is good evidence that a higher FENO level in steroid-naïve patients at the time of diagnosis indicates better responses to treatment with inhaled ICS (51). FENO measurement is also

utilised to excellent effect in the monitoring of compliance with treatments, particularly the use of inhaled corticosteroids. FENO should suppress in asthmatics who are compliant with their inhaled corticosteroid regimens and non-suppression of FENO can identify nonadherence with treatments (52). Therefore FENO assessment does play an important role in the profiling of asthmatics and in the monitoring of disease, if not in the diagnosis of the condition.

There is certainly a place for thorough assessment of multiple physiological parameters in both the diagnosis and the management of asthma and this is reflected in evolving asthma guidelines.

4.2 Hypothesis

Multiple measures of airway physiology are characteristically altered in asthma. These alterations form part of the diagnostic criteria for the disease as we have discussed, and also can inform us regarding the phenotype of asthmatic patients. Physiological phenotyping can in some cases inform on the utility of various treatment options but despite the importance of these tests, they tend to be under used. It is unclear as yet how the microbiome interacts with airway physiology, particularly in the context of treatment courses such as antibiotics. We believe that thorough and robust assessment of multiple lung function tests contribute to our understanding of this disease process and furthermore that this type of profiling is essential in any investigation of the respiratory microbiome. We aim to construct thorough physiological profiles of this asthmatic cohort.

To fulfil this aim, the specific objectives of this chapter were:

Establish full physiological profiles for this cohort by assessing spirometry, lung volumes, diffusion capacity, fraction of exhaled nitric oxide, end tidal carbon dioxide, and skin prick allergy testing.
Repeat this assessment after a course of oral levofloxacin to assess for any significant changes.

4.3 Results

4.3.1 Confirmation of asthma: spirometry and bronchial provocation

20 patients underwent detailed lung function testing at the outset of the study period. 7 were deemed to be non-asthmatic controls based on lack of demonstrable bronchodilator response or bronchial hyperresponsiveness. 13 patients were found to be asthmatic due to a combination of their lung function results and suitable clinical history. 6 subjects (46%) were diagnosed based on positive bronchodilator response >12% and 200ml reversibility. Mean % reversibility was 17.33% and mean absolute reversibility was 346.66ml. In the case where bronchodilator reversibility was not present on spirometry, patients then went on to have methacholine challenge testing performed. 7 patients (54%) were diagnosed due to positive bronchial challenge test results with a mean PD20 of 0.5mg methacholine.

In terms of the detailed physiological assessments, full spirometry was performed and accompanied by lung volume measurement via plethysmography. All patients had FEV_1/FVC ratio of less than 70% indicating obstructive airways disease. All similarly had concave flow-volume loops. Diffusion capacity for carbon monoxide was also assessed. Mean results for the group are outlined in table 4.1.

Table 4.1: Baseline spirometry, lung volumes, and diffusion capacity

Parameter	**Mean value (l) (range)**	**Mean % predicted (%) (range)**
FVC	4.05 (1.88 – 5.72)	96.57 (79 – 124)
FEV_1	2.96 (1.2 – 5.13)	82.85 (49 – 127)
TLC	5.95 (4.37 – 8.22)	101.01 (81 – 120)
RV	2.16 (1.48 – 3.43)	117.54 (75 – 159)
DLCO	__	84.46 (62 – 122)

4.3.2 Fraction of exhaled nitric oxide

Fraction of exhaled nitric oxide was measured as outlined in the methods section and patients were asked to withhold their inhaled corticosteroids for 72 hours prior to testing. All patients completed this test and the mean result was 38 parts per billion indicating mild levels of eosinophilic inflammation (normal level <25ppb). The range spanned from 13 ppb to 203 ppb. 4 patients had levels greater than 50ppb indicating severe eosinophilic inflammation, 3 patients had levels of 25-50ppb indicating mild to moderate eosinophilic inflammation, and 6 patients had normal FENO as illustrated in table 4.2.

Table 4.2: FENO results per subject, mean of 3 test attempts represented

Subject ID	**FENO (ppb)**
1	14
2	163
3	32
4	24
5	18
6	16
7	13
8	25
9	203
10	20
11	60
12	95
13	38

4.3.3 End tidal carbon monoxide

End tidal carbon monoxide measurement took place during the same testing session in order to assess for any co-existing hyperventilation overlay. Mean results were 34mmHg, a normal result lying between 35mmHg and 45mmHg. This indicated a slight tendency towards hyperventilation overall. The range of results spanned from 22mmHg to 42mmHg with 3 patients demonstrating low end tidal CO_2 measurements. Results are illustrated in table 4.3.

Table 4.3: $ETCO_2$ results per subject. Respiratory rate also represented

Subject ID	$ETCO_2$ mmHg	Respiratory Rate
1	38	17
2	40	18
3	36	16
4	33	18
5	28	19
6	42	22
7	22	18
8	41	17
9	36	18
10	38	19
11	37	17
12	38	20
13	36	18

4.3.4 Skin prick allergy testing

Skin prick allergy testing was undertaken on all patients after a washout period of 4 days with regard to oral antihistamine preparations. There were no adverse events noted during administration of the test or immediately after testing had been completed. Allergens tested included dog, cat, horse, cow, dust-mite, tree, weed,

and grass. 1 patient self-reported a previous episode of anaphylactoid reaction to horse dander and this allergen was therefore withheld from her panel. 1 patient of the cohort did not exhibit any reaction to the allergens tested. 3 patients reacted to single allergens, and the remainder mounted responses to multiple allergens. Please see the full outline of the results in the following table, 4.4.

Table 4.4: Skin prick allergy test results per subject

Subject ID	**Allergen(s)**
1	Cat
2	Dust mite
3	Cat, dog
4	Cat, dog, cow, dust mite, grass
5	Grass, weed, tree
6	Dust mite, grass, weed, tree
7	Cat, dog, dust mite, grass, weed, tree *horse not tested due to previous anaphylactoid reaction
8	Cat, dog, grass
9	Cat, dog, horse, dust mite, grass, weed
10	Dust mite, tree
11	Dust mite
12	Negative
13	Dust mite, grass

4.3.5 Subgroup analysis by eosinophil status

We conducted sub group analysis of airway physiology parameters according to subjects' pre-antibiotic eosinophil count. Eosinophils were considered elevated if $>0.4x10^9$/L. 5 patients in the cohort had elevated eosinophil levels while 8 had normal levels. There were significant differences in baseline physiology when patients were stratified in this way. FEV_1 (absolute value) and FVC (absolute value)

were found to be lower in those with elevated eosinophil levels. DLCO also appeared lower in this group. The results are illustrated in the following table, 4.5.

Table 4.5: Comparison of airway physiology in normal eosinophil subjects vs elevated eosinophil subjects

Parameter	**Elevated eosinophils >0.4x10^9/L**	**Normal eosinophils <0.4x10^9/L**	**p-value**
FEV_1 (l/min)	1.96	3.37	0.0208
FEV_1%	72.2	94.87	0.056
FVC (l/min)	3.07	4.46	0.0286
FVC%	94.6	104.37	0.204
TLC (l)	5.24	6.4	0.125
TLC%	102.2	100.37	0.814
RV (l)	2.25	1.67	0.456
RV%	128	111	0.386
DLCO%	72.4	92	0.20
FENO (ppb)	55.8	55.25	0.988
$ETCO_2$ (mmHg)	34	36.82	0.386

Analysis was also undertaken to assess for correlation between eosinophil count and physiological parameters using linear regression modelling. This was undertaken using all the aforementioned parameters and there were no statistically significant relationships uncovered. Figure 4.1 illustrates this correlation assessment for FEV_1%, FVC%, and FENO.

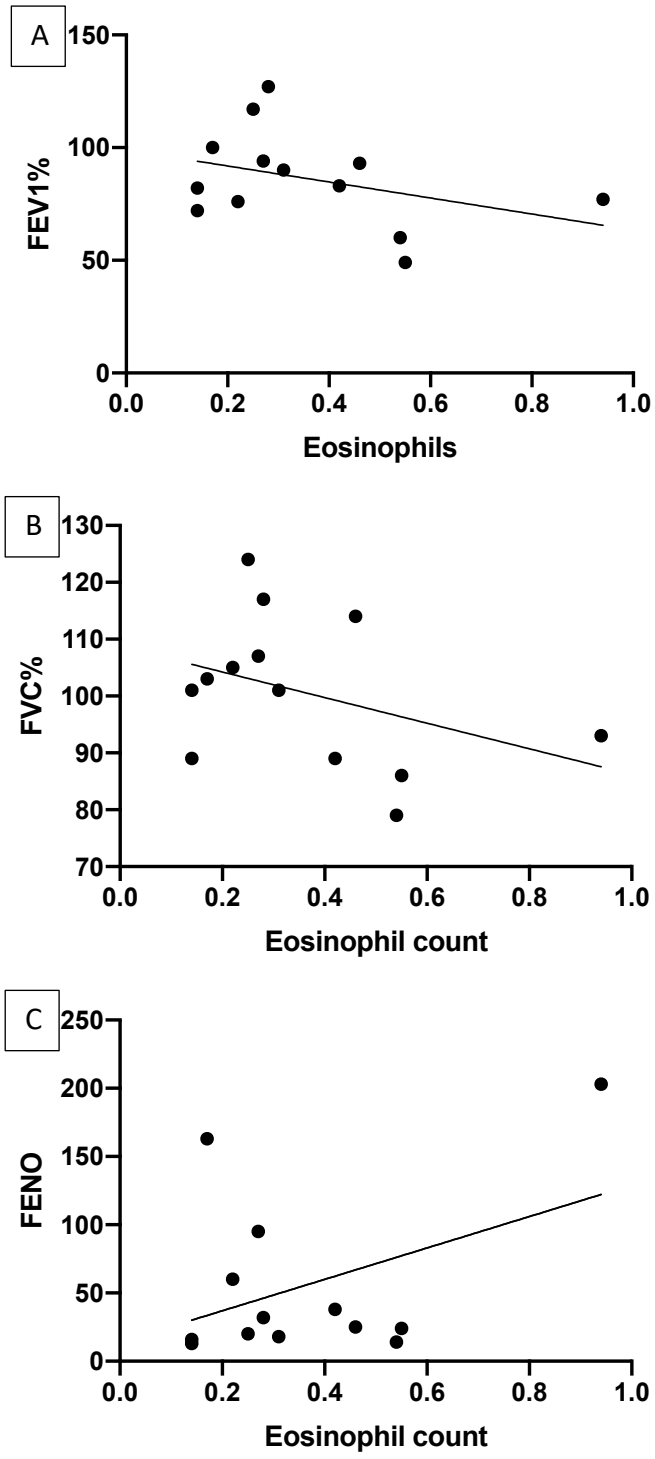


Figure 4.1: Physiological parameters correlated with eosinophil count

A: Eosinophil count correlated with FEV_1%, not statistically significant p=0.208

B: Eosinophil count correlated with FVC%, not statistically significant p=0.195

C: Eosinophil count correlated with FENO, not statically significant p=0.154

4.3.5 Repeated measures of airway physiology

All of the above tests were repeated 8 weeks after the antibiotic course, just prior to repeat bronchoscopy. The same methods were employed including the same inhaler washout protocol. The below table summarises the mean results pre- and post-antibiotic. All data were analysed for normality and found to be normally distributed therefore Student's t-test was applied and the p-values are outlined in the last column of table 4.6.

Table 4.6: Repeated measurement of airway physiology and comparative statistics

Parameter	Pre antibiotic	Post antibiotic	p-value
FEV_1 (l/min)	2.96	2.89	0.95
FEV_1%	82.85	79.71	0.82
FVC (l/min)	4.05	4.06	0.71
FVC%	96.57	95.71	0.87
TLC (l)	5.95	6.16	0.88
TLC%	101.01	103.28	0.90
RV (l)	2.16	2.32	0.80
RV%	117.54	128.42	0.73
DLCO%	84.46	90.57	0.67
FENO (ppb)	38	26	0.057
$ETCO_2$ (mmHg)	34	36	0.48

There were no observable differences in the skin prick allergy testing results when repeated.

The same patients who were diagnosed as asthmatic due to positive bronchodilator response again showed positive bronchodilator response at 8 weeks. The magnitude of the reversibility seen was slightly less, 236.7ml post antibiotic vs 346ml, p = 0.0299.

Bronchial hyperresponsiveness was assessed in the same 7 patients who had it originally measured and there was no significant difference, PD 20 was 0.4mg methacholine pre antibiotic vs 0.49 mg methacholine post antibiotic.

When airway results were stratified by eosinophil status, there were similarly no differences in the parameters as measured pre- and post- antibiotic course as illustrated in tables 4.7 and 4.8. We considered eosinophils to be elevated if $>0.4x10^9$/L. 5 patients had elevated eosinophils and 8 patients had normal eosinophils. Pre antibiotic and post antibiotic PFTs were similar in both groups.

Table 4.7: Repeated measurement of airway physiology and comparative statistics in those subjects with elevated eosinophil level $>0.4x10^9$/L

Parameter	Pre antibiotic	Post antibiotic	p-value
FEV_1 (l/min)	1.96	2.04	0.55
FEV_1%	72.2	75.2	0.537
FVC (l/min)	3.07	3.16	0.49
FVC%	94.6	97.4	0.43
TLC (l)	5.25	4.79	0.36
TLC%	102.2	101.2	0.84
RV (l)	2.25	1.99	0.33
RV%	128.0	112.2	0.348
DLCO%	72.4	89.4	0.383
FENO (ppb)	55.8	53.0	0.177
$ETCO_2$ (mmHg)	34.0	34.4	0.846

Table 4.8: Repeated measurement of airway physiology and comparative statistics in those subjects with normal eosinophil level $<0.4x10^9$/L

Parameter	Pre antibiotic	Post antibiotic	P-value
FEV_1 (l/min)	3.37	3.31	0.516

Parameter	Pre antibiotic	Post antibiotic	P-value
FEV_1%	94.87	92.02	0.393
FVC (l/min)	4.46	4.44	0.73
FVC%	104.37	103.12	0.57
TLC (l)	6.4	6.63	0.127
TLC%	100.37	101.3	0.50
RV (l)	1.67	38.36	0.74
RV%	111	116.87	0.354
DLCO%	92	86.37	0.133
FENO (ppb)	55.25	38.75	0.086
$ETCO_2$ (mmHg)	36.82	37.75	0.46

4.4 Discussion

These results indicate the complexity of airway physiology assessment in asthma. Firstly it must be noted that this was an extensive panel of lung function assessments which took place in a single sitting and was demanding both physically and logistically for subjects. Both the pre antibiotic and post antibiotic assessments required patients to attend for roughly two hours to complete testing. Multiple medications were withdrawn in the days prior to testing in order to adhere to best practice principles as outlined in the methods section. No subjects reported adverse effects while not on their inhaled medications for these days. There were no adverse reactions or complications throughout the testing period and we thank the physiologists of the Respiratory Pulmonary Function Laboratory of Connolly Hospital for their expertise.

The group showed very similar results in their baseline measurements. FEV_1 mean was favourable at 82.25% predicted indicating overall a mild level of airflow obstruction. The range in which the results fell was expansive (49-127% predicted) indicating that subjects demonstrated inter-group heterogeneity of obstructive airflow limitation. As mentioned previously, not all patients demonstrated reversible airflow obstruction on administration of inhaled bronchodilator. Those who were not reversible went on to undergo bronchial provocation testing where airway hyperresponsiveness was observed, confirming their asthma diagnosis. Total lung capacity, residual volume, and diffusion capacity were unremarkable for the cohort. Spirometry parameters did not differ significantly post antibiotic course despite the fact that asthmatic patients are known to have variable airflow limitation and that this is often reflected in serial spirometry measurements. As discussed previously this inherent variability can form part of diagnostic algorithms for asthmatic patients. There are likely a number of reasons for the finding that spirometry remained overall unchanged during the study. The study cohort were a group of objectively diagnosed, treated, and well controlled asthmatics in whom little interval change would be expected with regard to spirometry measurements. Additionally all medications remained unchanged for the duration of the study

period. Finally the 8-10 week study period would not have allowed for the confounding effects of seasonal change to effect measurement parameters. Therefore it is unsurprising that spirometry remained relatively constant in the cohort.

Fraction of exhaled nitric oxide continues to be used in clinical practice in the workup and particularly in the follow-up of asthma, especially in those with an eosinophilic phenotype. Results were very varied in this study with results ranging from 13ppb to 203ppb. 4 patients (30.7%) had levels greater than 50ppb indicating severe eosinophilic inflammation, 3 patients (23%) had levels of 25-50ppb indicating mild to moderate eosinophilic inflammation, and 6 (46.1%) patients had normal FENO. Those with elevated FENO levels did not have significantly different spirometry parameters or allergic profiles than those with normal levels. Those with elevated FENO were all treated with inhaled corticosteroid, an intervention widely expected to lower FENO. Mean FENO decreased from 38ppb to 26ppb at the end of the study period, this result approached statistical significance, p=0.057. There is conflicting evidence regarding the impact of antibiotic courses on FENO across a range of respiratory illnesses. It is widely held that FENO does not vary predictably or reproducibly after antibiotics therefore its use is not encouraged in times of exacerbation. It is therefore unknown whether this reduction in FENO could be related to the intervention. What is more likely, and well described in the literature, is that an element of subject bias has occurred. Due to the knowledge that they are enrolled in an observational trial, subjects are known to occasionally change their behaviour to act in a way they think is expected by the researcher. In this case it could be commented that use of inhaled corticosteroid became more stringent and regular for subjects during the period of the trial as a result of aforementioned subject bias. This observation is made more likely in our study design as visits to the research team were frequent and there was a high level of interaction between patients and the hospital setting.

End tidal carbon dioxide measurement was included in our trial protocol to assess for the confounding effects of hyperventilation on asthmatic airways. $ETCO_2$

measurement is ubiquitous in the intensive care setting and has become commonplace in the management of cardiac arrest when it can be used to assess the quality of CPR manoeuvres. $ETCO_2$ is felt to be a reliable proxy for arterial CO_2 measurement, and one which is markedly less invasive. The gradient between $ETCO_2$ and $PaCO_2$ tends to increase with age and increasing pulmonary dead space. Elevated $PaCO_2$ in blood is known to be associated with a bronchodilator effect therefore it has been postulated logically that the reverse could be true of low $PaCo_2$. The relationship between $PaCO_2$ and bronchoconstriction has been studied in various trials but results are conflicting and research centres mainly around animal models rather than the potential downstream clinical effects in our patients. We postulate that low $PaCO_2$ could be associated with the cardinal symptoms of shortness of breath, wheeze, and cough seen in those patients referred for workup of asthma and that in the absence of objective reversible airflow obstruction hyperventilation could be attributed as a causative factor for symptoms. Hyperventilation is also known to compound the symptoms of asthma and is thought to complicate asthma in both the acute and chronic state. In our study mean $ETCO_2$ results were 34mmHg, a normal result lying between 35mmHg and 45mmHg. This indicated a slight tendency towards hyperventilation overall. The range of results spanned from 22mmHg to 42mmHg with 3 patients demonstrating low end tidal CO_2 measurements. This did not change significantly after the intervention. The relationship between $PaCO_2$ and asthmatic airways is largely uncertain but $ETCO_2$ measurement remains a non-invasive measure of $PaCO_2$ and can be employed in the physiological profiling of asthmatic patients to assess for the potential compounding effect of hyperventilation on asthmatic symptoms.

The majority of our cohort (85%) were considered to be atopic due to the presence of rhinosinusitis, eczema, or both. All of these patients self-reported allergies to various agents and therefore it is no surprise that the proportion of positive skin prick allergy test results was so high. One patient from the cohort had a negative test result when exposed to a standard panel of allergens. Three patients reacted to a single allergen while the remainder of the cohort demonstrated reactions to multiple topically applied allergens. The profile of skin prick results did not change

at all after antibiotics and again this is unsurprising. Atopic asthma continues to be the most common form of asthma in all ages and identification of problematic aeroallergens is important for our patients in terms of avoidance strategies and in terms of potential treatment strategies such as sublingual immunotherapy (SLIT).

4.5 Conclusion

Thorough physiological assessment of asthmatic patients can infer valuable information that standard isolated assessment of spirometry may overlook. This study suggests that not all asthmatic patients readily demonstrate reversible airflow obstruction and that addition of bronchial provocation assessment can prove crucial in the diagnostic workup of asthma where there is clinical concern for the condition. This observation raises the possibility of a distinct phenotype of asthmatic patients who do not show bronchodilator response on spirometry but readily demonstrate airway hyperresponsiveness and this observation requires further research in larger cohort studies. FENO and $ETCO_2$ are considered to be supplementary tests with a narrow range of indications but they can be used to good effect to strengthen the diagnosis of asthma and also to monitor response to treatment interventions. Assessment of allergic status is vital and can have an impact on our patients quality of life through allergen avoidance and immunotherapies. These markers of airway physiology seem to be reproducible over time in the cohort despite addition of antibiotics and this shows them to be a reliable set of parameters of airway physiology.

Chapter 5: Respiratory Microbiome

5.1 Introduction

The respiratory microbiome is altered in asthmatic patients (83). This has been clear from the very first assessment of the respiratory microbiome, albeit assessed in relatively small numbers (83). Proteobacteria members, particularly *Haemophilus* species, were found to be dominant in asthmatic airways compared to controls. This finding has been borne out in subsequent studies (88, 95, 146-148). This expansion in members of the Proteobacteria phylum is associated with an overall reduction in bacterial diversity in the community. Expansion of particular community members can be associated with various markers of disease severity. For example, in one of the earliest studies into the respiratory microbiome of asthmatic patients, Huang et al. found that the relative abundances of members of the *Comamonadaceae, Sphingomonadaceae,* and *Oxalobacteraceae* families correlated highly with the degree of bronchial hyperresponsiveness (88). Green et al studied 28 patients with treatment-resistant asthma and discovered that *Moraxella* and *Haemophilus* were dominant and that these species correlated well with longer asthma duration, worse post-bronchodilator FEV_1 (% predicted), and higher sputum neutrophil content (149). Taylor et al. also observed a high correlation between expanded *Haemophilus* and *Moraxella spp* and neutrophilic airways (148).

Direct causality has not yet been established in the case of these observations, however much work is ongoing to discover the mechanisms by which microbial dysbiosis may lead to allergies and asthma. It has long been observed that children who lack exposure to bacteria in early life have a higher risk of developing allergic diseases later in life (71). In a study which assessed bacterial content of the home and correlated this against risk of asthma, asthma prevalence was shown to be lower in those with elevated levels of *Bifidobacteriaceae* and *Clostridia* cultured from windowsills (150). A separate study showed that reduced exposure to Firmicutes and Bacteroidetes predisposed to atopy and wheeze in children living in inner city areas (151). The vast differences in the prevalence of asthma between Amish and Hutterite farmers, two groups who share similar genetic ancestries but

differ in their farming practices, seems to be accounted for by differential microbial exposure in youth (72). Studies which directly assess the microbiome, rather than correlate asthma prevalence with environmental exposures, seem to concur with the above observations, though they rely mainly on faecal and nasal sampling. In one study nasal dysbiosis was associated with asthma in that asthmatic children displayed lower diversity than non-asthmatics due mostly to expanded *Moraxella* species (152). Gastrointestinal tract dysbiosis characterized by reduction in the relative abundance of *Lachnospira, Veillonella, Faecalibacterium,* and *Rothia* is associated with increased risk of development of atopic diseases including asthma in later life (115). Children who's upper airways are colonized in the first year of life with *Haemophilus, Streptococcus,* and *Moraxella* are more likely to develop asthma (153).

Mechanistically there are a number of observations which may begin to untangle these relationships. Many of these potential mechanisms are gleaned from mouse-model studies. Mice treated with cephalosporin antibiotics develop gut overgrowth of *Candida* species and it is found that these mice develop a CD4 T-cell mediated allergic airway response to subsequent *Aspergillus fumigatus* exposure, whereas mice not treated with the same antibiotic do not react to the *Aspergillus* exposure (154). These same mice are found to have elevated eosinophilis, enhanced IL-5 and IL-13 production, higher serum IgE levels, goblet cell hyperplasia, and mastocytosis (155). When *Acinetobacter lwoffii* and *Lactococcus lactis* are administered intranasally to mice, induction of the Th1 cytokine IL-12 can attenuate T_H2/T2 allergic responses (156). In a similar experiment, when *Staphylococcus sciuri* is administered to mice sensitized with ovalbumin and house-dust mite, lung inflammation is ameliorated via inhibition of both T_H1 and T_H2 pathways through activation of TLR2 and NOD2 (nucleotide-binding oligomerization domain-containing protein 2) immune receptors (157). These three aforementioned bacterial species are farm-derived and these observations help to shed light on the possible mechanisms by which rich and varied early-life bacterial exposure may protect against asthma. Bacteria commonly associated with human systems seem to be similarly implicated. When *Lactobacillus johnsonii* which is a commensal in

the human vagina and GI system is supplemented orally, there is significant suppression of IL-4, IL-5, and IL-13 with protection against allergen challenge (114). Interestingly germ-free mice develop significantly exaggerated T_H2 responses compared to mice with a normal microbiome when both are challenged with ovalbumin, demonstrating increased airway eosinophilia, hyperresponsiveness, and mucus hypersecretion (158). These observations would need to be assessed in humans in order to correctly extrapolate the immunological processes by which certain components of the microbiome could protect against allergy and asthma and work is ongoing in this field.

The effects on the microbiome of various treatment strategies employed in the management of asthma is not well understood. One study found significant differences between the microbiota of steroid-naïve atopic asthmatics and steroid-using atopic asthmatics (95). The steroid-naïve cohort were found to have enriched *Haemophilus, Neisseria, Fusobacterium,* and *Porphyromonas* and this mirrors an earlier study which found that Proteobacteria members such as *Haemophilus, Neisseria,* and *Moraxella* were expanded in those who had never used corticosteroids (147). *Tropheryma whipplei*, an a*ctinobacteria*, has been seen to be enriched in sputum samples of a cohort of severe asthmatics and is postulated to be increased in abundance due to increased corticosteroid use (159). It seems therefore that inhaled corticosteroid use logically has an impact on the composition of the microbiome but it is unclear whether this effect is due more to the fact that severe asthma is associated with increased inhaler usage.

The impact of antibiotics on the microbiome is less well understood, particularly in the context of asthmatic patients. A large study evaluating the role for long term azithromycin in the management of COPD found that although azithromycin (taken daily for 8 weeks) did not alter bacterial burden on BAL samples performed at baseline and post treatment, there was significant reduction in α-diversity compared with placebo (160). On in-vivo analysis it was found that the treatment group expressed lower levels of inflammatory cytokines such as tumour necrosis factor, IL-13, and chemokine ligand-1 but elevated bacterial metabolites including

glycolic acid, indol-3-acetate, and linoleic acid. Ex-vivo analysis further documented that these bacterial metabolites blunted lipopolysaccharide-induced macrophage generation of the aforementioned pro-inflammatory cytokines thus demonstrating that this antibiotic affects anti-inflammatory bacterial metabolites that may contribute to its therapeutic effect (160). Following on from this study, a similar trial was conducted to evaluate the safety and efficacy of long term macrolide use in asthmatic patients, finding that adults with persistent symptomatic asthma experience fewer exacerbations and improved quality of life when treated with long-term azithromycin (161). An arm off this large study evaluated the effect of azithromycin on airway microbiota and antibiotic resistance genes using paired sputum samples at baseline and post 48 weeks of antibiotic use (162). Investigators found that bacterial burden did not change but that there was a significant reduction in *Haemophilus influenzae* load and overall community diversity. A significant increase in macrolide resistance genes was also observed. The combination of reduced diversity and increased resistance paired with overall reduction in asthma exacerbation and improved quality of life led investigators to call for further investigation in the field. To our knowledge there have been no other direct investigations into the relationship between the antibiotics used to treat asthmatic patients and the asthmatic microbiome.

Many of the aforementioned studies are limited somewhat by sample size and lack of longitudinal data. Bronchoscopic sampling of the airway microbiome is invasive and does not lend itself well to repeated sampling among large cohorts of patients. For this reason many studies rely on small numbers of subjects. There is ongoing debate regarding the optimal sampling method in terms of microbiome assessment. Certainly sputum analysis and bronchoscopic sampling are the two most commonly used methods and bronchoscopic analysis is fast becoming the preferred method, despite initial concern regarding the potential for contamination. It has been shown that pharyngeal and nasal contamination is low in this procedure as serial lavages performed during the same procedure do not result in progressively more dilute bacterial burden, as would be expected in the case of contamination (87).

It is generally agreed that there is good correlation between oropharyngeal and lower respiratory tract samples when sequencing techniques are applied (85). It has been found that when oropharyngeal swabs were compared with bronchoscopy-obtained endobronchial brush samples, community composition was indistinguishable but that bacterial burden was lower in lower airway samples (85). Findings such as this have given rise to the theory that microbial immigration via microaspiration and microbial elimination via cough and mucociliary clearance are responsible for the topography of the healthy microbiome (86). This has been manipulated often in the literature such that oropharyngeal swab sampling is accepted as a proxy for more invasive methods of bronchoscopic evaluation.

In the knowledge that the lungs are not sterile and that in fact a rich microbiome exists in the respiratory tract, multiple observations have been made regarding the characterization of this microbiome, the interactions between microbe and host in health and disease, and the effects of treatments on community composition. However in contrast to the more expansively studied area of gastrointestinal microbiome, many basic questions remain unanswered. The direction of causality in this research area has yet to be established, namely whether the intrinsic microbiome alterations observed in various disease states occur as a consequence of disease or contribute to them. Data from studies which relate early life microbial exposure to the development of atopic diseases seem to suggest that microbiome alterations may be causative of disease. This has yet to be explored in depth in human cause-and-effect trials, though mouse-model trials are proving informative. It is hypothesized that any causative relationships between the microbiome and asthma would involve differential immune regulation and yet this too requires further study. Longitudinal analysis of this system is rare for some of the reasons explored above, and so it is not known whether the respiratory microbiome maintains stability over time in health and in disease states. Furthermore it is not known whether the various treatments used in these diseases can dysregulate any stability that may be observed. Though some treatments, mainly ICS, have been shown to be associated with enhanced dysbiosis, it is not clear whether this

dysbiosis occurs as a direct consequence of the treatments or whether it reflects a more difficult disease which in turn demands the use of these treatments.

5.2. Hypothesis, Aims and Objectives

Longitudinal analysis of the respiratory microbiome is rare for some of the reasons outlined in this chapter's introduction. It is rarer still in asthmatic patients and there is little known about the response of the respiratory microbiome to treatments such as antibiotics. We hypothesized that the bacterial burden of the respiratory microbiome as assessed using lung brush and oropharyngeal samples would not change significantly over time in a cohort of asthmatic patients. We aimed to assess the bacterial burden of respiratory samples over time in asthmatics.

To fulfil this aim the specific objectives of this chapter were:
Establish the bacterial burden of oropharyngeal throat swab samples and endobronchial protected brush samples in a cohort of asthmatic patients.
Explore how the bacterial burden changed in repeated samples obtained after exposure to an antibiotic.

5.3. Results

5.3.1. Samples

Lung brush samples and oropharyngeal/throat samples (OTS) were both considered to be airway samples and these will therefore be discussed together in this chapter.

OTS swabs were collected from patients immediately prior to the first bronchoscopy, just in advance of topical anaesthetic being applied to the throat in the bronchoscopy procedure room. They were then repeated in the clinic setting on return visits at 2 and 4 weeks after the antibiotic had been taken, at the same time as faecal and skin samples were being collected. Bacterial burden is known to be high on throat swabs when compared with skin swabs and many other methods of biological sampling, therefore sampling was conducted to be as quick and comfortable as possible for patients. The final OTS swab was taken prior to second bronchoscopy in the same manner as the first sample.

Covered lung brushes were employed as the device of choice in endobronchial sampling. This was performed in a standardised manner and involved 2 brush samples of the left upper lobe (LUL) and 2 brush samples of the left lower lobe (LLL). LLL samples were obtained first in order to minimise any overspill of disrupted mucosa from the LUL. Brush samples were processed and stored in the manner outlined in the methods section.

Of the 13 patients in the cohort, 11 successfully completed both bronchoscopies. 4 brush samples were obtained from each of these patients meaning that the final sample number of lung brushes available for downstream analysis was 88. One patient could not return for second bronchoscopy due to scheduling difficulty arising from a family bereavement. The other patient presented for second bronchoscopy as planned but was deemed to be unfit for same due to intercurrent illness causing her oxygen saturations to be suboptimal to safely proceed. She did submit to throat, skin swab, and faecal sampling on that day. Swabs were labelled and immediately frozen to -80^{o}C and were stored in the freezer until transfer.

OTS swabs were collected from all 13 patients at the initial bronchoscopy and from 12 of the 13 patients scheduled for second bronchoscopy, see above. 3 patients did not attend for one of their planned interval clinic sessions and so did not have OTS swabs (or skin and faecal samples) collected at these points.

Samples were then couriered to the Asmarley Genomics Laboratory in Imperial College London using dry ice to maintain their temperature. Once received they were inspected by a member of the research team. All samples, OTS swabs and brushes, were intact with no damage to their casings. One sample however was unlabelled and could not be stored according to tissue-tracking guidelines in the UK. Therefore this sample was disposed of and could not be used for downstream analysis. Thus the final number of brush samples for assessment was 88 (100% of anticipated samples) and of OTS samples was 47 (90.4% of anticipated samples).

5.3.2. DNA extraction

DNA extraction was undertaken according to the protocol outlined in the methods section. Only slight differences in technique were required for the extraction process of the brush samples, these are outlined also in the methods section. All brushes (n=88) and swabs (n=47) successfully underwent DNA extraction and precipitation. As with all biological samples in this study a double extraction technique was used in order to maximise the yield of genetic material from the samples to allow greater accuracy in downstream analysis.

5.3.3. Nanodrop spectrophotometry

Spectrophotometry was employed to assess the purity of the extracted DNA prior to qPCR analysis. Table 1 outlines the results for the 88 lung brush samples.

Table 5.1: Nanodrop Spectrophotometry results per sample

Sample ID	ng/µl	260/280	260/230
010274	168.06	2.01	1.77
010483	159.67	1.97	1.68
011953	106.17	1.96	1.3
011965	89.75	1.94	1.58
010035	105.31	2.02	1.67
010393	149.07	1.96	1.66
011923	164.04	1.87	1.3
011935	217.72	1.97	1.73
011093	109.77	1.9	1.72
011102	51.42	1.77	0.88
010068	12.38	1.4	0.48
011126	78.21	1.84	1.33
010183	226.15	1.95	1.95
011983	224.98	1.97	1.76
011995	125.26	1.94	1.56
010363	137.39	1.96	1.61
010513	107.23	1.91	1.41
010123	151.05	1.95	1.59
011893	176.58	1.92	1.6
010304	149.15	1.91	1.3
010423	62.18	1.89	1.19
011801	197.63	2	1.7
010153	269.67	1.67	0.92
010251	191.5	1.94	1.73
010273	170.87	2	1.82
010484	273.16	1.96	1.83
011954	80.81	1.87	1.08
011966	209.66	1.95	1.8

Sample ID	ng/µl	260/280	260/230
010394	147.9	1.86	1.44
011924	251.25	1.96	1.84
011936	252.35	1.99	1.86
011094	450.4	1.94	1.93
011108	249.3	1.95	1.85
010069	10.17	1.29	0.39
011137	70	1.88	1.32
010184	144.06	1.94	1.67
011984	547.86	1.96	2.01
011996	224.05	1.98	1.81
010364	125.53	1.99	1.72
010514	79.7	1.92	1.43
010124	259.28	1.97	1.73
011894	197.64	1.96	1.72
010303	199.97	2.01	1.67
010424	153.79	1.79	1.22
011802	208.53	1.91	1.76
010154	189.9	1.9	1.67
010249	168.01	2.04	1.77
010276	207.45	1.98	1.81
010485	262.23	1.97	1.88
011955	264.75	1.96	1.82
011967	190.99	1.95	1.8
010039	145.99	2.13	1.04
010395	213.75	1.96	1.81
011925	489.45	1.95	1.89
011938	312.5	2	1.94
011091	192.89	1.94	1.77
011095	139.39	1.89	1.73
010070	770.7	1.97	2.07

Sample ID	ng/µl	260/280	260/230
011132	159.32	1.93	1.63
010185	244.51	1.95	1.75
011986	352.9	1.97	2.04
011998	190.4	1.99	1.95
010365	362.14	1.95	1.89
010515	114.78	1.98	1.58
010125	179.07	2.01	1.7
011895	162.27	1.99	1.87
010305	169.11	2.03	1.74
010425	51.12	1.95	1.3
011803	105.78	1.97	1.58
010155	253.37	1.95	1.64
010248	155.89	2.03	1.67
010275	143.94	1.97	1.74
010486	177.34	1.94	1.66
011956	85.15	1.91	1.29
011968	142.45	1.96	1.59
010045	61.42	1.87	1.33
010396	205.6	1.94	1.47
011926	225.17	2.02	1.85
011937	376.23	1.97	1.9
011097	284.33	2.01	1.99
011099	174.11	1.91	1.77
010071	454.83	1.96	1.96
011138	146.05	2.01	1.73
010186	163.36	1.95	1.62
011985	250.75	1.56	1
011997	179.62	1.96	1.76
010366	199.45	1.95	1.77
010516	272.53	1.98	1.87

The mean estimated biomass of the lung brush samples was 196.9ng/µl. Mean 260/280 ratio was 1.93, and mean 260/230 ratio was 1.62. This indicated that biomass was high enough to allow for successful qPCR analysis. Protein contamination (as estimated by 260/280 wavelength ratio) was likely to be low as a result of 1.93 is above the optimal threshold of 1.8. There was likely some salt contamination as the 260/230 ratio of 1.62 did not cross the optimal threshold of 2.0. However overall it was estimated that the integrity of these samples was favourable enough to proceed to downstream analysis. The same process was applied to the purified DNA samples from OTS swabs. The results are outlined in table 5.2 below.

Table 5.2: Nanodrop spectrophotometry results per sample

Sample ID	Ng/µl	260/280	260/230
011964	147.17	2	1.62
012089	51.2	2.03	1.25
010271	22.32	2.05	0.72
010481	35.84	1.53	0.73
013402	18.17	1.53	0.59
011951	133.73	1.99	1.5
013252	200.33	2.09	1.8
010391	54.15	1.72	1.02
010034	145.72	2.02	1.61
011921	272.22	2.1	2.13
011933	31.53	2.22	1.13
013222	191.35	1.97	1.67
013229	140.16	2.06	1.68
011089	58.69	1.88	1.05
013132	42.27	1.61	0.74
013139	40.49	1.95	1.17
010061	44.01	1.91	1.09

Sample ID	ng/µl	260/280	260/280
011120	46.22	1.8	1.09
013042	56.67	1.65	0.87
010181	36.3	1.67	0.92
013102	53.37	1.81	1.08
013108	31.18	1.61	0.65
011993	24.9	1.46	0.64
011981	17.73	1.5	0.55
013282	20.19	1.34	0.57
013289	26.35	2.19	0.89
010361	97.5	1.49	0.75
010511	75.45	1.76	1.07
013372	42.73	1.86	1.22
010121	65.79	1.85	1.08
011891	18.01	1.97	0.94
013192	71.13	1.99	1.44
013205	30.43	1.84	0.9
010301	26.16	1.73	0.78
010421	43.17	1.67	0.86
013341	46.29	1.6	0.92
013348	37.96	1.47	0.62
011811	277.52	2.08	1.82
013072	170.61	1.94	1.44
013078	40.38	2.14	1.43
010151	59.39	1.66	0.94
010241	79.86	1.83	1.03
013161	46.77	1.62	0.95
013168	119.07	1.46	0.68
011964	142.27	1.9	1.62
012089	49.2	2.03	1.45
010271	42.32	1.95	0.72

The mean estimated DNA concentration for these OTS swabs was 74.98ng/μl. Mean 260/280 ratio was 1.81 indicating a favourably low likelihood of protein contamination. Mean 260/230 ratio was 1.09 which indicated a likelihood of salt contamination, presumed to be due in part to swab material. After discussion with the laboratory group it was decided that these samples were of sufficient integrity to proceed to downstream qPCR analysis.

5.3.4. 16s rRNA Real time Quantitative Polymerase Chain Reaction

qPCR was undertaken as the next step in analysis and employed the ViiA 7 Real-Time qPCR system as previously. qPCR reaction mix preparation followed the steps outlined in the methods section. 5μl of extracted DNA was used per well. The standard curve was again generated using decreasing known concentrations of amplified *Vibrio natregens* and a control reaction containing sterile molecular grade water was also run. All reaction mixtures whether they contained standard, control, or sample, were run in triplicate. qPCR cycles were set as per those detailed in the methods chapter.

All samples were run through these qPCR conditions, i.e. DNA from 88 lung brushes and from 47 OTS swabs. Initial test runs were completed in order to identify the optimal dilution for each sample type in order to give most accurate results. The optimal dilution for lung brushes was found to be 1:100 and the optimal dilution for OTS samples was 1:40. Results were then back-calculated to give results as 16s rRNA copy number per μl and copy number per swab/brush. All samples generated adequate results for quantification of 16s rRNA copy number. The runs were deemed to be efficient with the slope of the standard curve found to be 0.98 for brush samples and 0.99 for OTS samples.

Mean 16s rRNA copy number for the brush samples was 5.278×10^5/μl of extracted sample and 3.139×10^7/brush. OTS swab copy count was expectedly higher at an average copy count of 1.518×10^8/μl and 9.112×10^9/swab. Unfortunately due to

time constraints in the laboratory, streptococcus specific qPCR could not be performed on the airway samples.

5.3.5. Longitudinal analysis of qPCR results

11 of the 13 patients completed both bronchoscopies therefore longitudinal analysis of the lung brush samples was limited to these 11 subjects.

As discussed, not all sample sets were complete in terms of the OTS samples. Mixed effects modelling was used as the statistical test of choice to account for missing data points. 8 subjects returned complete data sets so these were also analysed using Friedman's test. The below table, 5.3, illustrates the data points available for analysis of OTS swab results.

Table 5.3: 16S rRNA copy count per sample in available OTS samples. Gaps in data set as illustrated

	Sample 1, n=13	**Sample 2, n=12**	**Sample 3, n=11**	**Sample 4, n=11**
1	9.4293096e+007	1.807888e+007	1.272782e+007	6454509.5
2	3.6972035e+008	4.740794e+008		9.75266e+007
3	4.4786576e+008	2.935605e+007	5.761570e+007	1.11306e+008
4	1.3942593e+009	3.6941657e+008	5.995172e+008	9.50227e+007
5	6.2734036e+007	5386536.5	3.0589402e+007	1.0420581e+007
6	4.738258e+007		5.0041216e+007	5.187866e+007
7	3.901422e+007	1.19382712e+008	1.9553854e+007	
8	7838086.5	2341088.75	3.7729852e+007	9580961
9	8273540.5	4.845074e+007		1.8494996e+007
10	9.6671448e+007	9.3667744e+007	4.8725776e+007	1.8130946e+007
11	3468623.25	79316.3984	261790.75	1.4730709e+007
12	9.4193056e+008	3.14136832e+008	1.43340496e+008	
13	5857098.5	1271980.63	304163.938	6.2989292e+007

Wilcoxon's matched-pairs signed rank test was used to analyze for differences in bacterial count between samples taken at the first and second bronchoscopies, i.e. before and after the antibiotic, as data were found to be non-parametric. Left lower lobe samples and left upper lobe samples were analyzed separately. Line graphs were used to represent the individual data sets and box plots represent the mean data. There were no statistically significant differences observed in total bacterial count per LLL sample over the study period, p=0.51 (figure 5.1). There were no statistically significant differences observed in total bacterial count per LUL sample over the study period, p=0.89 (figure 5.2).

Data were stratified by eosinophil count to assess for any differences in bacterial burden by sub group analysis. Eosinophils were considered to be elevated if $>0.4x10^9$/L. Applying this criteria, 3 of the 11 patients (27%) had elevated eosinophils. There were no statistically significant differences in bacterial burden as measured as 16s rRNA copies/μl in either the elevated or normal eosinophil groups, across both LUL and LLL samples (figure 5.3). Linear regression was also employed to assess for any correlation between eosinophil count and mean 16S rRNA copy count per brush for each patient (figure 5.4). There were no statistically significant relationships observed.

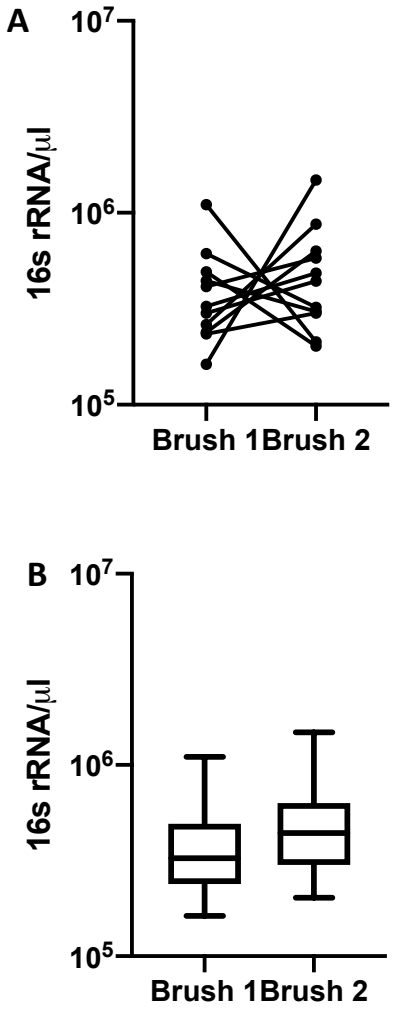


Figure 5.1.: 16s rRNA/μl in covered lung brush samples from left lower lobe

A. A. Line graph of full data set over time-scale, there was no significant change in bacterial load/μl over the study duration, p= 0.51
B. Box-and-whisker plot of full data set over time-scale, there was no significant change in bacterial load/μl over the study duration, p=0.51

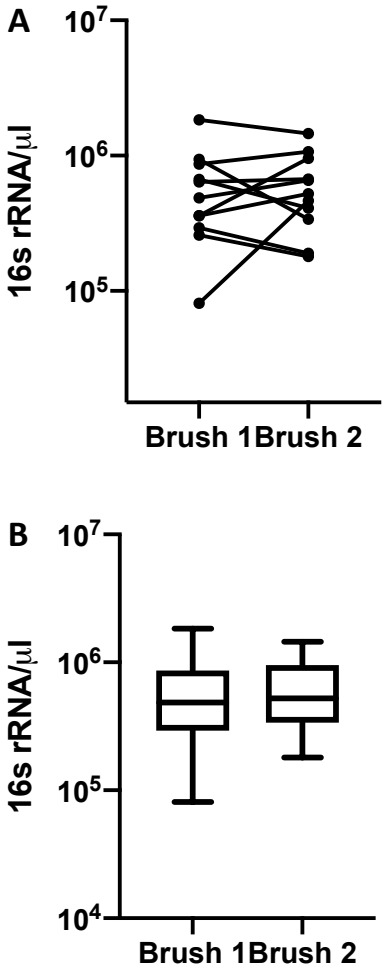


Figure 5.2.: 16s rRNA/μl in covered lung brush samples from left upper lobe

A. Line graph of full data set over time-scale, there was no significant change in bacterial load/μl over the study duration, p=0.89.
B. Box-and-whisker plot of full data set over time-scale, there was no significant change in bacterial load/μl over the study duration, p=0.89.

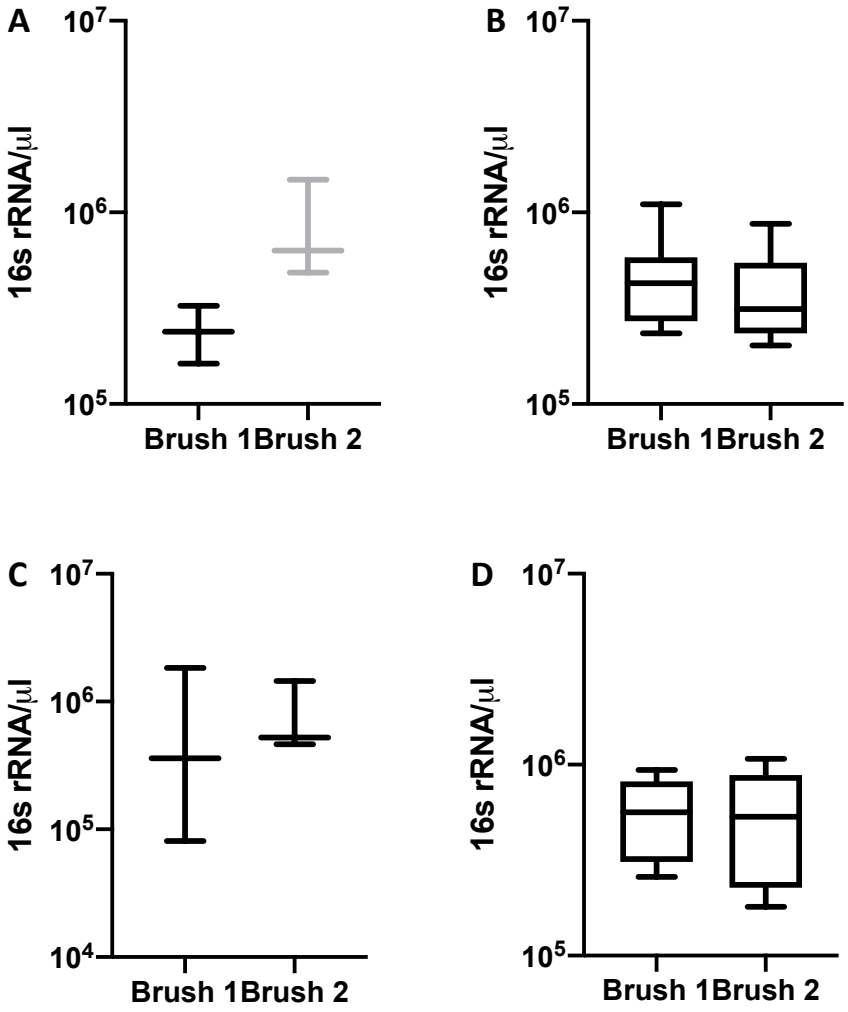


Figure 5.3.: 16s rRNA count/μl stratified by eosinophil count

A. Box-and-whisker plot of lung brush samples from **left lower lobe** in those with elevated eosinophils. There was no significant change in bacterial load/μl, p=0.25.
B. Box-and-whisker plot of lung brush samples from **left lower lobe** in those with normal eosinophils. There was no significant change in bacterial load/μl, p=0.74.
C. Box-and-whisker plot of lung brush samples from **left upper lobe** in those with elevated eosinophils. There was no significant change in bacterial load/μl, p=0.75.
D. Box-and-whisker plot of lung brush samples from **left upper lobe** in those with normal eosinophils. There was no significant change in bacterial load/μl, p=0.94.

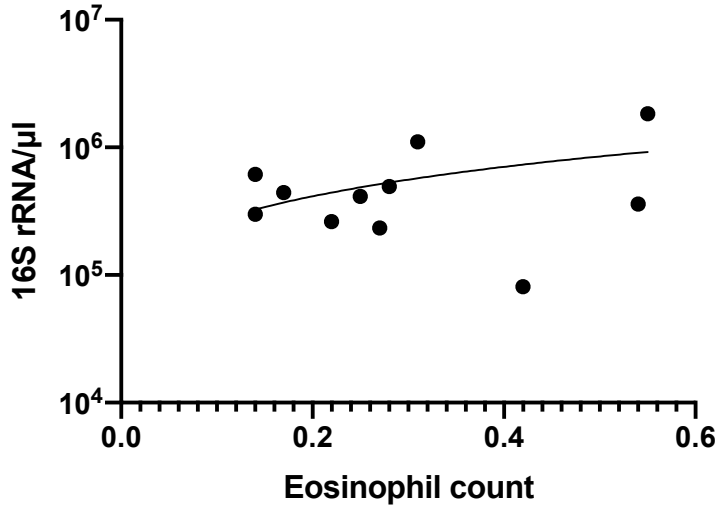


Figure 5.4: Eosinophil count v 16S rRNA count of brush samples

Linear regression correlating eosinophil count with mean bacterial burden of brush samples for each patient. There were no statistically significant relationships observed. P= 0.193

For analysis of the OTS results, total 16s rRNA count was assessed for all time points as laid out in table 3. Mixed effects modelling was applied to test for any differences between the mean bacterial count at each time point (figure 5.5), taking blank data points into account. Line graphs were again used to represent the individual data sets and box plots represent the mean data. There were no statistically significant differences observed in total bacterial count per sample over the time period, p=0.11. Post-hoc analysis using Holm-Sidak's multiple comparisons test revealed that there were no differences between the mean bacterial counts at any time point.

Friedman's test was assessed on completed data sets of 4 samples, n=8. There were no significant differences in mean bacterial load in the 4-sample data set, p=0.058 (figure 5.6). Dunn's multiple comparison test showed a significant decrease in bacterial burden between sample 1 and sample 2, p=0.042, but there were no significant differences at any other time points.

Data were stratified by eosinophil count to assess for any differences in bacterial burden by sub group analysis. Eosinophils were considered to be elevated if $>0.4x10^9$/L. Applying this criteria, 5 of the 13 patients (38.46%) who returned OTS swabs had elevated eosinophils. There were no statistically significant differences in bacterial burden of throat swabs as measured as 16s rRNA copies/μl in either the elevated or normal eosinophil groups, p=0.31 and p=0.266 respectively (figure 5.7). Linear regression was employed to assess correlation between eosinophil count and 16S rRNA copy count per swab for each patient (figure 5.8). The slope of the regression line was significant, p=0.0473, indicating that those with elevated eosinophil counts had elevated mean bacterial burden in OTS swabs for the duration of the study period.

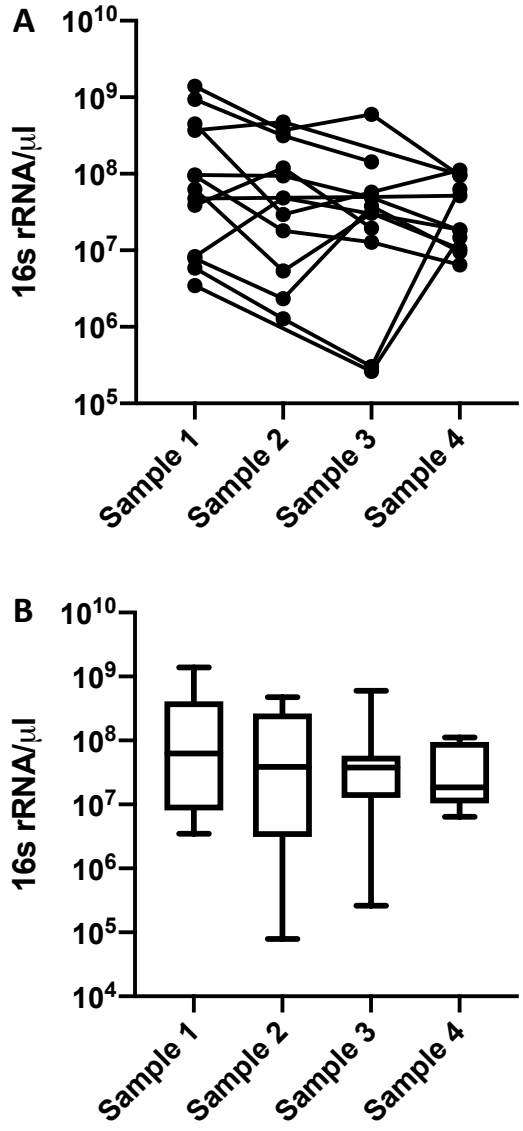


Figure 5.5.: 16s rRNA/μl in all OTS samples

A. Line graph of all data sets over time-scale, there was no significant change in bacterial load/μl over the study duration, p=0.11.
B. Box-and-whisker plot of all data sets over time-scale, there was no significant change in bacterial load/μl over the study duration, p=0.11.

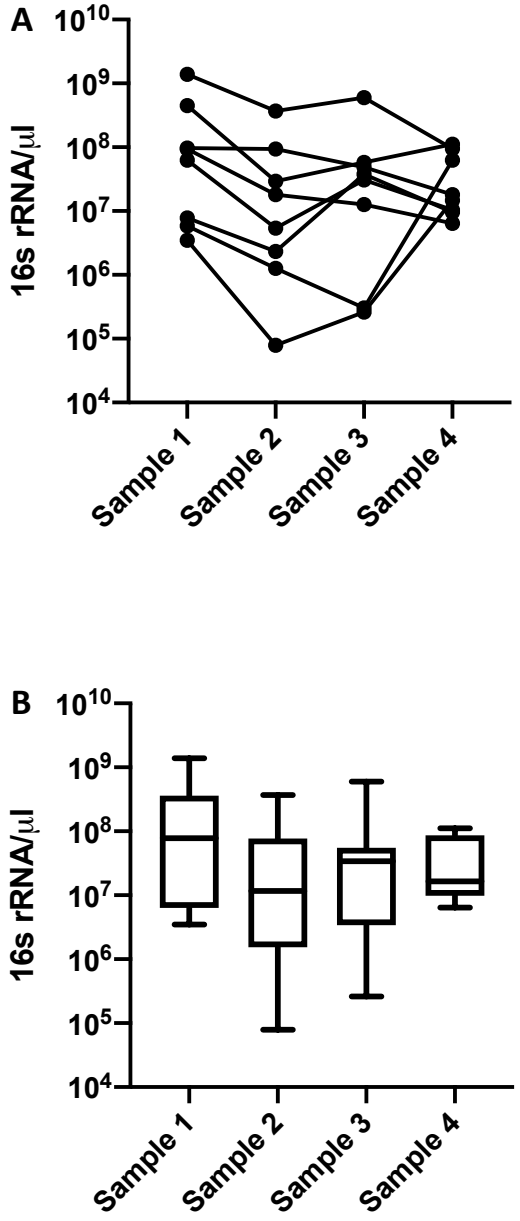


Figure 5.6.: 16s rRNA/μl in complete OTS sample sets only, n=8

A. Line graph of complete data sets only over time-scale, there was no significant change in bacterial load/μl over the study duration, p=0.058.
B. Box-and-whisker plot of complete data sets only over time-scale, there was no significant change in bacterial load/μl over the study duration, p=0.058.

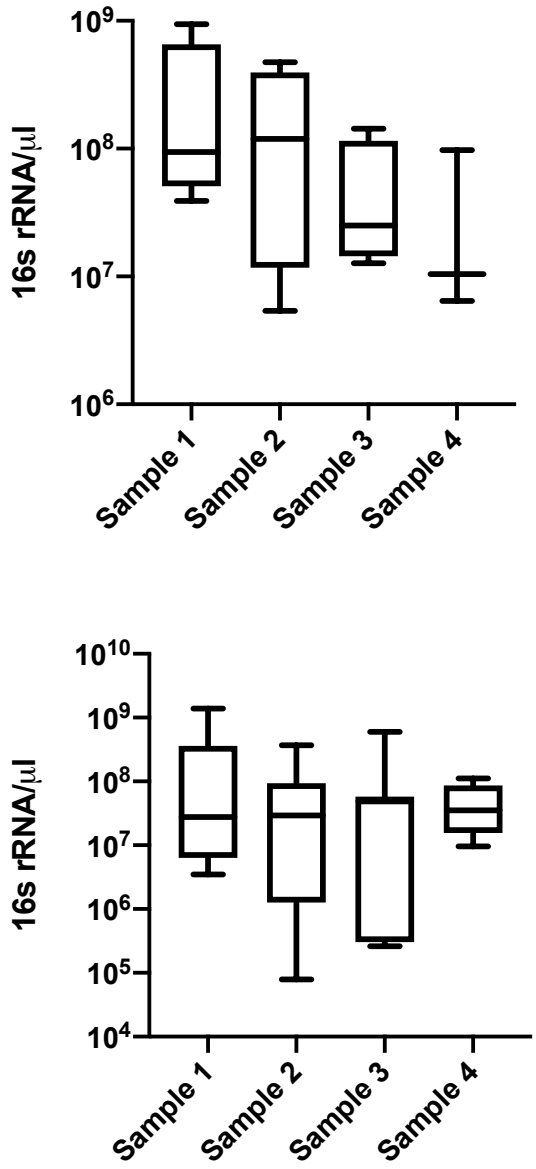


Figure 5.7.: 16s rRNA/μl in all OTS sample sets, stratified according to eosinophil level

A. Box-and-whisker plot of 16s rRNA count per OTS sample over time in subjects with elevated eosinophils >0.4x10^9/L , there was no significant change in bacterial load/μl over the study duration, p=0.31.

B. Box-and-whisker plot of 16s rRNA count per OTS sample over time in subjects with normal eosinophils <0.4x10^9/L , there was no significant change in bacterial load/μl over the study duration, p=0.266.

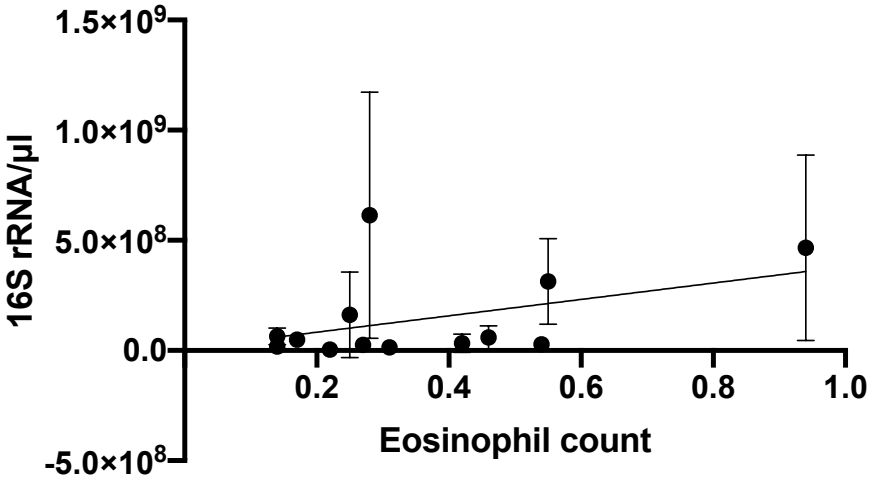


Figure 5.8: Eosinophil count v 16S rRNA count per OTS sample

Figure 5.8 illustrates the correlation curve between eosinophil count and the mean 16S rRNA copy count of swab samples for each patient. Error bars are included. There were no statistically significant relationships observed, p=0.0473.

5.4. Discussion

In this chapter a longitudinal assessment of the bacterial burden of the airways via assessment of 16s rRNA copy count in lung brush and oropharyngeal swab samples was conducted. Additionally this assessment incorporated use of an antibiotic in order to explore whether bacterial load changed meaningfully in response to one of the standard treatments for asthma. Longitudinal assessments of this kind are sparse in the literature for multiple reasons, mostly relating to patient participation in intense study protocols. Repeated measure assessments tend to rely on less invasive methods of sampling the microbiome such as sputum analysis and therefore are best conducted in those with bronchiectasis, cystic fibrosis, or COPD where expectoration is a prominent symptom (163). We assessed 13 patients with endobronchial sampling pre and post antibiotic and supplemented this airway assessment with oropharyngeal samples taken at regular intervals in between. To our knowledge this is the first assessment of its kind undertaken in patients with asthma specifically to assess the response of airway bacterial load.

11 of 13 patients completed both bronchoscopies. All 13 patients submitted to oropharyngeal testing and 47 of the intended 52 samples (90%) were returned. This was felt to be a favourable response rate. No patients suffered any adverse events during the time of sampling or bronchoscopy.

It appears that there are no differences in the bacterial burden of endobronchial lung brush samples between the index and follow up bronchoscopies. This result is independent of eosinophil status. There were no statistically significant differences in the bacterial burden of the full set of OTS swab samples at any time point in the assessment. There was however a trend towards significance when only complete data sets, those sets where four swabs were successfully returned, were analysed separately. Though overall the p-value was non-significant ($p=0.058$), post hoc analysis revealed a significant drop in bacterial burden between the first and second throat swabs, $p=0.0402$. Oropharyngeal swabs are widely felt to be representative of lower respiratory samples but are documented to have higher

biomass. This observation could point towards a similarly significant relationship in the lower respiratory tract that is not observable due to the biomass and relatively low numbers of the brush samples processed in this study. However further study would be required to test this relationship in a meaningful manner.

Additionally linear regression conducted to assess correlation between eosinophil count and mean bacterial burden during the study showed that those with lower eosinophil counts seemed to have lower bacterial burden in their oropharyngeal swab samples, p=0.0473. This fits with the above observation but the role of this relationship is unclear in a study population of this size. Further work would be required to assess whether this subgroup analysis is meaningful in terms of eosinophilic asthma being related to higher bacterial biomass in oropharyngeal microbiome communities.

Overall these results seem to tally with one of the only similar studies conducted recently as an arm off a larger trial to investigate the safety and efficacy of long term azithromycin in asthma. The index trial (AMAZES trial) showed long term azithromycin to be safe and effective for asthmatics, demonstrating reduced exacerbation frequency and improved quality of life when treated with oral azithromycin for 48 weeks (161). A subset of patients in this trial were asked to provide paired sputum samples to allow for analysis of the potential pleiotropic effects of azithromycin in this cohort (162). They assessed paired samples from 61 patients (n=34 placebo, and n=27 azithromycin) and found that total bacterial load did not change significantly when a patient is exposed to 48 weeks of oral macrolide. They did however show that on shotgun metagenomic sequencing, there was a reduction in the load of airway Haemophilus Influenzae in the treatment group compared to placebo and that this reduction led consequently to reduced α-diversity. They also showed that macrolide resistance genes were upregulated in the treatment group. Overall this provided conflicting evidence regarding the utility of long term macrolides in asthma.

Our results tally with those mentioned above in that we demonstrated that there is no significant change in overall bacterial burden of endobronchial and oropharyngeal airway samples in response to an antibiotic. However our study is limited by lack of access to sequencing equipment and so we could not proceed to assess for compositional changes in the samples, as the above trial did. It may be that similar reductions in diversity follow a standard antibiotic course in the short to medium term, but further studies will be required to test this hypothesis.

5.5. Conclusion

In summary the experiments in this chapter suggest that there is no meaningful change in the bacterial burden of airway samples in response to levofloxacin antibiotic. This has not been studied before in asthmatics. In addition, the asthmatic patients included in this trial had been carefully selected for inclusion based on a strict criteria of physiological measures as outlined in prior chapters. These results tally with those of the aforementioned AMAZES sub-trial which seems to be the most similar in structure to our study. Those investigators demonstrated that the bacterial burden in sputum samples of asthmatics remained stable after long term exposure to azithromycin. Further investigation is warranted in larger cohorts and sequence data would complete the results we have demonstrated.

Chapter 6: Gastrointestinal Microbiome

6.1 Introduction

The human gastrointestinal tract is home to a large and diverse microbiome with the metagenome of the gut estimated to contain more than 3 million genes, outnumbering human genes by the order of 150 to 1 (164). The GI microbiome, while diverse, is dominated by the phyla Firmicutes and Bacteroidetes in health (165), with Actinobacteria, Proteobacteria, and Verrucomicrobia contributing in lesser proportions (103). There is significant compositional heterogeneity between the microbiome of individuals but an individual's microbiome will remain relatively stable longitudinally (166). Intestinal microbiota play multiple vital roles in the healthy gut including protection against pathogen overgrowth (167), vitamin biosynthesis (168), toxin elimination (169), and perhaps most vitally ensuring continued maturation and education of host immune defenses (170). Development of the gut microbiome begins in utero and can be influenced by multiple factors such as the mode of delivery and use of prenatal antibiotics (171, 172). Diversity expands rapidly from infancy to early childhood but then slows by 5 years of age so that children continue to have lower microbial diversity than adults (173), gradually expanding diversity through adolescence to reach a steady-state in adulthood.

Dysbiosis of the gut microbiome has been implicated in the pathogenesis and propagation of multiple disease processes including atherosclerosis (174), diabetes mellitus (175), obesity (176), and certain inflammatory diseases such as rheumatoid arthritis (177) and inflammatory bowel disease (178). Consequently, attempts to normalize the microbiota in the gut have yielded therapeutic response in some of these areas, most notably in the treatment of recurrent refractory *Clostridium difficile* infection with faecal transplantation (179). Dysbiosis in turn is caused by environmental factors. Diets high in fat and sugar can induce dysbiosis (180) and are well established to promote the development of obesity, cardiovascular disease, and diabetes. Alcohol consumption can significantly alter the composition of intestinal flora. Sporadic alcohol use has been shown to reduce bacterial diversity in an intake-dependent manner (181). Meanwhile chronic alcohol dependence has been linked to lower abundances of Bacteroidetes and elevated

abundances of Proteobacteria on colonic biopsy which correlates to higher levels of serum endotoxin in subjects (182).

However causality is not easily assessed with respect to the microbiome and there are multiple factors which challenge thorough assessment of the GI microbiome in disease. Many studies in the field are statistically underpowered case-control studies owing to the significant inter-individual variability of the microbiome as well as intra-individual variability when taking confounders such as diet into account. There is no agreed consensus regarding the optimal sampling method for explorations of the GI microbiome and so there is significant heterogeneity in the literature. Often assessment is obscured by the use of necessary medications for the conditions which are being studied thus impacting on the ability to draw meaningful conclusions (183).

The relationship between antibiotics and gut microbiome is better understood than that between antibiotic use and the respiratory microbiome. As mentioned, the GI microbiome is established in infancy and early childhood and increases in diversity during adolescence to eventually resemble the adult but this development can be altered by environmental factors. Antibiotics are one such factor that can delay the progression of the GI microbiome significantly (184). Antibiotics are routinely administered during caesarean section and can compound the intrinsic loss of vaginal microflora translocation associated with this delivery mode. Infants delivered via Caesarean are more likely to suffer MRSA infections (185), these infections in turn are associated with a microbiome that is unstable and low in diversity. Premature birth is associated with a vast expansion in Proteobacteria abundance compared with the Firmicute dominance of the full-term infant microbiome (184). Frequent administration of antibiotics in the preterm neonatal setting compounds this finding and increases Proteobacteria abundance further by relatively suppressing *Bifidobacterium spp* (186).

In adulthood no single microbial composition has been identified in health and it is established that there is significant inter-individual variation across all populations

studied. Furthermore it is difficult to predict the effects of factors such as antibiotic administration because pharmacokinetics and pharmacodynamics of any given drug can also vary significantly between individuals. Therefore it is generally agreed that repeated measures of the microbiome in a particular community upon exposure to an antibiotic is the best way to establish a pattern of response. For example repeated assessment of the faecal microbiome post administration of oral amoxicillin has been seen to cause significant disruptions to composition which persisted for up to two months post exposure (187). In addition to altering bacteriome composition, antibiotics can have lasting downstream effects including altering gene expression and metabolism of GI microbiota and this can in some states mimic disease. For example altered sugar metabolism can occur in patients treated with beta-lactams via induction of enzymatic pathways in a similar manner to the alterations observed in obese individuals (188).

It has been shown that altered microbiome composition in childhood can predispose to the development of atopy and asthma. Reductions in the relative abundances of *Rothia, Faecalibacterium, Veillonella,* and *Lachnospira* in GI samples during the first 100 days after birth were associated with a higher risk of asthma in later life (115). This important study found that the reduction in these taxa was associated strongly with dysregulation of enteric metabolites and a reduction in the level of faecal acetate. Interestingly investigators found that by supplementing these taxa in the airways of germ-free mice there was a significant improvement in airway inflammation in subsequent progeny. Reduction of *Lachnospira* has been reproducibly postulated as a risk for the development of asthma and particularly in cases where its reduction allows expansion of *Clostridium* species (189, 190).

These findings contribute to the current hypothesis of the Gut-Lung-Axis, namely the concept that constant ‘cross-talk’ between the lung and the GI tract has an impact on immune regulation and therefore inflammation. Short chain fatty acids (SCFAs) are the most extensively studied of the metabolites implicated in mediating this cross-talk and are vital for their ability to influence the function of multiple immune cells (191). SCFAs, through various signaling pathways including induction

of immune-suppressive IL-10 (192), modulation of Treg cell differentiation (193), and promotion of intestinal IgA production (194), act to limit inflammation in sites where the microbiome is rich and diverse. Conversely reductions in diversity of a microbial community can down-regulate microbial-derived SCFAs and potentially promote inflammatory disease.

The particular study of gut microbiota in asthmatic patients is in its infancy and early studies have suggested that diversity is reduced in adults with a diagnosis of bronchial asthma. One study found that *Faecalibacterium prausnitzii, Sutterella wadsworthensis* and *Bacteroides stercoris* were reduced which Clostridium species and *Eggerthella lenta* were expanded compared with controls (195). Less still is known about the changes conferred, if any, on the GI microbiome in asthmatic patients after antibiotic administration. Despite benefitting from decades of research in advance of the discovery of the respiratory microbiome, the determination of causality remains similarly elusive in the study of the gastrointestinal microbiome.

6.2 Hypothesis

Robust and thorough profiling of symptoms, physiologic measurement, and microbial burden in asthmatic patients may expand our current understanding of this common but complicated disease. The aim of this study was to integrate detailed assessment of airway physiology with assessment of microbial parameters in not just the lungs but also the skin and gastrointestinal system in a cohort of carefully selected asthmatics. Understanding the role that these microbial environments play in this disease is vital and of particular interest is the interplay between communities in response to treatments such as antibiotics.

To fulfil this aim the specific objectives of this chapter were:

Evaluate microbial density in the faeces of asthmatic patients.
Explore how faecal microbial density changes over time in response to a course of antibiotics.
Examine the specific streptococcus burden in patients treated with levofloxacin.

6.3. Results

6.3.1 Samples

Faecal samples were collected from patients as outlined in the methods section. All patients were coached with regard to sampling method. The initial sample was obtained prior to commencement of levofloxacin and usually also before the index bronchoscopy. In order to preserve patient comfort and encourage participation in the study, patients were offered the option to collect the sample in their own home and transport it to the lab. If this was the subject's preference, they were asked to either return the sample to the lab within 20 minutes of collection, or freeze the sample at home for up to 5 days and return it at a time convenient to them. Samples were requested at index bronchoscopy, review visit post conclusion of antibiotics, review visit 2 weeks thereafter, and at the final visit totaling 4 samples.

4 out of the 13 patients in the cohort successfully returned all samples, 5 patients returned 3 samples, one patient returned 2 samples, and 3 returned one sample. All patients gave a first index sample. This gave a sample number of 37 out of a potential 52 samples, 71.15% returned. The main reason cited for not returning samples was discomfort on behalf of the subject. All samples returned had been correctly frozen and stored by subjects and were deemed suitable for further analysis.

Timing of samples was uniform in the cohort and occurred at index bronchoscopy, 10 days and 2 weeks post index bronchoscopy (10 days was the duration of the antibiotic course), 2 weeks thereafter, and again at their 8 week second bronchoscopy.

Samples were couriered to the Asmarley Genomics Laboratory, Imperial College London, on dry ice and inspected on receipt. All were deemed to have remained suitably frozen during transportation and were eligible for downstream analysis.

6.3.2 DNA Extraction

DNA extraction was performed using a CTAB buffer-based reaction as outlined in the methods section. A double extraction technique was used to increase the yield of DNA in order to maximise the likelihood of successful downstream analysis. 200mg of faecal matter was aliquoted for use from each original faecal sample. Three extractions were performed without faecal matter present in order to act as control experiments during processing. These were labelled as control 1, control 2, and control 3. All samples underwent extraction successfully.

6.3.3 Nanodrop spectrophotometer Assay

Once purified DNA was extracted, small quantities were assessed for DNA concentration and purity using spectrophotometry. Table 6.1 illustrates the results of the nanodrop assay for all gastrointestinal samples.

Table 6.1: Nanodrop spectrophotometry results per sample and control

Sample ID	ng/µl	260/280	260/230
12095	4358.9	2.07	2.16
13036	1017.9	1.18	1.21
13066	1331.7	1.95	1.81
13079	2394.1	2.03	1.66
13085	4472.3	1.97	2.06
13096	4211.3	2.04	2.14
13126	2749.3	2.03	2.1
13133	1968.3	1.94	1.4
13140	2218.8	2.01	1.9
13145	3518.8	2.04	2.19
13162	5288.3	1.68	1.8
13169	3494.4	2.05	2.14
13175	5078.9	1.92	2.05
13186	4939.6	1.96	2.11
13193	5397.7	1.77	1.91
13199	5440.4	1.85	1.92
13206	2079.3	2.13	2.27
13216	1514.1	2.04	1.4

Sample ID	ng/μl	260/280	260/230
13223	5838.7	1.51	1.46
13230	1133.7	2.02	1.67
13236	5170.7	1.84	1.71
13246	3007.7	2.1	2.16
13253	1115.8	2.04	1.94
13259	1968.4	2.14	2.14
13276	3567.4	2.09	2.19
13283	3995.9	2.02	2.11
13290	2631.1	2.11	2.24
13296	4462	2.04	2.15
13306	3384.7	2.07	2.19
13313	3656	2.14	2.3
13320	3996.5	2.08	2.23
13326	5190.6	1.83	1.91
13342	5478.1	1.73	1.79
13366	4621.8	2	2.11
13373	3957	2.05	2.14
13396	2948.2	2.04	2.09
13403	5304.7	1.99	2.07
control 1	13.7	1.81	0.54
control 2	5.4	2.75	0.29
control 3	5.1	1.63	1.42

Table 6.1 outlines the results for the faecal sample nanodrop assays. This includes the three control samples, n=40. With controls excluded, mean DNA concentration was 3323ng/μl (SD 1690ng/μl), mean 260/280 ratio was 1.967 (SD 0.229), and mean 260/230 ratio was 1.877 (SD 0.43). Where there were duplicate values that varied widely, the assay was repeated and outlier discounted. These data were discussed with the genomic scientists in the laboratory and deemed to demonstrate sufficiently high DNA concentrations and sufficiently low protein and salt contamination that none needed to be re-extracted or re-processed and all were eligible for downstream analysis.

6.3.4 16S rRNA Real time Quantitative Polymerase Chain Reaction

qPCR was undertaken using the ViiA 7 Real-Time qPCR system and associated software (ThermoFisher Scientific). The universal 16srRNA primers used were 520**F**

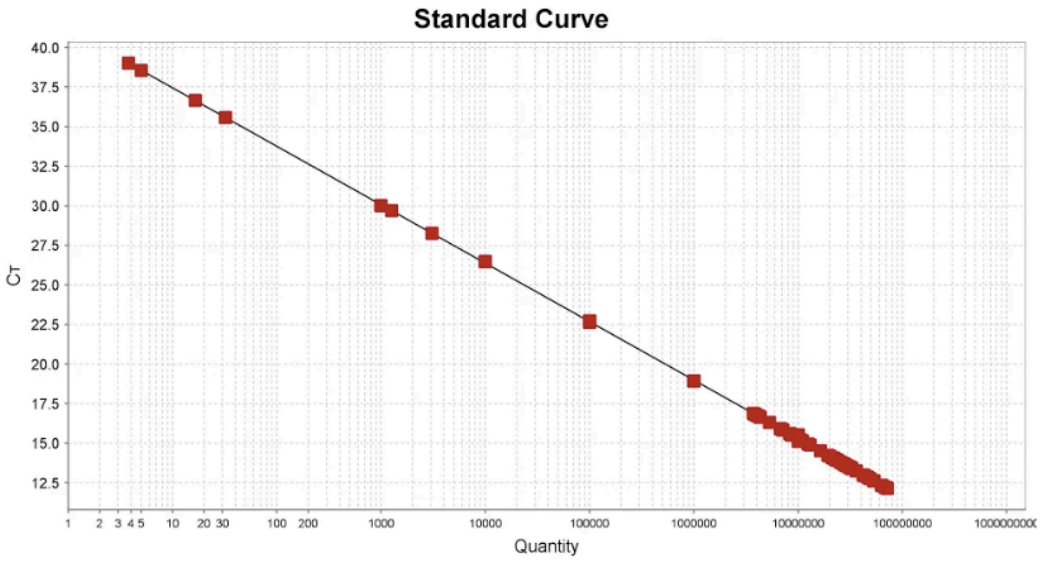
(5'-AYT GGG YDT AAA GNG -3') and 802**R** (5'-TAC NVG GGT ATC TAA TCC -3') and these were ordered from Eurofins MGW Operon. 96 well plates (MicroAMP Fast 96-well Reaction Plate 0.1mL) were constructed to contain 15µl of reaction mixture per well as outlined in the methods section.

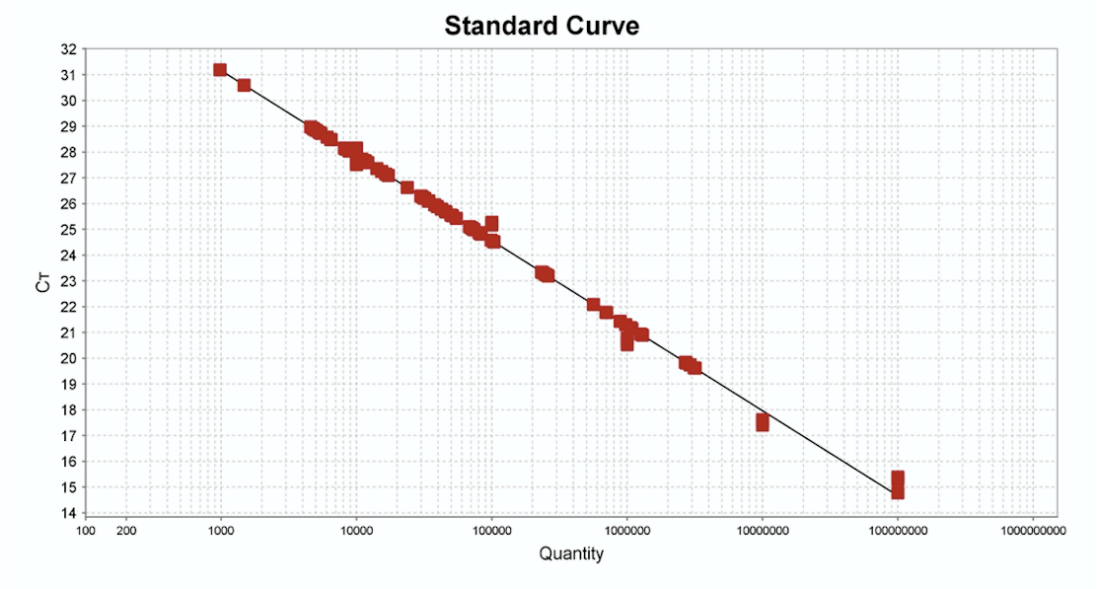
The 37 faecal samples were processed in this way as well as three control samples. Multiple runs were undertaken to find the optimal dilution required for an efficient run. 1:100 was found to be acceptable for the faecal samples as the biomass in faeces was so high. 1:10 dilution was preferred for the streptococcus run as the predicted specific biomass of streptococcus species was anticipated to be lower than total bacterial count. The qPCR runs were found to be reproducibly efficient as shown in the following standard curve, figure 6.1. Curve 1 represents the full faecal data set and curve two represents the streptococcus specific data set. Efficiency was calculated as the slope of the line and found to be 0.99 and 1.01 respectively.

Figure 6.1:

Curve 1: Standard curve of faecal sample 16S rRNA qPCR run, efficiency 0.99

Curve 2: Standard curve of faecal sample streptococcus qPCR run, efficiency 1.01





The mean 16s rRNA bacterial count for the total sample cohort was $2.09569x10^{12}$/g of faecal sample and $4.40132x10^{8}$/µl of extracted sample. 16s rRNA load per control sample averaged at $1.7841x10^{5}$ copies per control sample. Streptococcus counts were predictably lower at a mean of $1.0679x10^{6}$/µl of extracted sample.

6.3.5 Longitudinal analysis of qPCR output

Because not all sample sets were complete, statistical analysis of the change in bacterial count was conducted in two ways. Complete datasets of three samples (n= 9) and four samples (n=4) were assessed using Friedman's test to assess for any statistically significant differences between the mean bacterial count along to study timeframe. Mixed effects modelling was employed to assess the full dataset which included missing values. The data points are illustrated below in table 6.2.

Table 6.2: 16S rRNA copy count/μl of extracted sample, missing data points illustrated

	Sample 1, n=13	Sample 2, n=10	Sample 3, n=9	Sample 4,n=4
1	2.08522e+012			
2	1.9787e+012	3.31332e+012	2.79976e+012	
3	3.84422e+012			
4	3.42024e+012	6.57209e+011	1.57646e+011	
5	7.97602e+011	2.3157e+012	2.87744e+012	
6	1.11909e+012	1.44161e+013	4.08784e+011	3.27493e+011
7	6.58023e+011	2.36575e+012	9.29047e+011	9.12463e+011
8	8.44307e+011	7.30161e+011	4.22163e+011	
9	1.72257e+011	5.13051e+012	1.13284e+012	9.1692e+011
10	1.93149e+012	6.48601e+011	1.88707e+012	1.46899e+012
11	5.23907e+012			
12	8.64329e+011	2.26451e+012		
13	2.85385e+012	2.73669e+012	2.1807e+012	

Total 16s rRNA count was assessed for all time points and mixed effects modelling was applied to test for any differences between the mean bacterial count at each time point (figure 6.2). Line graphs were used to represent the individual data sets and box plots represent the mean data. There were no statistically significant differences observed in total bacterial count per sample over the study period, p=0.244. Bacterial count was given as 16s rRNA copies/μl of sample.

Friedman's test was assessed on completed data sets of 4 longitudinal samples (n=4) and those which returned 3 samples (n=9). Data for the 3-sample sets are represented in figure 6.3 and data for the 4-sample set are represented in figure 6.4. Post-hoc analysis was conducted using Dunn's multiple comparisons test to

compare the means at each time point in order to assess for any statistically significant differences across the study period.

There were no significant differences in mean bacterial load in either the 3-sample set or 4-sample set, p=0.397 and p=0.432 respectively. Dunn's multiple comparisons test went on to reveal no individual statistically significant difference between mean bacterial density at each time point.

Data were assessed for any differences when stratified by eosinophil count. Linear regression was employed to assess for correlation between eosinophil count and bacterial load as given by 16S rRNA count/μl of extracted faecal sample (Figure 6.5). There were no statistically significant relationships observed.

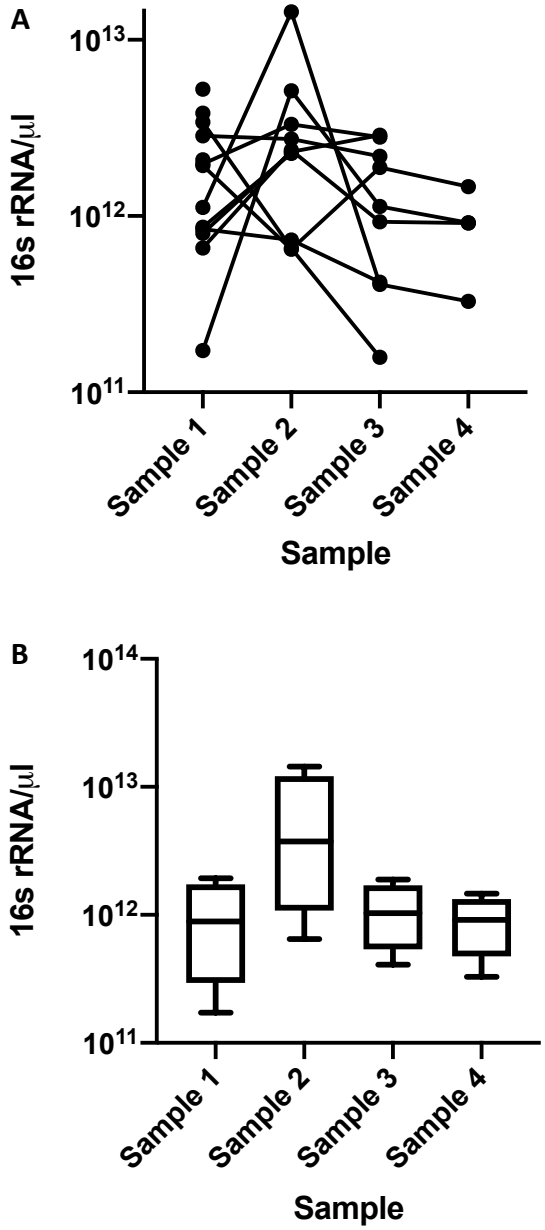


Figure 6.2: 16s rRNA/μl in all faecal samples

A. Line graph of full data set over time-scale, there was no significant change in bacterial load/μl over the study duration, p=0.244.
B. Box-and-whisker plot of full data set over time-scale, there was no significant change in bacterial load/μl over the study duration, p=0.244.

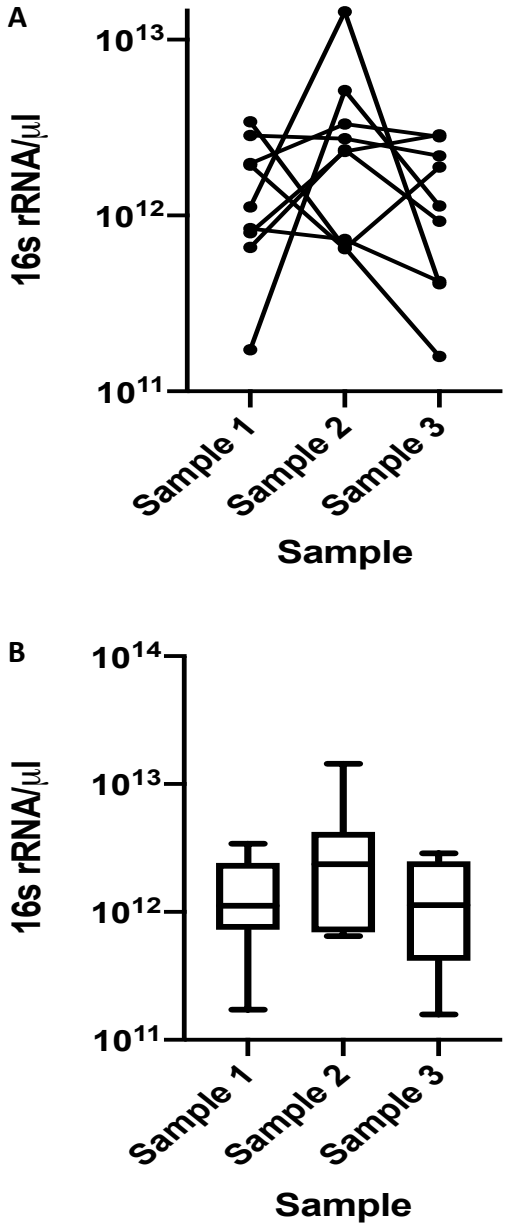


Figure 6.3: 16s rRNA/μl in those subjects who returned 3 faecal samples

A. Line graph of 9 three-sample data sets over time-scale, there was no significant change in bacterial load/μl over the study duration, p=0.397.
B. Box-and-whisker plot of 9 three-sample data sets over time-scale, there was no significant change in bacterial load/μl over the study duration, p=0.397.

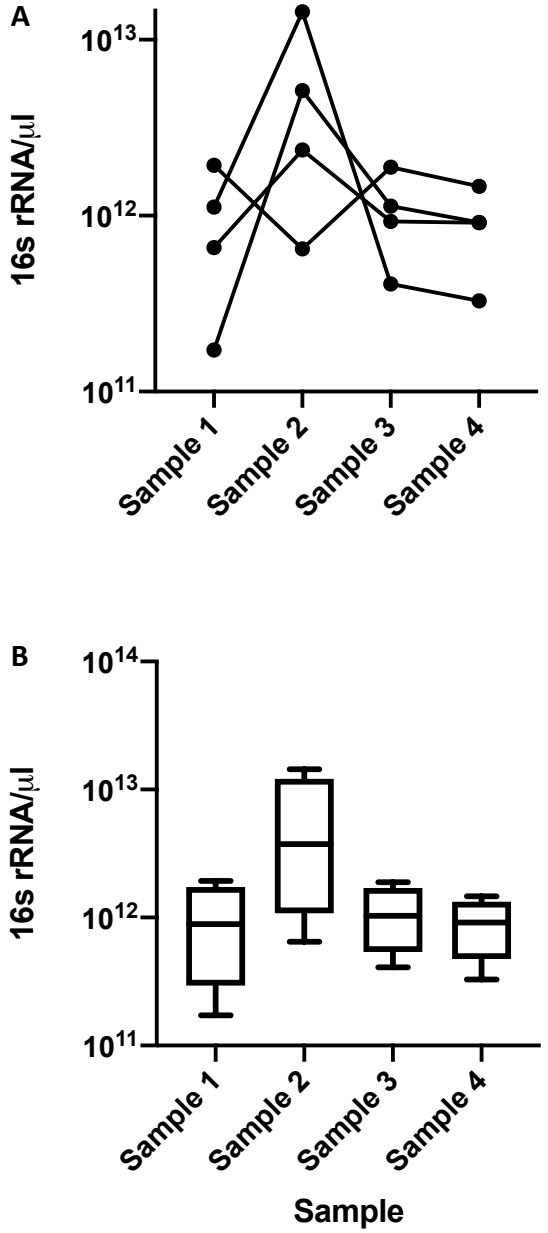


Figure 6.4: 16s rRNA/μl in those subjects who returned all 4 faecal samples

A. Line graph of 4 completed four-sample data sets over time-scale, there was no significant change in bacterial load/μl over the study duration, p=0.432.
B. Box-and-whisker plot of 4 completed four-sample data set over time-scale, there was no significant change in bacterial load/μl over the study duration, p=0.432.

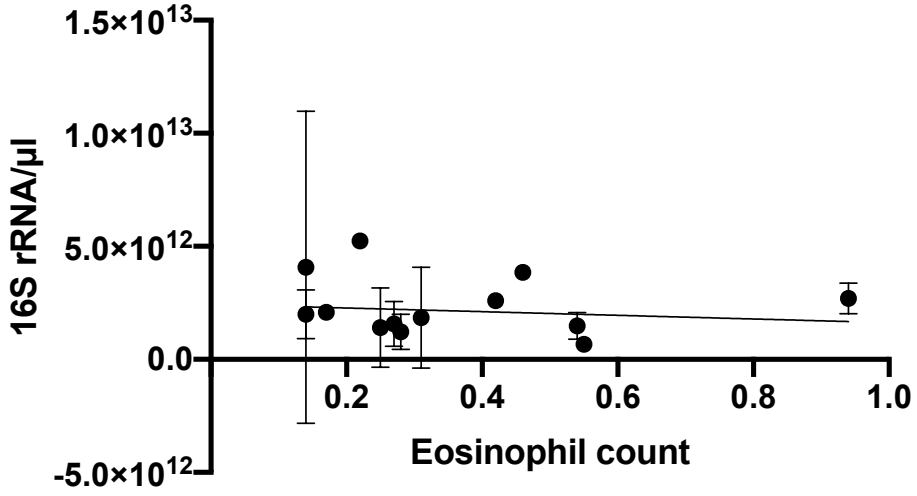


Figure 6.5: Eosinophil count v 16S rRNA copy count in faecal samples

Figure 6.5 illustrates the correlation curve between eosinophil count and mean 16S rRNA/µl of extracted faecal samples for each patient. Error bars around the mean are included. There were no statistically significant relationships observed.

6.3.6 Streptococcus Real time Quantitative Polymerase Chain Reaction

Levofloxacin is a medication used frequently to treat respiratory tract infections. It is a quinolone which has strong activity against streptococcus species. To increase the utility of our experimental protocol it was decided to proceed with streptococcus-specific qPCR assessment of samples. This was carried out in a very similar way to the 16s rRNA assay with subtle differences.

Part of the methionine aminopeptidase (*map*) gene has been shown to allow accurate quantification of only members of the Streptococcus genus in extracted DNA samples. Primers were designed to amplify this specific part of the *map* gene and were 23 base pairs in the forward prime direction ($^{5'}$GCWGACTCWTCTTGGGCWTATGC$^{'3}$) and 24 base pairs in the reverse prime direction ($^{5'}$TTARTAAGTTCYTTCTTCDCCTTG$^{'3}$). *Streptococcus mitis* (strain DSMZ-12643) was used as the standard in this assay, compared with *Vibrio natregens* used in the 16s rRNA assay. The qPCR reaction was made up in the manner outlined in the methods section. Once again a 1:100 dilution of the faecal DNA was established as optimal for the run. Strep counts were back-calculated accordingly to give final output data of strep copies/μl.

In a similar way to analysis of the 16s rRNA data, mixed methods analysis was carried out on the entire dataset to account for missing results. Repeated measures ANOVA was used to analyse completed three-sample and four-sample data sets to assess for differences in mean strep count/μl at the various time points in the study period. The data points are illustrated below in table 6.3.

Table 6.3: Streptococcus copy count/μl of extracted sample, missing data points illustrated

	Sample 1, n=13	**Sample 2, n=10**	**Sample 3, n=9**	**Sample 4, n=4**
1	172342.076			
2	106275.299	168839.8	146548.2	
3	106499.841			
4	202523.254	40171.1338	95582.344	
5	221742.92	198097.2	145463.66	
6	107166.375	2326022.46	2315233.32	5621642.6
7	2051212.36	222789.104	2785731.24	2248672.06
8	278361.67	106493.314	812895.604	
9	29827.0202	465899.6	505041.942	654788.976
10	814480.004	297592.8	3286636.22	388141.088
11	1103771.87			
12	102122.324	295825.462		
13	4823695.5	815212.744	5300002.14	

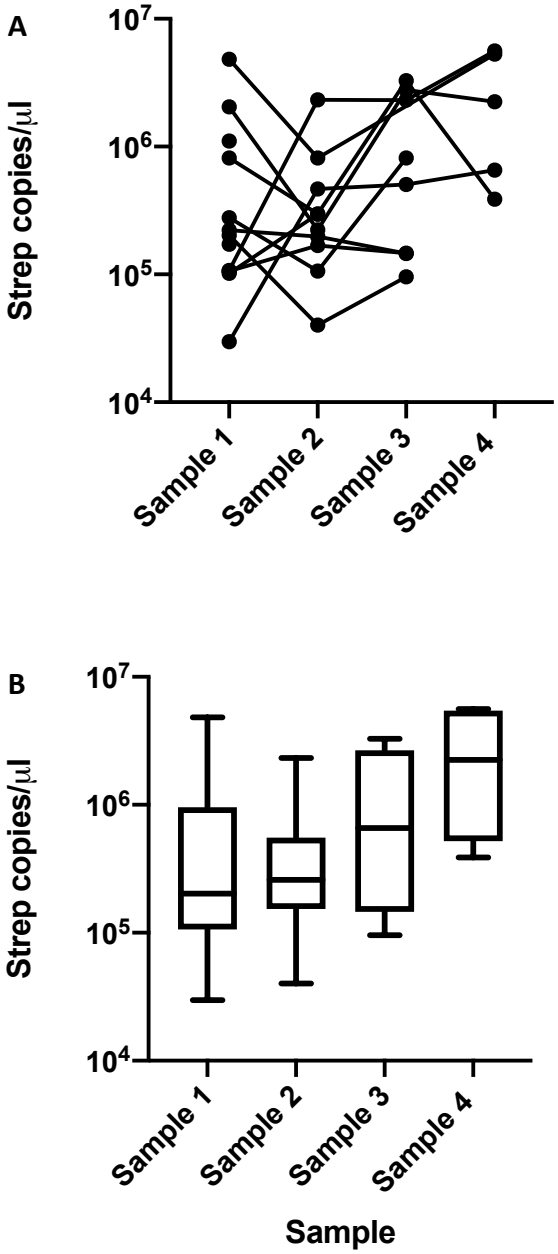
Total strep count was assessed for all time points and mixed effects modelling was applied to test for any differences between the mean strep-specific count at each time point (figure 6.6). Line graphs were used to represent the individual data sets and box plots represent the mean data. There were no statistically significant differences observed in total strep count per sample over the study period, p=0.0872. Strep count was given as strep copies/μl of sample. Post hoc analysis using Tuckey's multiple comparisons test equally did not detect any significant differences in the mean values per time point.

Friedman's test was assessed on completed data sets of 4 longitudinal samples (n=4) and those which returned 3 samples (n=9). Data for the 3-sample sets are represented in figure 6.7 and data for the 4-sample set are represented in figure

6.8. Post-hoc analysis was conducted to compare the means at each time point to assess for any statistically significant differences across the study period.

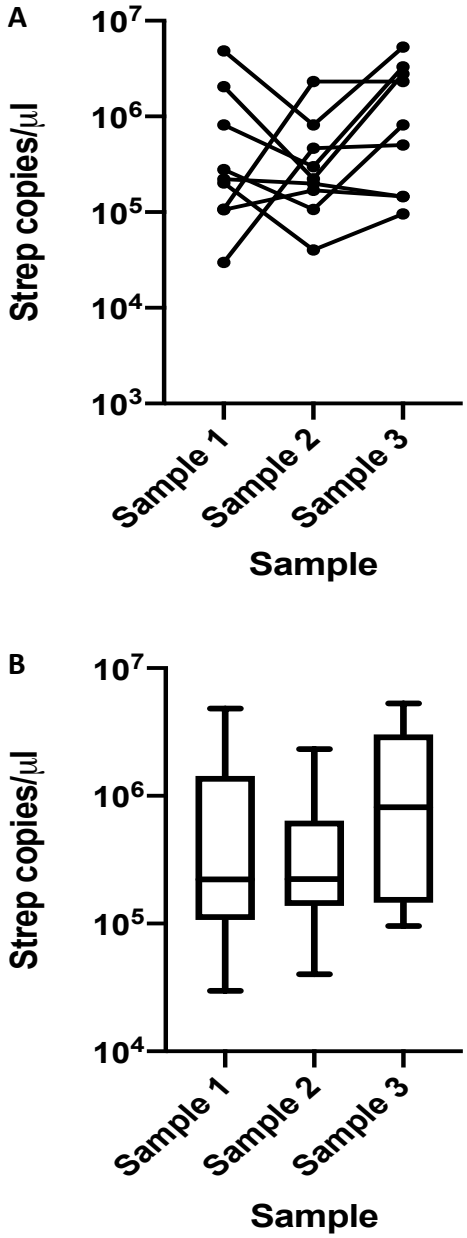
There were no significant differences in mean streptococcus load in either the 3-sample set or 4-sample set, p=0.278 and p=0.158 respectively. Dunn's multiple comparisons test went on to reveal no individual statistically significant difference between mean streptococcus density at each time point.

Data were assessed for any differences when stratified by eosinophil count. Linear regression was employed to assess for correlation between eosinophil count and streptococcus count/μl of extracted faecal sample (Figure 6.9). There were no statistically significant relationships observed.



6.6: Strep copies/μl in all faecal samples

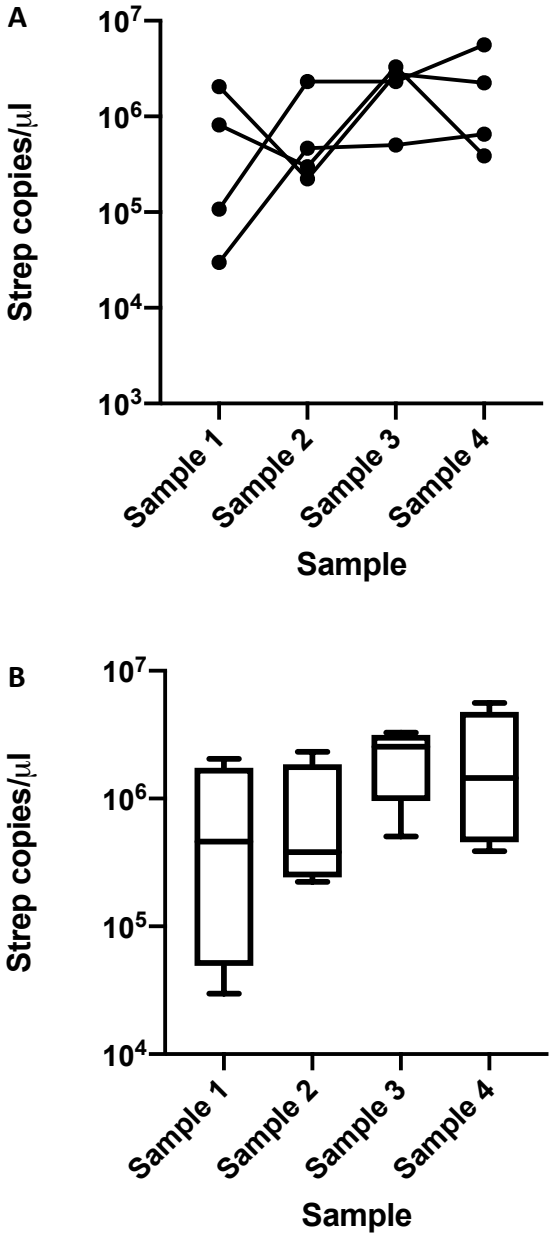
A. Line graph of all data sets over time-scale, there was no significant change in streptococcus load/μl over the study duration, p=0.0872.
B. Box-and-whisker plot of all data sets over time-scale, there was no significant change in streptococcus load/μl over the study duration, p=0.0872.



6.7: Strep copy/μl in those subjects who returned three faecal samples

A. Line graph of 9 three-sample data sets over time-scale, there was no significant change in streptococcus load/μl over the study duration, p=0.278.

B. Box-and-whisker plot of 9 three-sample data sets over time-scale, there was no significant change in streptococcus load/μl over the study duration, p=0.278.



6.8: Strep copy/µl in those subjects who returned all four faecal samples

A. Line graph of 4 completed four-sample data sets over time-scale, there was no significant change in streptococcus load/µl over the study duration, p=0.158.

B. Box-and-whisker plot of 4 completed four-sample data sets over time-scale, there was no significant change in streptococcus load/µl over the study duration, p=0.158.

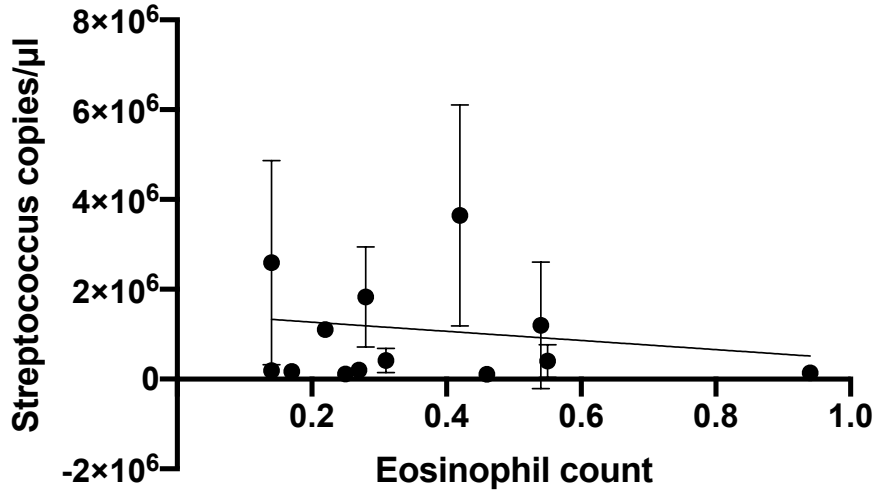


Figure 6.8: Eosinophil count v streptococcus count in faecal samples

Figure 6.8 illustrates a correlation curve between eosinophil count and streptococcus copy number/µl of extracted faecal sample for each subject. Error bars were included. There were no statistically significant relationships observed.

6.4 Discussion

In this chapter a detailed quantitative analysis of bacterial load and streptococcus-specific bacterial load in the faeces of adult asthmatic patients exposed to an antibiotic has been performed. In addition this quantification has been assessed in a longitudinal manner and to our knowledge this type of assessment has not been undertaken to date in asthmatics. This assessment has been carried out on adult asthmatics who fulfil strict physiological criteria in relation to their diagnosis. Assessments of this type are invaluable contributions towards robust analysis of the microbiome in asthmatic patients, not only in the context of the respiratory microbiome, but in the establishment of the concept of 'whole body microbiome'. In the introduction we have explored the evidence for substantial cross-talk along the gut-lung axis (191, 192, 194, 195) mediated by microbiome-induced metabolites such as short chain fatty acids, and as such the importance of GI assessment in asthmatic microbiome studies cannot be understated.

This study demonstrated that faecal bacterial load maintains relative stability in asthmatics after administration of an antibiotic. There were neither statistically significant changes in mean bacterial density per time point nor statistically significant changes in mean streptococcus-specific bacterial density per time point. Furthermore post hoc analysis of mixed effects modelling and repeated measures ANOVA testing failed to show any signals towards significant change at each point in time. This would suggest that the GI bacteriome is not overtly sensitive to levofloxacin in an asthmatic patient. Qualitative sequence data would be required to confirm this finding. qPCR is a quantitative rather than qualitative measurement of bacterial composition but can infer valuable conclusions relating to bacterial density, particularly if employed in repetitive measures over time.

71.5% of intended faecal samples were successfully gathered in the course of this study. This is in contrast to 100% return of lung brush, skin swab, and oropharyngeal swab samples. The main reason cited by subjects for failing to return requested samples was discomfort and unease with the process. This is intrinsic

and intuitive with regard to faecal samples, particularly as patients in some cases were required to store samples in home freezers. However we feel the response rate would have been lower again if patients had been required to provide the sample in hospital at the time of review, this has been borne out in the literature (196). Studies have shown faecal sample return rates to be lower than for other biological samples (197) so this is not a unique finding to our study. Mixed effects modelling was employed statistically to minimise interruptions by missing data.

6.5 Conclusion

In summary the experiments in this chapter suggest that the bacterial burden of the gastrointestinal system remains relatively constant in asthmatic patients after exposure to levofloxacin. Levofloxacin is an antibiotic with a strong activity against streptococcus species but streptococcus burden was also found to remain relatively stable over the study period. There were no statistically significant differences in the mean 16s rRNA or streptococcus count at any time interval in the study. This was true for the entire data set as analysed by the mixed effects model, and for three-sample and four-sample sets as analysed using Friedman's test.

Chapter 7: Skin Microbiome

7.1 Introduction

The skin is the largest organ of the body and functions to serve as a protective physical barrier against exogenous insults. In carrying out this role it acts as an interface with the outside environment, continually exposed to harsh conditions and invading pathogens. The skin plays host to millions of microbes and these are thought to play pivotal roles in augmenting the function of this organ (198). In a similar manner to other microbiome communities, if there is imbalance between host commensals and pathogens, for example in response to a break in the barrier's integrity, skin disease and occasionally systemic disease can occur.

Multiple features of the skin contribute to select for specific colonies of microorganisms which adapt to the particular area which they inhabit and this is borne out in culture-dependant studies in past literature (199, 200). The skin surface has great topographical variance due to multiple differences in anatomical composition. For example, invaginations such as those in the groin, axilla, and toe webs are higher in humidity and temperature, therefore preferentially contributing to growth of thermophilic bacteria (*S. aureus*, gram-negative bacilli etc)(117). In contrast, areas such as the back and chest which are rich in sebaceous glands are associated with expansion of lipophilic species (*Malassezia spp*. and *Propionibacterium spp*.)(117). The skin of the arms and legs are exposed to more widely varied temperatures which tend to fluctuate and so these areas have fewer organisms. Additionally multiple host factors such as age and gender can have vast effects on community composition (201).

Molecular and genomic techniques have allowed for more precise characterisation of the skin's microbiome than the aforementioned culture-based assessments and have begun to untangle this complex microbial environment. The skin microbiome in health is dominated by members of the phyla Actinobacteria, Firmicutes, Bacteroidetes, and Proteobacteria in varying abundances according to topographical location (118). *Corynebacterium* and *staphylococcus* species are the dominant bacteria in skin folds such as the umbilicus, axilla, gluteal crease, and antecubital fossa (202) and through processing of apocrine secretions these

bacteria are responsible for the malodour of human sweat (119). Dry skin areas have the greatest diversity and in one study these areas have been seen to have greater phylogenetic diversity than that of the gut and skin in the same individual (202). Actinobacteria, Proteobacteria, Firmicutes and Bacteroidetes are present in these sites with varying relative abundance (118, 203) as are gram negative organisms which, prior to molecular analysis, were thought to colonise skin exceedingly rarely and usually considered to be contaminants from the gastrointestinal system (117). *Propionibacterium spp* dominate the sebaceous skin of the back along with *Betaproteobacteria* and *Flavobacteriales* and although these three taxa are also found on the antecubital fossa, their relative abundance is reduced in favour of expanded *Staphylococcus spp* (203). Intra- and inter-personal variation is high with intrapersonal temporal variation in community composition appearing to be higher with regard to the skin than the gut or oral cavity (202). Allowing for this observation however it is generally accepted that there is longitudinal stability of the skin microbiome as a whole in healthy subjects (204).

Dysbiosis of the skin microbiome can predispose to certain skin conditions and dysbiosis in turn is driven by factors such as lifestyle, age, hygiene, and nutrition (120). Exogenous insults such as cosmetics and chemotherapy can also have disruptive effects to balance in the community (121, 205) including those designed to treat skin disorders. In skin where *Staphylococcus aureus* overtakes other components of the microbiome in abundance, skin infection can occur and lead to resistant bacterial infections, namely impetigo. Dandruff is associated with scalp skin where the proportion of *Malassezia restricta*, a commensal basidiomycetous yeast, is expanded (206). *Propionibacterium acnes* is an Actinobacterium who's normal function is to protect against *S. aureus* and methicillin-resistant *S. aureus* (207) but which can cause acne in areas where its relative abundance to other members of the community is high.

Work is ongoing to fully characterise the functional impact of the skin microbiome, particularly relating to potential interactions with the immune system. There is evidence that migration of regulatory T cells to the skin in the neonatal period is partially dependant on commensal microbes (208). In turn these regulatory T cells

are responsible for mediation of immune tolerance to commensal skin bacteria (209). Complement may be regulated in the skin by commensal bacteria and other components of the microbiome (210). IL-1 signalling may also be modulated by commensal skin bacteria suggesting a role for the microbiome in the mediation of some inflammatory skin conditions (211).

Atopic dermatitis (AD) or eczema is a chronic inflammatory skin condition and is one of the most common skin conditions in paediatric populations (212). It is one of the atopic diseases and is most commonly the first presentation in the 'atopic march', the sequential development of allergic diseases during early childhood which often culminates in asthma in affected patients (213). It is known that both structural abnormalities of the skin and immune dysregulation play a vital role in the pathogenesis of this disease (214), but there is emerging evidence that dysbiosis of the skin microbiome may also be at play. AD is characterised by a vast overpopulation of *Staphylococcus aureus* in the areas of skin which are affected (122), thereby reducing the relative abundance of other skin commensals and leading to a reduction in overall microbial diversity The development of AD is interestingly associated with reduced levels of *Staphylococcus aureus* in infancy (215), the presumption being that presence of *S. aureus* as a skin commensal infers immune-mediated protection against the development of this condition, though further studies are needed to confirm this hypothesis. In order to manipulate this finding into a therapeutic target, some groups are investigating the role for probiotics in supplementing commensal strains to selectively out-compete the overpopulated *S. aureus* and thereby repopulating the skin with beneficial strains. This has been shown to be an effective strategy with topical supplementation of *Vitreoscilla filliformis* significantly reducing *S. aureus* colonisation, improving patient scores, and objectively improving AD (according to the validated SCORAD assessment tool) in patients independent of age (123). Other supplementation strategies of interest include use of coagulase negative *staphylococcus* species (216) and use of the patients own commensal skin bacteria in moisturiser (123), though results are awaited.

The skin microbiome has not been extensively investigated in asthmatic patients

therefore little is known about any community dysbiosis independent of potentially coexistent AD. The respiratory and gastrointestinal microbiome is altered in asthmatic patients compared with controls but it is not known whether this is also the case regarding the skin microbiome. AD is closely associated to asthma and often coexists, even in adult populations (217) and is also known to be associated with an altered gut microbiome (218) along the so-called 'Skin-Gut Axis'. This seems to be similar to the 'Gut-Lung Axis' in asthma in that the development of AD is associated with the immunomodulatory effect of the altered gut microbiome (218). It is likely that there is some level of interplay between all three environments but this has not been studied in asthmatic patients.

Knowledge of the skin's response to antibiotics is similarly under-explored in terms of the microbiome. The impact of systemic antibiotics on the skin microbiome has been well studied in the areas of acne and rosacea but not extensively in other areas. This research pertains mainly to topical antibiotics rather than to systemic use. There are no studies evaluating the skin microbiome in asthmatic patients in response to systemic antibiotics.

7.2 Hypothesis and aims

In order to thoroughly assess the microbiome of an asthmatic patient we believe it is essential to study other microbiome compartments in order to build a comprehensive picture. It is logical, if unstudied, that these microbial environments interact on some level in this complex disease. It is of particular interest in a clinical sense as to how these communities respond to and equally impact on treatment courses such as antibiotics. We hypothesise that the bacterial burden of the skin will change in a similar manner to the bacterial burden of airway and gastrointestinal samples in asthmatic patients exposed to an antibiotic and we aim to assess this.

To fulfil this aim the specific objectives of this chapter were:

Evaluate microbial density on the skin of asthmatic patients.
Explore how skin microbial density changes over time in response to a course of antibiotics.
Examine the specific streptococcus burden in patients treated with levofloxacin.

Results 7.3

7.3.1 Samples

Skin swab samples were collected by investigators in the manner outlined in the methods section. For each sample visit, swabs were obtained from both antecubital fossae and both axillae. Prior to these samples being taken, a control swab was obtained by dipping the swab in the sterile water used to pre-moisten the sample swabs. Each collection generated therefore 5 swabs for extraction, possible 20 swabs per patient over 4 collections. The final number of collected swabs is shown in table 7.1.

Table 7.1: Skin samples obtained through the study period

Sample type	**Intended number per patient**	**Intended total**	**Final total**
Control skin swab	4	52	50
Left axilla skin swab	4	52	50
Right axilla skin swab	4	52	50
Left antecubital skin swab	4	52	50
Right antecubital skin swab	4	52	50
Total	20 per patient per visit	260	250

The collection rate was 96% for skin swabs, n=50 per site. One patient missed their third visit to the research team for swab collection and one patient did not attend for second bronchoscopy. Otherwise this was considered to be an adequate return rate for skin samples.

Timing of samples was uniform in the cohort and occurred at index bronchoscopy, 10 days and 2 weeks post bronchoscopy, 2 weeks thereafter, and again at their second bronchoscopy 8 weeks after index.

Samples were couriered on dry ice to the research lab in London and immediately frozen in storage. They were inspected prior to defrosting for downstream DNA extraction and one sample was found to have a fractured casing around an intact swab. This was flagged with the research team and the decision was made to exclude it from downstream analysis as these would have been significant contamination of the sample. Thus the number of samples for DNA extraction was 249.

7.3.2 DNA extraction

Due to the large number of skin swab samples for these patients it was decided to conduct a test-batch analysis in order to decide which sample of the 4 topographical areas yielded the highest biomass. Swabs were obtained in the same method from 4 members of the lab research team. Swabs underwent DNA extraction, nanodrop spectrophotometry, and qPCR analysis as outlined in the methods chapter. It was found that biomass, while low on all swabs obtained, was higher in swabs taken from the right axilla and thus allowed for accurate qPCR analysis. Therefore after discussion, it was decided to only proceed with full analysis of the 50 right axillary swabs. 4 control swabs were randomly selected for analysis with these axillary swabs.

As with the faecal samples, double extraction was performed on the swabs to maximise the yield of genetic material in order to allow for accurate qPCR analysis. All selected swabs underwent extraction successfully.

7.3.3 Nanodrop spectrophotometry

Spectrophotometry was employed to assess the purity of the extracted DNA prior to qPCR analysis. Table 7.2 outlines the results.

Table 7.2: Nanodrop spectrophotometry results per sample

Sample ID	ng/µl	260/280	260/230
13033	19.4	1.5	0.43
13039	8.4	1.63	0.31
13045	17.9	1.53	0.45
13064	15.74	1.58	0.41
13070	25.2	1.38	0.54
13076	19.9	1.69	0.45
13083	14.3	1.57	0.32
13093	20.7	1.74	0.68
13099	45.2	1.5	0.74
13105	22.6	1.35	0.49
13123	10.2	1.84	0.37
13129	15.1	1.32	0.45
13136	17.7	1.52	0.46
13143	10.3	2.34	0.55
13153	5.8	1.54	0.32
13165	33.1	1.44	0.67
13172	25.9	1.49	0.39
13183	11.4	1.67	1.59
13189	20.9	1.43	0.45
13196	18.9	1.61	0.52
13202	37.9	1.53	0.4
13213	15.9	1.49	0.41
13219	11.4	2.17	0.4
13226	24.6	1.47	0.43
13233	19	1.6	0.39
13243	12.3	1.58	0.37
13249	9.7	1.61	0.34
13256	10.3	1.48	0.37
13263	25.7	1.47	0.63
13273	17.9	1.75	0.46
13279	27.8	1.48	0.41
13286	16.9	1.46	0.47
13293	13.5	1.59	0.58
13303	12.7	1.55	0.42
13309	4.5	1.29	0.43
13316	14.8	1.47	0.46
13323	11.3	1.48	0.4
13329	26.5	1.48	0.42
13333	18.9	1.61	0.36
13338	26.2	1.49	0.54
13345	36.6	1.45	0.56
13351	18.8	1.42	0.5

Sample ID	ng/μl	260/280	260/230
13363	11.7	1.63	0.35
13369	17.2	1.5	0.5
13376	8.3	1.9	0.51
13393	32.1	1.54	0.66
13399	20.4	1.48	0.5
13406	19	1.51	0.41
13412	15.5	1.5	0.4
(control) 13095	24.1	1.35	0.48
(control) 13101	14.1	1.43	0.8
(control) 13141	17	1.65	0.39
(control) 13145	8.6	1.56	0.46

The mean estimated biomass of the swab samples (with control samples excluded) was 18.56ng/μl, significantly lower than that of the faecal samples (3323ng/μl), $p < 0.0001$. Mean 260/280 ratio was 1.56 and mean 260/230 ratio was 0.482. These results again were significantly different from those of the faecal samples (1.967 and 1.877 respectively). This indicated that there was significant potential salt and protein contamination. However the presence of the swab material itself in samples likely accounted for this. After discussion it was decided to proceed with downstream qPCR analysis even though the samples were of estimated low biomass on nanodrop assay.

7.3.4 16s rRNA Real time Quantitative Polymerase Chain Reaction

qPCR was undertaken as the next step in analysis and employed the ViiA 7 Real-Time qPCR system as previously. qPCR reaction mix preparation followed the steps outlined in the methods section. 5μl of extracted DNA was used per well. The standard curve was again generated using decreasing known concentrations of amplified *Vibrio natregens* and a control reaction containing sterile molecular grade water was also run. All reaction mixtures whether they contained standard, control, or sample, were run in triplicate. qPCR cycles were set as per those detailed in the methods chapter.

All samples were run through these qPCR conditions however 2 samples from the same subject were found to have too little biomass to cross the amplification

threshold and therefore generate results. Therefore 47 samples generated qPCR results.

The mean 16s rRNA copy count for the total sample cohort was $6.892x10^7$/swab or $1.148x10^6$ /μl of extracted sample. Mean 16s rRNA copy count in the control swab cohort (n = 4) was $8.379x10^4$/swab and $1.396x10^3$/μl of extracted swab. As expected, mean bacterial count was significantly lower in the control group, p = 0.0108.

Streptococcus-specific qPCR was attempted on a random cohort of 5 of the extracted skin swab samples. The average streptococcus count per swab was 9.59 copies per swab. This was felt to be due to the relatively low biomass of the swab samples. It was decided after discussion that this streptococcus count would be too low to yield any meaningful results on comparative analysis and so streptococcus-specific qPCR was not performed on the swab cohort.

7.3.5 Longitudinal analysis of qPCR output

As again, similar to the faecal data set, not all sample sets were complete, statistical analysis of the change in bacterial count was conducted in two ways. The complete datasets of four samples (n=10) were assessed using Friedman's test to assess for any statistically significant differences between the mean bacterial count along to study timeframe. Mixed effects modelling was employed to assess the full dataset which included missing values. The data points are illustrated below in table 7.3.

Table 7.3: 16S rRNA copy count per sample, missing data points illustrated

	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**
1		136375.957		
2	6.5929695e+007	1.14140353e+7	1.29688566e+07	1748394.73
3	1.29871463e+07	209957.578	485656.641	
4	54676.5601	337610.479	2029460.63	20538.9331
5	207853.374	1981716.56	1273442.46	713186.895
6	8278.01239	9809819.06	5.15615138e+007	467221.641
7	1476893.79	687479.238	7938119.06	8.62969575e+007
8	347483.086	89181.9214	272237.256	1408737.77
9	3.2675571e+008	1382616.56	1.58413095e+008	8.7934752e+008
10	1922149.1	2826662.11	440742.539	1895001.21
11	2337532.27	7.5486654e+008	3.6427443e+008	3.0087393e+008
12	4071018.28	1.1518245e+007		4499047.03
13	4377907.03	4793557.5	1.79892585e+008	2.66150794e+007

Subject 1 is incomplete as 2 swabs did not cross the amplification threshold for detection and one swab was damaged prior to DNA extraction therefore was not processed due to contamination risk. Subject 3 and subject 12 both missed an interval appointment therefore have incomplete data sets. Therefore 47 datapoints make up the cohort set.

Total 16s rRNA count was assessed for all time points and mixed effects modelling was applied to test for any differences between the mean bacterial count at each time point (figure 7.1). Line graphs were used to represent the individual data sets and box plots represent the mean data. There were no statistically significant differences observed in total bacterial count per sample over the study period, $p=0.542$. Bacterial count was given as 16s rRNA copies/μl of sample.

Friedman's test was assessed on completed data sets of 4 longitudinal samples (n=10, figure 7.2). Post-hoc analysis was conducted to compare the means at each time point to assess for any statistically significant differences across the study period. There were no significant differences in mean bacterial load in the 4-sample set, p=0.589. Dunn's multiple comparisons test went on to reveal no individual statistically significant difference between mean bacterial density at each time point.

Data were assessed for any differences when stratified by eosinophil count. Linear regression was employed to assess for correlation between eosinophil count and bacterial load as given by 16S rRNA count/μl of extracted skin swab for each patient (Figure 7.3). There were no statistically significant relationships observed.

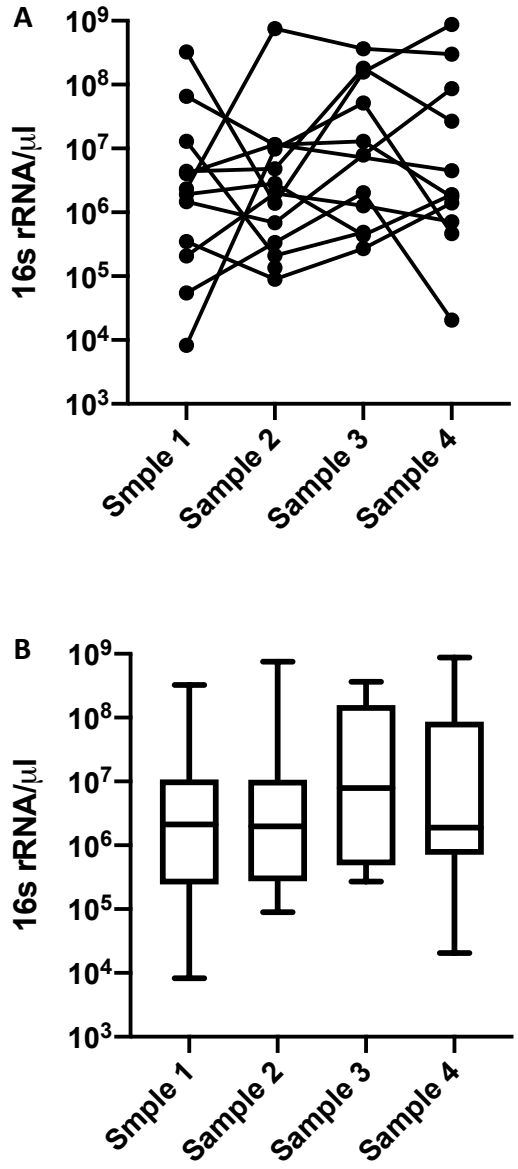


Figure 7.1: 162 rRNA/swab of all data

A. Line graph of full data set over time-scale of study. There were no significant changes in bacterial load/swab over the study duration, p=0.542.
B. Box-and-whisker plot of full data set over time-scale of study. There were no significant changes in bacterial load/swab over the study duration, p=0.542.

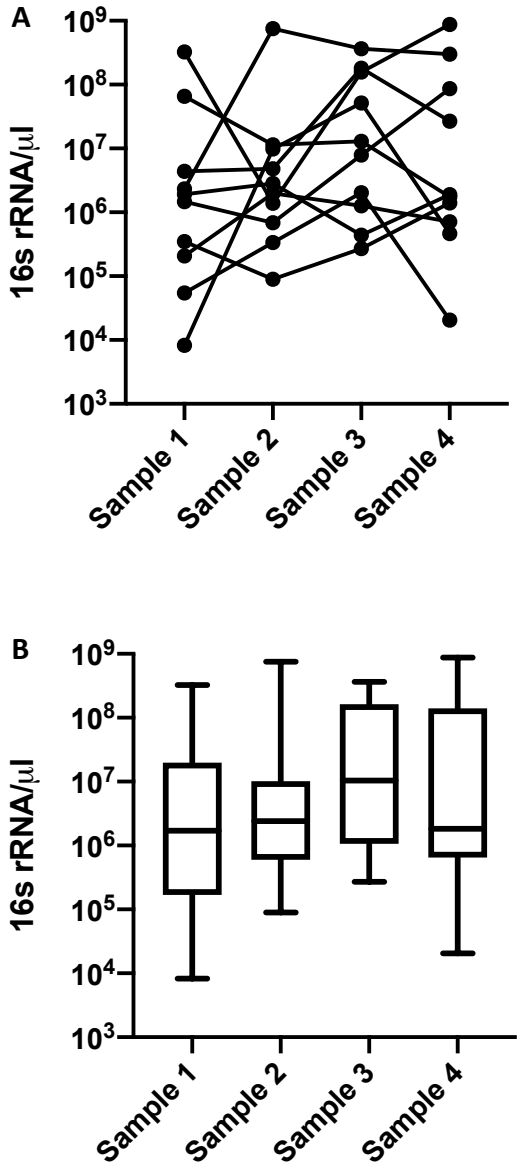


Figure 7.2: 16s rRNA/swab in full data sets only

A. Line graph of 10 four-sample sets over time-scale, there were no significant changes in bacterial load/swab over the study duration, p=0.589.

B. Box-and-whisker plot of 10 four-sample sets over time-scale, there were no significant changes in bacterial load/swab over the study duration, p=0.589.

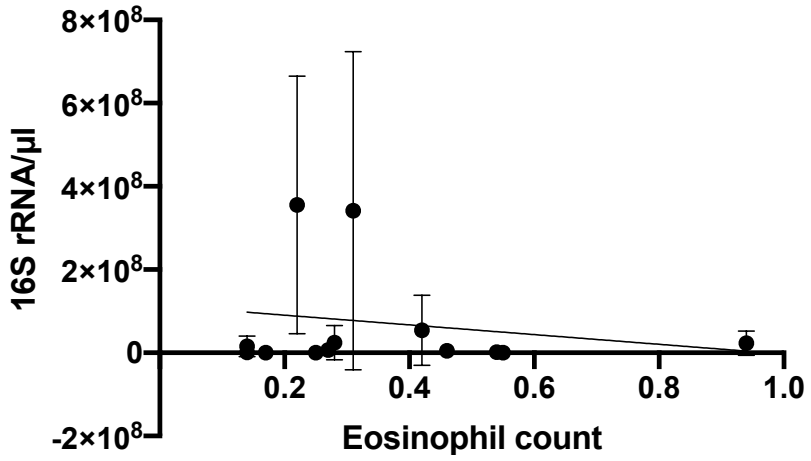


Figure 7.3: Eosinophil count v 16S rRNA/µl in skin swab samples

Figure 7.3 illustrates correlation between eosinophil count and 16S rRNA/µl of extracted skin swabs for each patient. Error bars are included. There were no statistically significant relationships observed.

7.4 Discussion

This chapter contained a comprehensive longitudinal assessment of the bacterial burden of the skin in asthmatic patients exposed to levofloxacin antibiotic. This assessment took place over an 8 week period during which patients repeatedly presented for skin swab collection. Though repeated-measure assessment of the skin microbiome is common in the literature, to our knowledge this has never been conducted in an asthmatic cohort. The patients included had been selected based on a combination of clinically relevant symptoms and demonstrable reversible airflow limitation. Careful patient selection adds strength to any relationships observed on data analysis and avoids any potential towards regression dilution bias. Longitudinal assessments of this type are important in the area of microbiome research as they can inform us regarding the stability of the system over time and in response to treatments.

This assessment revealed that the bacterial burden of the skin does not change significantly in response to systemic antibiotic treatment. Unfortunately we discovered that biomass was too low in skin swab samples to successfully run a streptococcus-specific analysis of the bacterial burden over time. Full sequence data would complete the picture and assess for compositional shifts in response to the antibiotic.

96% of the intended sample set were successfully collected, 2 patients did not attend for one review visit each. One swab was lost to contamination as detailed, and two samples did not have sufficient biomass to cross the threshold for detection on qPCR analysis therefore the number of swabs eligible for data analysis was 48, 92% of the potential cohort. There is no agreement in the literature as to the optimal method for collection of skin samples (219), however skin swab analysis as performed in the manner outlined in the methods section is the one most commonly employed (118, 198, 202, 203, 210). The greatest limitation of this method is that the conditions of each swab attempt (such as pressure, duration, and direction) cannot be well controlled or reliably reproducible and this has been

shown to affect collection efficiency (220). In order to control somewhat for this, all swabs from all subjects were collected by one individual over the study period. The observation that there were no distinct changes in bacterial burden in this cohort lends credibility to the concept that swab sampling was done in a reproducible manner.

7.5 Conclusion

The skin harbours a distinct microbiome which is known to be altered in atopic disease. There is little known about the skin microbiome in asthma and in particular there is little known regarding the response of the skin microbiome to antibiotics in asthmatic patients. We have shown that overall bacterial burden does not change meaningfully in asthmatic patients exposed to systemic levofloxacin over an 8 week period. The collection rate was good in this cohort and mixed effects modelling was employed for statistical analysis in cases where data points were absent. This analysis and ANOVA assessments confirmed that the 16s rRNA copy count in these samples remained overall stable during the study period despite exposure of subjects to an antibiotic. Further sequence analysis would complete this picture and assess for compositional shifts in the community.

Chapter 8: General Discussion

8.1. Asthma Diagnosis

Asthma is a common condition which poses a wide range of significant burdens to patients and health care systems. It is a common cause of morbidity for patients and can also be associated with significant mortality, according to WHO at least 250000 people die prematurely every year from complications of the disease (221). It is one of the leading causes of lost labour days in first world countries (222) and imposes significant burdens on healthcare systems in terms of financial cost and resource utilisation. It is also a contentious issue in the area of therapeutic use exemptions for high level competitive sports (223, 224). The diagnosis of asthma is not straightforward and there is a high level of diagnostic uncertainty involved, owing largely to the intermittent nature of the disease. When we consider the difficulty involved with a certain diagnosis in combination with the knowledge of the above burdens which the disease imposes on patients and health care systems, it is clear that correctly diagnosing patients who present with the symptoms of asthma is vital.

There is a wealth of evidence to suggest that asthma tends to be over-diagnosed in all populations if confirmatory pulmonary testing is not employed as part of the diagnostic process (15, 129, 225). This has important ramifications for patients. Attribution of symptoms to a condition such as asthma without objective evidence of reversible airflow limitation could lead to missed opportunities regarding identification of the correct underlying diagnosis. This also has knock on effects for investigative research as it introduces an element of observer bias and leads to the potential for regression dilution bias whereby the strengths of any observed relationships are attenuated if the exposure variable (in this case asthma) is incorrectly measured.

This study enrolled a cohort of patients who, in addition to complaining of the classical symptoms of asthma, demonstrated evidence of altered airway physiological parameters. Patients were enrolled when there was a strong clinical suspicion of asthma and conducted detailed lung function assessments. Patients

were considered asthmatic if they demonstrated reversible airflow limitation signalled by a change in FEV_1% predicted of >12% and 200ml or demonstrated significant airway hyperresponsiveness as indicated by a reduction in FEV_1% predicted of >20% on incremental inhalation of methacholine during bronchial provocation testing. Applying this criteria, a cohort of 13 asthmatics were enrolled.

In Chapter 3 we outlined that 71 patients were initially enrolled and screened as above with spirometry and reversibility, or with bronchial challenge test if no reversibility was observed. All had their diagnosis of asthma assigned to them by either their primary care physician or by the respiratory clinic in Connolly Hospital. 27 of the 71 assessed (38.1%) did not have airway physiology results which were consistent with asthma, nor had they any prior evidence of reversible airflow limitation on chart review. These patients underwent immediate clinical review and through thorough history taking and examination were assigned alternative diagnoses. 10 patients were diagnosed with gastroesophageal reflux disease, 13 with rhinosinusitis, 3 with hyperventilation syndrome, and 1 with vocal cord paradox. All patients were entered in to a regular clinical review schedule, inhalers were withdrawn, and patients were observed for any return of asthmatic-type symptoms despite adequate treatment of their underlying diagnosis. No patients reported symptoms necessitating re-testing of lung function or reintroduction of inhalers over one year of clinical review.

These results question the validity of physician-diagnosed asthma . a significant proportion of patients enrolled in this study did not have asthmatic airways on assessment of their lung function and all maintained good health when alternative diagnoses were assigned and treated. This proportion is similar to that quoted in previous literature (15). Results such as these make a strong case for increased availability of spirometry at the primary care level so that objective physiological criteria can be applied to the diagnosis of asthma for patients.

8.2. Asthma airway physiology

Other lung function tests can help to streamline the diagnosis of this condition and inform us regarding the phenotypic profile of patients. Fraction of exhaled nitric oxide assessment, end tidal carbon dioxide measurement, and skin prick allergy testing can help to build a physiological profile of asthmatics which can further inform treatments choice. Elevated FENO can be used with great effect as a marker of non-compliance with inhaled corticosteroids, $ETCO_2$ can help us to diagnose hyperventilation syndromes which commonly co-exist with and complicate asthma, and skin prick testing can inform us regarding allergen avoidance strategies and sub-lingual immunotherapies. These are tests which are generally only available in tertiary centres but which are very useful when applied.

We have evaluated all patients in this asthmatic cohort with this combination of lung function tests thereby establishing robust physiological profiles of the group. Firstly with regard to diagnosis, 6 subjects (46%) were diagnosed due to bronchodilator response. Mean % reversibility was 17.33% and mean absolute reversibility was 346.66ml. In the case where bronchodilator reversibility was not present on spirometry, patients then went on to have methacholine challenge testing performed. 7 patients (54%) were diagnosed due to positive bronchial challenge test results with a mean PD20 of 0.5mg methacholine. Next all patients underwent assessment of fraction of exhaled nitric oxide. The mean result was FENO of 38ppb (range 13 – 203) indicating a tendency toward mild eosinophilic inflammation in the group. 4 patients had levels greater than 50ppb indicating severe eosinophilic inflammation, 3 patients had levels of 25-50ppb indicating mild to moderate eosinophilic inflammation, and 6 patients had normal FENO. In terms of $ETCO_2$ measurement, 3 patients demonstrated low end tidal carbon dioxide levels consistent with concomitant hyperventilation. Only one patient of the cohort did not mount a response to allergen testing on skin prick, 3 patients reacted to single allergens, and the remained reacted to multiple allergens. When these various parameters were stratified according to eosinophil status, there were no appreciable differences. Spirometry results did differ significantly with patients who

had elevated eosinophil levels more likely to have reduced FEV_1 (p=0.0208) and FVC (p=0.0286). As this study involved repeated measures post antibiotic, all airway physiology assessments were repeated 8 weeks after the index measurement. There were no statistically significant changes noted in any single parameter and this was independent of eosinophil status. FENO levels did change and this approached significance (38ppb vs 26ppb, p=0.057).

In this way we can see that the major parameters of airway physiology combine to produce a useful clinical phenotype of asthmatic patients. These measures seem to be unchanged when assessed repeatedly, despite the addition of an intercurrent short-course of oral antibiotic and this is reflected in the literature. There are not many studies pertaining to repeated assessment of lung function after clinical interventions such as courses of antibiotics, but it is felt that airway physiology will remain relatively constant at this interval despite intervention. We postulate that the reduction in FENO was reflective of the increased awareness of their condition which occurs to most subjects enrolled in a study to investigate their disease. Subject bias such as this is often associated with increased compliance which could in turn have caused the observed reduction in FENO levels.

Overall this study reflects the utility and relative ease of building a physiological phenotype of asthmatic patients. There is a distinct role for this type of assessment not only in aiding diagnosis of this complex disease, but also in terms of streamlining treatment choice and monitoring of compliance to the treatments prescribed.

8.2 Integrative microbiome assessment in asthma

The respiratory microbiome is altered significantly in asthma (83). This finding was uncovered in the index exploration of the respiratory microbiome in 2010 and has been replicated in similar studies of the asthmatic microbiome since then. There is reduced microbial diversity observed in asthmatic samples due to expanded populations of species such as *Haemophilus* and *Moraxella* and other members of the Proteobacteria genus. The functional impact of this alteration is not clear, although certain parameters of the microbiome have been linked with various clinical markers (88, 149). The mediators of these relationships are not well identified or understood though there is emerging evidence to suggest that airway inflammation is ameliorated by certain bacterial species through inhibition of some classical asthma-related pro-inflammatory cascades (156, 157). Equally it seems that germ free mice exhibit exaggerated airway responses when inoculated with ovalbumin (158). This evidence gives credence to the hygiene hypothesis, namely that some level of early life microbial exposure is protective against allergic disease. However much of this evidence is based on mouse model trials, therefore it is difficult to extrapolate to asthmatic populations just yet.

The effects of many of the treatment strategies used to manage asthma are not well understood in terms of the microbiome with much discordance in the literature. Equally the role of antibiotics is not well documented. One recent study found that bacterial density does not change over time after an antibiotic exposure but that community composition and diversity did change meaningfully (162). A previous study evaluating azithromycin in a COPD cohort suggested that in-vivo findings of reduced inflammatory cytokines and elevated bacterial metabolites were responsible for the observation that azithromycin reduced exacerbation frequency in this cohort (160). Though the use of long term azithromycin has been shown to reduce exacerbation rates in asthma (161), long term use has also been associated with significant increases in macrolide resistance patterns (162). As we can see there is no generalised agreement in terms of antibiotic use in asthma and much less regarding the potential role for the microbiome in this area.

The gastrointestinal microbiome is has been the topic for high level research for a much longer time than the respiratory microbiome has, therefore there is a relative wealth of information in this area. The gut is dominated by the phyla Firmicutes and Bacteroidetes in health and dysbiosis is commonly associated with pathogenesis and propagation of multiple disease patterns, not only those diseases which locally affect the gut (174-177). Dysbiosis is caused mainly by environmental factors such as diet and alcohol consumption. There are similarly clear relationships between the gastrointestinal microbiome and asthma. Dysbiosis in infancy and childhood can predispose to the development of atopy and asthma (115). Dysbiosis can in turn be induced in infancy by antibiotics and even birth mode (108, 112). There is evidence for cross-talk between the microbiota of the airways and GI tract along the "Gut-Lung-Axis". This is the concept that cross-talk between these systems has an impact on immune regulation and therefore inflammation. This cross-talk seems to be mediated by short chain fatty acids which act via multiple various signalling pathways to limit inflammation in areas where there exists a rich and diverse microbiome (116). Despite the clear links between the gut and lung microbial communities, there are few studies which have sampled these concurrently to inform on direct relationships between the systems.

The skin is also home to a rich and diverse microbiome which contributes to its function as a barrier to external environments. The topographical variance of the skin is greater than in other microbiome systems and this is owing to differences in anatomical composition (124). There is evidence that the skin microbiome impacts on the immune system (208). There is certain evidence that the microbiome of the skin has significant effects on the development of atopic dermatitis, a condition closely related to asthma (122, 123). In addition, AD is known to be associated with an altered GI microbiome. There is little direct evidence for any role of the skin microbiome in relation to the risk of asthma development but it is likely, given particularly the evidence in terms of eczema, that there is some level of interplay between all three systems.

In order to capitalise on this gap in the literature, we designed a study that would assess microbiome outcomes in all three systems in an asthmatic cohort. We believed it was important as firstly, these relationships had not been assessed concurrently before, and secondly integrative studies such as this are vital in terms of any study of the microbiome. It is not known whether microbiome systems exist independently of each other or are related, most likely through immunomodulatory properties and cross talk. Though work is continually ongoing to answer this question, concurrent assessment of multiple microbiome systems has never been undertaken in asthma. We paired this concurrent assessment with assessment of airway physiology in an attempt to link microbiome outcomes to airway physiology markers of asthma, as has been done previously. We assessed all these measures after intervention with an antibiotic as this was another area which was not robustly studied prior to our study design.

Chapters 5, 6, and 7 detail the analysis of the respiratory, gastrointestinal, and skin microbiome systems respectively. 221 biological samples were gathered from the 13 patients overall and these included lung brush samples, throat and skin swab samples, and faecal samples. Mean 16s rRNA copy number was $3.139x10^7$ per lung brush, $9.112x10^9$ per throat swab, $2.095x10^{12}$ per gram of faecal sample, and $9.892x10^7$ per skin swab. The relative difference in bacterial burden tallies with reported levels for each sample type in the literature. Faecal samples are expected to have the largest bacterial burden, with the bacterial counts of throat swabs, skin swabs, and lung brushes decreasing sequentially. When longitudinal analysis was conducted into the bacterial burden of samples at different time points along the study period, it was clear that there were no statistically significant differences in the mean values at each of these time points across all sample types. Sub group analysis showed this to be independent of eosinophil count in all but oropharyngeal samples. The relevance of the trend towards significance on regression analysis of 16S rRNA copy count/μl of extract throat swab when correlated with eosinophil count ($p= 0.0473$) is unclear in this context. It seems that those with elevated eosinophil count have higher mean bacterial burden on oropharyngeal sampling than those with lower eosinophil counts. The sample size of this group means that

subgroup analysis is difficult to assess and we would apply caution in drawing meaningful conclusions from isolated significant results such as this.

Levofloxacin is an antibiotic which shows excellent efficacy against streptococcus species, particularly in the lung. We were able to assess streptococcus-specific qPCR counts in faecal samples which revealed that there were no significant differences in the streptococcus burden of faeces after levofloxacin.

This seems to agree with the results from the AMAZES trial and its sub-trial which investigated long term azithromycin use in asthma (162). The researchers also found that there were no significant changes in bacterial count on paired sputum samples before and after 48 weeks of oral azithromycin. We have shown this on a shorter term and with interval proxy measurement of the lower airway using oropharyngeal samples as well as using samples from other microbiome systems.

8.3 Strengths and limitations

This study employed detailed physiological testing to confirm asthma diagnosis in subjects prior to progression for bronchoscopy. In this way there were clear diagnostic criteria applied to patients and the recruited cohort were certain asthmatics. In our practice this had the previously discussed benefit of identifying patients who had been labelled as asthmatic but who did not fulfil criteria therefore prompting review and investigation to establish alternative diagnoses and treat there accordingly. This level of patient selection also reduced potential towards regression dilution bias, an error to which many asthma trials can be open.

This was a robust assessment of the microbiome across multiple systems which have shown to be potentially involved with each other in previous studies but which had not been studied concurrently. This was done alongside significantly detailed profiling of each subject's airway physiology and so provided a detailed integrative picture of the impact of antibiotics on asthmatic patients.

The study population was a small one. The literature on the asthmatic microbiome is composed of studies with varying sample sizes, generally ranging from single figure study cohorts to just over 100 patients. As such, it was impossible to run sample size calculations. In general it seems that those studies which utilise bronchoscopy as the sampling method of choice have fewer participants and those which involve less invasive sampling such as sputum collection have higher numbers. Our study combined multiple sampling methods and repeated these measures frequently over a relatively short space of time, generating a large biobank in terms of sample numbers. Bronchoscopy and faecal sampling would be considered quite invasive and throat and skin swabs less so. Overall this required huge input from our subjects and was quite an intensive study period for them. For this reason, sample size was limited. In addition, due to the intensity of this protocol for patients, they were given direct phone access to the research team. This allowed them to communicate any concerns, adverse effects of the antibiotic, or intercurrent illnesses to the team and allowed the team to freely arrange

suitable times for follow up visits. This contact was unrestricted and so for operational reasons, worked best with a relatively small cohort.

Unfortunately it was not possible to assess the *Streptococcus*-specific qPCR data for airway samples. Biomass of the skin swabs was too small to allow for assessment of *streptococcus* count in this sample group also. This is a limitation of the study as this analysis was available for one sample type but not for others. As levofloxacin is an antibiotic with strong activity against *streptococcus* species, this information would perhaps have shed light on potential differences or similarities between all the microbiome compartments assessed in this study in terms of how the *Streptococcus* burden changed.

The initial plan for this study was to progress all biological samples to undergo 16S rRNA sequencing in order to fully investigate the composition of the microbiome and to assess for compositional shifts throughout the study period related to the administration of the antibiotic. Unfortunately due to external factors this was not possible. Brexit and the knock-on effects on funding for studies such as this one meant that sequencing was not available within the scope of this MD. The decision was made to use the data available to us at the time of completion of the laboratory placement which represented a novel approach to assessment of the microbiome as previously discussed. This study therefore relied on 16s rRNA qPCR assessment alone. qPCR assessment limits the conclusions of this study to quantitative assessment of the bacteriome rather than the full microbiome as it does not take viral, fungal, or protozoal components of the microbiome into account. Even within assessment of the bacteriome, this analysis is quantitative and does not provide qualitative assessment of the compositional stability or lability of these samples over time. Without this additional information it is difficult to address the central hypothesis of this thesis, namely that the microbiome compartments studied are related in their response to systemic antibiotics. Even though the bacterial burden of these compartments have been shown to maintain stability over the time course of this study, it is possible that compositional shifts occurred which were not assessed.

It is hoped that these samples, which remain in storage at the lab in London, will undergo full sequencing assessment in the near future to potentially unravel the relationships between the various microbiome compartments in a more meaningful manner, as was the original intention of this study. These will certainly be exciting areas for future research in the field and we would hope that any further research would be conducted in a similarly integrative manner to this study.

8.4 Conclusions

The central hypothesis of this thesis posed two questions: whether the microbiomes of the airways, skin, and gastrointestinal system are related and whether these systems exhibit stability over time. This study was constructed and conducted with these questions in mind but these are multi-faceted queries regarding complex systems which are intrinsically difficult to measure. Studies of the microbiome are renowned to be open to multiple sources of confounding. They require the use of invasive sampling methods which can be demanding of subjects. Assessment technologies can be difficult to access and expensive to run. There is a lack of agreement as to the optimal sampling methods which would generate the most accurate representation of the microbiome.

To minimise the effects of these factors we aimed to increase the accuracy of our patient selection. Asthma is a condition which is difficult to diagnose. We have explored in detail the effects which an incorrect asthma diagnosis can have on patients and on the integrity of studies such as this. As such, we set out to enrol only patients who demonstrated a combination of respiratory symptoms suggestive of asthma and objective evidence of reversible airflow limitation. In doing so, we found that 38.1% of our patients who had been diagnosed with asthma based on clinical criteria did not fulfil physiological diagnostic criteria for asthma. This would suggest that judicious use of physiological testing could improve the accuracy of this diagnosis and allow the diagnosis of alternative conditions where applicable. We found that addition of ancillary lung function tests, namely FENO, $ETCO_2$ and skin prick allergy testing, provided additional evidence that the correct diagnosis had been made and enhanced patient profiling throughout the study.

In order to address the aforementioned central hypothesis we conducted a concurrent assessment of the airways, skin, and gastrointestinal system. This is a trial design that has not been attempted previously and as such is a novel and innovative approach to untangle some of the fundamental unanswered questions of this area. Direct comparison of the composition of these compartments was not

possible under the remit of this particular trial as sequencing was not possible. It is hoped that sequence data will complete the information we can gather from these samples through future collaboration with the research group in Imperial College, London. However the assessment that was conducted has shed light on some characteristics of the microbiome. It appears from this study that there are no meaningful differences in the bacterial burden of these systems over time in response to an antibiotic. This finding was common to all systems even when subgroup analysis was conducted and suggests that these compartments are in fact related, as is hypothesised in the literature.

The implications of this study are unclear and there is certainly a need for larger explorative trials in this area. Sequence data would address whether there are community compositional changes after exposure to an antibiotic despite stability of bacterial burden, as seen in limited other studies. The study of this area is in its infancy and trials such as this add to the research pool and contribute to unravel the complex entity of the respiratory microbiome and its relationship with asthma.

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Appendix 1

Full exclusion criteria:

- Asthmatic subjects must be non-smokers or ex-smokers with <5 pack-years smoking
- No morbid obesity (BMI>30)
- No diagnosis of rheumatoid arthritis (RA), allergic broncho-pulmonary aspergillosis (ABPA), or Churg Strauss syndrome
- No confounding drugs (beta-blockers, ACE inhibitors, anti-asthma immune modulators) other than steroids
- No antibiotics within 4 weeks
- No acute exacerbation of asthma within 4 weeks
- No history of upper or lower respiratory infection (including common cole) within 4 weeks of baseline assessments
- No confounding occupations (such as baking)
- No significant vocal cord disorder (VCD)

Appendix 2

Full patient information sheet and consent forms. One copy for patient notes, one copy for research database, one copy for Imperial College to be shipped with samples

The Microbiome of Asthmatic and Normal Airways: Patient Information Sheet

What is the purpose of the study?

Asthma is a condition that affects one in ten adults in Ireland and the United Kingdom. Asthma affects the bronchial tubes that carry air in and out of the lungs. These airways are narrowed by swelling and by the contraction of the muscle cells in their walls.

Asthma and allergies have a strong relationship to infections. The normal human airways and lungs contain microbes such as bacteria and moulds that are collectively called "The microbiome". A normal microbiome may promote the health of the airways. The microbiome can be disturbed in patients with asthma, and this may cause swelling and inflammation in the airway linings.

We want to understand the differences between the microbes that are present in healthy and asthmatic airways, so that we can develop better treatments for the disease. We will also find out whether smoking alters the microbial population in the lungs.

We will reach the airways by studying them through a bronchoscope. This is a safe procedure where a thin flexible tube is passed into the large bronchial tubes and samples from their lining are obtained. These samples are called biopsies and brushings.

We will take samples during the bronchoscopy to identify all of the organisms present and to show how much inflammation and damage is present in the airways. We will extract genetic material (DNA and RNA) from the human cells to the discover differences in the activity of genes between normal persons and people with asthma.

We will also take a sample of blood to measure the level of allergic antibodies and to look for changes in white cells that may accompany asthma.

All tests are used in routine clinical practice and have proven safety records. All information you share with us will be completely confidential.

Before you decide to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and to discuss it with others if you wish. Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. You do not have to take part, and if you do not wish to, please be assured that the medical care that you receive will not be affected.

Why have I been chosen?

We are asking you to take part in this study because you have asthma, or because you are either a non-smoking and or a smoking normal healthy volunteer.

Do I have to take part?

It is up to you to decide whether or not to participate. 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. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

Prior to agreeing to take part in this study, you will be provided with this information sheet to read. After consideration of this information (usually one week), if you wish to participate, you will be asked to attend a clinic visit, during which time, the study doctor will discuss the study and answer any questions you may have. If you are happy to proceed, we will ask you to sign a consent form in order to confirm that you have understood this information sheet and are happy to participate. If you participate, we will then ask you to make an appointment to have the following tests done.

Medical history and questionnaire

You will be asked questions about your medical condition and to fill up a standard questionnaire regarding asthma and smoking.

Breathing Tests

You will be asked to perform routine diagnostic breathing tests to find out how your lungs are functioning. For some of the tests, you may be asked to stop certain inhaler(s) for 24-48 hours before doing the breathing tests. It will be possible to use alternative inhalers and you will be given precise information on this.

Blood tests

We will take a sample of blood (5 teaspoonfuls; 30 ml) for the following tests:
We will measure the numbers and types of white cells in your blood. A further blood test will see if you have an allergic predisposition, by measuring the level of allergic antibodies.
We will extract DNA from the blood to see if there are any genetic or epigenetic variants that predispose to asthma, and we will assess markers of inflammation that include molecules called proteins and messenger ribonucleic acid (mRNA). Messenger RNA can help us understand how genes function. These molecules can affect asthma and may relate to what we find in the lung samples.
We will also measure how your blood clots prior to carrying out the bronchoscopy. If you are taking blood thinning medication, such as warfarin, you should not take part in the study.
The above tests should take 60 minutes to complete.
We will then book you for the bronchoscopy test. The test itself will take 20 to 30 minutes to complete, but you will stay in the bronchoscopy unit between 9 am and 2 pm. It is advisable to take the whole day off.

Bronchoscopy

Following your clinic review, you will be sent an appointment for a bronchoscopy. This is carried out in the Bronchoscopy Unit, at Connolly Hospital Dublin, You will be asked not to eat or drink anything from midnight prior to the bronchoscopy.
A bronchoscopy is routinely used for the diagnosis of lung conditions. It involves passing a thin fibreoptic tube (made up of many small glass fibres which transmit

light and it has a small camera at the end) via your nose or mouth, into the air passages of your lung. This allows the doctor to examine directly the large air passages of your lung.
We will spray a local anaesthetic called lignocaine (the same as the local anaesthetic used by dentists) on to the back your throat, into your nose and onto your voice box, so that the bronchoscope can be easily inserted into the air passage without discomfort. This is not a general anaesthetic. We will insert a small needle into a vein on the back of your hand or arm. We will give you a sedative medication (midazolam) into this needle. This medication will make you more relaxed and drowsy for the duration of the test. You may also be given salbutamol (a drug that opens up the airways) to inhale from a nebuliser after bronchoscopy.
We would like to retrieve the cells that are in the lung by the bronchoscope in three ways.
(1) First, we will also use a fine brush to collect some cells from the wall of the airways via the bronchoscope. This will be done 6 times.
(2) Second, we will take 8-10 samples of the airway lining called biopsies using biopsy forceps, which is not painful as there are no pain nerves in the airways.
(3) Thirdly, if the bronchoscopy has gone smoothly, we may wash out the cells from a small segment of the lung, by a procedure known as broncho-alveolar lavage. The lavage may introduces 120 mls of fluid into the lung, of which 60ml is recovered. It is safe and well tolerated by patients.
The bronchoscopy lasts approximately 20-30 minutes. After the bronchoscopy, you will be monitored for one to two hours in the recovery area within the endoscopy department. Therefore, in total you may spend up to 4 to 5 hours in hospital. You will be seen by the research doctor prior to discharge home. As you may be given a sedative medication, it is important that a responsible adult friend or relative accompanies you. You should not drive, use heavy machinery or drink alcohol for the rest of that day.
Bronchoscopies will only be performed by the doctors of the research team. In the unlikely event of our finding an abnormality during the course of this study, you will be referred to the appropriate Consultant for further assessment.

What else do I have to do?

You should continue to take your regular medication as usual. We may recommend that you stop some of your medication before the bronchoscopy, especially if you are on any blood thinning medication and your blood tests may be rechecked.

How will my donated samples be used?

The samples taken at bronchoscopy will be used to assess the amount of inflammation in your airways and whether there is infection within the lung. Blood samples will be used to extract RNA and DNA, and the serum will be kept for later analysis. . Your samples may be sent to other research collaborators for analysis but they will not be able to identify you as the samples will be anonymized – they will lack personal details, including your name and your address. In addition, your samples may be stored in a secure freezer for a maximum of 15 years for future analysis.

How will information stored on the computer be used?

Your anonymised medical information and any results will be put on a computer and stored in a secure electronic database. Some information may be sent to other research collaborators but as for the samples this information will be anonymized. When processing and storing personal information we will comply with the relevant laws to protect the confidentiality of research participants.

What is the drug or procedure that is being tested?

There is no drug being tested in this research.

What are the alternatives for diagnosis or treatment?

You can continue to be followed up for your asthma as usual by your GP or hospital practitioner. They will do routine investigations to manage your asthma and exacerbations based on their normal medical practice.

What are the side effects of any treatment received when taking part?

No treatment is being investigated in this follow up cohort.

What are the possible disadvantages and risks of taking part?

Blood tests will only be taken by trained personnel. Occasionally you may find some minor bruising. The breathing tests should not cause any discomfort, although some patients find that this test causes coughing.

Bronchoscopy is a standard diagnostic procedure and will be undertaken by an experienced doctor of the clinical team. It is a well-established procedure used on a routine basis at Connolly Hospital.

The small needle inserted into a vein may cause some minor bruising. Your mouth and throat may remain numb just in the same way as your mouth would after a dental procedure. As the bronchoscope is inserted you may feel some irritation in your throat and coughing. During the procedure there may be further coughing caused by irritation of the airways. You should feel much better within a few hours of the end of the procedure although your throat may remain sore for a couple of days. You may possibly notice a few flecks of blood in your spit. These discomforts will wear off and disappear within 2 hours or so.

You should not eat or drink for at least 2-3 hours, in order to keep food or liquids from accidentally entering the windpipe or lungs. Occasionally, following the test people get a 'flu'-like reaction with a fever. This only lasts a few hours and can be helped by taking paracetamol.

Sometimes the procedure can lead to a worsening of your asthma, usually in the form of increasing wheeze, cough and shortness of breath. If this were to happen during or after the bronchoscopy, you will be given prompt treatments for this. These treatments include the use of bronchodilators such as Ventolin given from a nebulizer. If necessary, we may keep you in Hospital for further observation for your recovery.

A rare complication (1 in 2000) of bronchoscopy is pneumothorax. This is a collapse of the lung that is treated by inserting a tube through the chest wall that allows the collapsed lung to expand. Another rare complication is significant bleeding. Obviously, if you were unfortunate enough to experience these complications you would be given the appropriate treatment in hospital.

What are the possible benefits of taking part?

We hope that you will benefit from the increased contact with specialist respiratory physicians and discussing your lung condition. They may suggest different treatments for your condition.

The donation of your samples will be crucial to understanding the process of asthma. Your participation and donation of samples may also provide benefit to future patients with asthma and related diseases, by increasing the possibility of developing new treatments.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Any other information about you which leaves the hospital will have your name and address removed so that you cannot be identified from it.

Your general practitioner will be informed of your participation. If the tests we will be performing during the study reveals something that is abnormal, we will inform your general practitioner.

What will happen to the results of the research study?

The results of this research will be presented at national and international medical conferences and published in medical journals within the next three years. Medical abstracts are usually available from the internet at no cost. Medical journals can provide full publications at a cost. You will be able to find publications on the internet, or we will provide copies to you of the relevant publications. You will not be identifiable in any report or publication.

Contacts for Further Information

Thank you for taking the time to read this information sheet. If you decide to participate in this study, you will also be given a copy of the signed consent form to keep.

Respiratory & Sleep Diagnostics Department
Connolly Hospital
Blanchardstown
Dublin 15
01-6466162

Consent

Patient copy

Name of Researcher: .....

If you agree with each sentence below, please INITIAL the box

1. I confirm that I have read and understand the information sheet dated August 2015 (version 1.0) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from Connolly Hospital. I give permission for these individuals to have access to my records. ☐
4. I agree to donate blood and samples taken at bronchoscopy to Connolly Hospital for the purposes of this study. ☐
5. I agree to give my permission for the samples to be used for future research. ☐
6. I agree to my GP being informed about my participation in this study. ☐
7. I agree to take part in the above study. ☐

Please print and sign your name below and add today's date (The participant must date his/her own signature):

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Researcher	Date	Signature
_____	_____	_____
Name of Person taking consent*	Date	Signature

*if different from researcher

Consent

Hospital notes copy

Name of Researcher: .....

If you agree with each sentence below, please INITIAL the box

1. I confirm that I have read and understand the information sheet dated January 2016 (version 1.0) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from Connolly Hospital. I give permission for these individuals to have access to my records. ☐
4. I agree to donate blood and samples taken at bronchoscopy to Connolly Hospital for the purposes of this study. ☐
5. I agree to give my permission for the samples to be used for future research. ☐
6. I agree to my GP being informed about my participation in this study. ☐
7. I agree to take part in the above study. ☐

Please print and sign your name below and add today's date (The participant must date his/her own signature):

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Researcher	Date	Signature
_____	_____	_____
Name of Person taking consent*	Date	Signature

*if different from researcher

Consent

Name of Researcher: .....

If you agree with each sentence below, please INITIAL the box

1. I confirm that I have read and understand the information sheet dated Jan 2016 (version 1.0) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from Connolly Hospital. I give permission for these individuals to have access to my records. ☐
4. I agree to donate blood and samples taken at bronchoscopy to Connolly Hospital for the purposes of this study. ☐
5. I agree to give my permission for the samples to be used for future research. ☐
6. I agree to my GP being informed about my participation in this study. ☐
7. I agree to take part in the above study. ☐

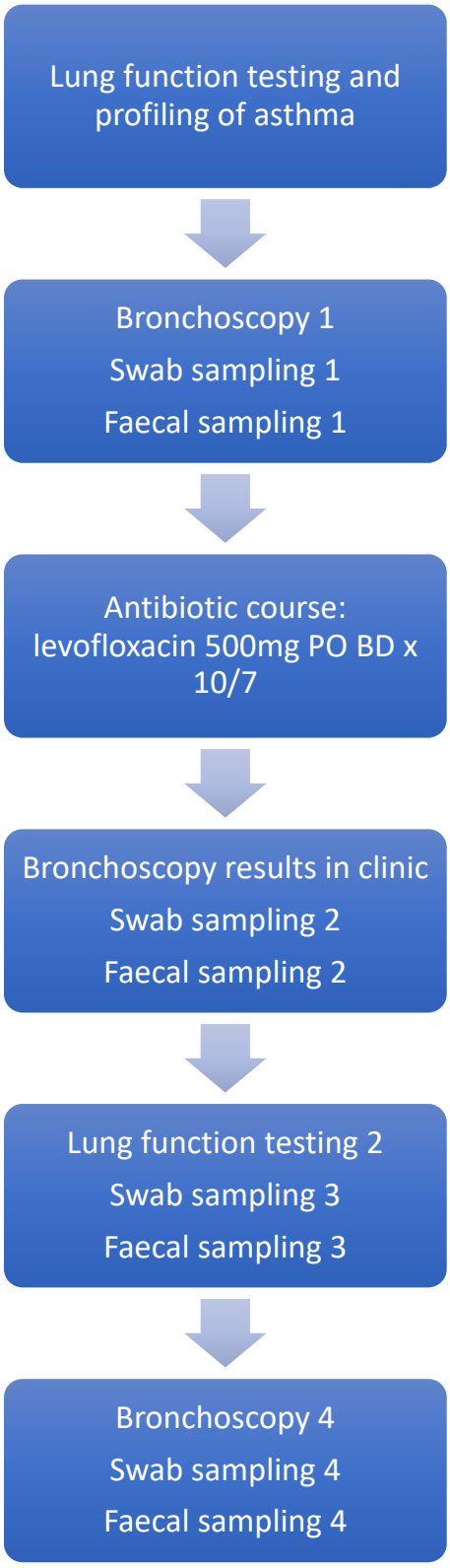
Please print and sign your name below and add today's date (The participant must date his/her own signature):

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Researcher	Date	Signature
_____	_____	_____
Name of Person taking consent*	Date	Signature

*if different from researcher

Appendix 3

Flow chart of patient visits



Appendix 4

Additional patient information sheet and consent form

Respiratory & Sleep Diagnostics Department, Connolly Hospital, Blanchardstown, Dublin 15

The Microbiome of Asthmatic and Normal Airways: Additional Patient Information Sheet and consent form

You have undergone extensive lung function testing with us as part of this research and you have been diagnosed with asthma. We have already gathered samples from bronchoscopy testing and blood sample analysis with the aim of identifying the organisms present in your lungs and the degree to which they are causing any damage or inflammation in your airways.

In a given year many patients with asthma need treatment with a course of standard antibiotics as part of their routine care; this may have already occurred for you. In the event of such treatment we can offer to repeat lung function tests, bronchoscopy, and blood tests as part of your routine asthma management. With your permission, we can again send samples to the Imperial College in London for expert analysis, as occurred with your first bronchoscopy.

This repeat testing, if indicated, would allow us to assess whether any changes in the organisms in your lungs are present over time. We would also be able to assess whether standard antibiotics you might receive during the natural course of your treatment can change the profile of organisms in your lungs in a meaningful and lasting way.

Some recent information suggests that organisms outside the lung especially in the gut may impact on lung function. Therefore we also aim to assess the organisms present in skin and gut samples to see whether any differences in the organisms in your lungs occur throughout other body systems. With this aim in mind, we would add a skin swab taken at the time of your bronchoscopy (painless cotton-tipped swab rubbed against the inside of your elbow) as well as faecal samples taken at your convenience. We would provide you with a standard container and ask that you return a faecal sample to us at your convenience.

If you are happy to proceed we will ask you to sign the below consent form. All other information supplied in the original patient information leaflet also applies to this phase of the study.

Contacts for Further Information

Thank you for taking the time to read this information sheet. If you decide to participate in this phase of the study, you will also be given a copy of the signed

consent form to keep. Your follow up and clinical care will not be affected should you chose to decline to participate.
Respiratory & Sleep Diagnostics Department
Connolly Hospital

Consent

Patient copy

Name of Researcher: .....

If you agree with each sentence below, please INITIAL the box

1. I confirm that I have read and understand the information sheet dated May 2017 (version 2.0) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from Connolly Hospital. I give permission for these individuals to have access to my records. ☐
4. I agree to donate blood and samples taken at bronchoscopy to Connolly Hospital for the purposes of this study as well as additional skin and faecal samples. ☐
5. I agree to give my permission for the samples to be used for future research. ☐
6. I agree to my GP being informed about my participation in this study. ☐
7. I agree to take part in the above study. ☐

Please print and sign your name below and add today's date (The participant must date his/her own signature):

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Researcher	Date	Signature
_____	_____	_____
Name of Person taking consent*	Date	Signature

*if different from researcher

Document Celfire_3

The Microbiome of Asthmatic and Normal Airways

[redacted]

Celtic Fire
Site: Dublin
Study I.D: 693
Date: _____
Initials: _____

Questionnaire

Height _____

Weight _____

Confirms to inclusion and exclusion criteria ☐

Case ☐

With repeat ☐

Control ☐

PATIENT DETAILS

1. Study ID
2. SEX (1=M, 2=F)
3. Date of Birth (DDMMYYYY) / /
4. Ancestry (1=European/Celtic, 2=Asian, 3=Other)
5. Occupation

I am going to ask you some questions, mainly about your breathing. I should like you to answer yes or no, or give a number whenever possible.

SHORTNESS OF BREATH

6. Are you troubled by shortness of breath when hurrying on level ground or walking up a slight hill? (1=Yes, 2=No)
7. Do you find it difficult to keep pace with other people of your own age when you walk?
8. Have you ever been woken by an attack of shortness of breath?

   If yes:

   a. How many times has this happened in the last year?

WHEEZE AND TIGHTNESS

9. Have you ever had attacks of shortness of breath with wheezing?

   If yes:

   a. How many times has this happened in the last year?

10. Has your doctor ever told you that you have had an attack of asthma?

    If yes:

    a. How many times has this happened in the last year?

11. What age were you when you had your first asthma attack?

SMOKING

12. Have you ever smoked regularly? (This means at least one cigarette a day OR one ounce of tobacco a month, for at least a year)

    If yes:

    a. How old were you when you started smoking regularly?

Document Celfire_3

13. If you have given up smoking, how many years ago did you last gave up? ☐

14. Over the time that you have smoked, how many cigarettes a day would you usually smoke on average? ☐

MEDICATIONS

What medications are you taking? (list)

15. Beta-adrenergic bronchodilators?* (**interpreted by interviewer*) ☐

16. Inhaled corticosteroids* ☐
    a. High dose?
    b. Low dose?

17. Inhaled combinations* ☐
    a. High dose?
    b. Low dose?

18. Oral corticosteroids?* ☐
    c. Name?
    d. Dose?

19. Any other medications (1=Yes, 2=No) ☐

20. Have you taken antibiotics within the last six weeks? ☐

OTHER MEDICAL CONDITIONS

21. Do you suffer from hayfever? ☐

22. Do you suffer from an itchy rash on the insides of your elbows or knees (eczema)? ☐

23. Do you have any other medical conditions (please list) ☐

24. Record the presence of conditions relevant to bronchoscopy, including vocal cord dysfunction, hypertension, rhinitis, nasal polyps, sinusitis, GORD, eczema, diabetes, and anticoagulant therapy. ☐